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The structure of the α-galactosidase gene loci in *Thermus brockianus* ITI360 and *Thermus thermophilus* TH125

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Abstract The *Thermus thermophilus* TH125 α-galactosidase gene, *agaT*, and flanking sequences were cloned in *Escherichia coli* and sequenced as well as flanking sequences of the previously cloned *agaT* from *Thermus brockianus* ITI360. Different structures of putative α-galactosidase operons in the two *Thermus* strains were revealed. Downstream of and overlapping with the α-galactosidase genes of both strains, a gene was identified that is similar to the galactose-1-phosphate uridylyltransferase gene (*galT*) of *E. coli* and *Streptomyces lividans*. Upstream of the *agaT* of *T. brockianus* ITI360, four open reading frames were observed. The deduced translation products displayed similarity to components of bacterial binding protein-dependent transport systems and a β-galactosidase. No galactoside utilization genes were identified upstream of *agaT* in *T. thermophilus* TH125. The inactivation of the α-galactosidase genes of both strains by insertional mutagenesis led to an inability to use melibiose or galactose as a single carbohydrate source. An attempt was made to isolate a gene encoding the enzyme responsible for para-nitrophenyl-(pNP-) β-galactoside hydrolyzing activity in *T. thermophilus* TH125. A gene designated *bglT* was cloned and expressed in *E. coli*. The inactivation of the *bglT* gene led to 55% reduction of the pNP-β-galactoside hydrolyzing activity in the mutant strain in comparison to the wild type.

Key words Thermophilic bacteria · Galactoside utilization genes · Insertional inactivation

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Introduction

Bacteria of the genus *Thermus* constitute the largest group of nonsporulating, aerobic heterotrophic thermophiles, containing several different species (Williams 1992). They have been isolated from neutral to alkaline terrestrial and marine hot springs around the world, and even from deep-sea hydrothermal vents (Marteinsson et al. 1995). *Thermus* bacteria have gained interest because of the biotechnological potential of their thermostable enzymes. Hence, many *Thermus* genes have been cloned and expressed in *E. coli*. Further, the application of *Thermus* strains as hosts for "in vivo protein engineering" has been demonstrated. For example, *Thermus thermophilus* was successfully applied by the selection of thermostable variants of 3-isopropylmalate dehydrogenase (Tamakoshi et al. 1995; Kotsuka et al. 1996).

We have been studying thermostable α- and β-galactosidases from various thermopilic bacteria, with reference to required properties for industrial application (Ganter et al. 1988; Fridjonsson et al. 1999a,b). Our intention was to study the evolutionary potential of various galactosidase genes by cloning them into a thermophile and by selecting thermostable enzyme variants. Such an attempt requires an examination of the galactoside utilization of the thermophile and the inactivation of the host genes involved.

Recently, we described the cloning and the inactivation of the α-galactosidase gene (*agaT*) from *Thermus brockianus* strain ITI360 (Fridjonsson et al. 1999b). Sequence analysis indicated the existence of a β-galactosidase gene upstream of *agaT*. For a potential future application of *T. brockianus* as a host strain for "in vivo protein engineering" of galactosidases, we decided to study the galactosidase activity in this strain further as well as the organization of the galactosidase genes (in a potential operon). Because this strain has a low transformation efficiency (Fridjonsson et al. 1999b), another strain, *Thermus thermophilus* TH125, which displays a high transformation capacity (Koyama et al. 1986; Hidake et al. 1994), was concurrently examined with respect to the same potential application. This work included an investigation of the corresponding enzyme

activities in the strain as well as isolation and characterization of the relevant genes and their genomic context.

This article describes differences in the organization and regulation of galactosidase genes in *T. brockianus* ITI360 and *T. thermophilus* TH125; the inactivation of the α-galactosidase gene in TH125; and the consequences of α-galactosidase gene inactivation in both strains. Further, the cloning and inactivation of a gene encoding a β-glycosidase in *T. thermophilus* TH125 is described.

Materials and methods

Bacterial strains and plasmids

The *Thermus brockianus* strain ITI360 was obtained from a collection of thermophilic bacteria, Idntaeknistofnum Islands, IceTec. *Thermus thermophilus* TH125 [*trpB*5] was generously provided by T. Hoshino. The *Escherichia coli* strains TAP90 [*supE*44, *supF*58, *hsdR*, *pro*, *leuB*, *thi*-1, *rpsL*, *lacY*1, *tanA*1, *recD*1903::minitet] (Patterson and Dean 1987) and HB101 F'*lac* [::Tn*1739 tnpR*] *supE*44, *hsdS*20, (r_B^-, m_B^-), *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*-5, *mtl*-1 F′ [::Tn*1739* Cm^R *lac*] (Altenbuchner 1993) were used as hosts for λ-RES phages and RES plasmids, respectively. *E. coli* JM109 [*supE*44Δ(*lac-proAB*), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1] (F'*traD*36, *proAB*, *lac*1^qZΔM15) (Vieira and Messing 1982) was used as a host for sequencing plasmids. The λ-RESIII, pBTac1, and pUC18/19 are described elsewhere (Altenbuchner 1993; Brosius et al. 1981; Viera and Messing 1982, respectively).

Media, culture conditions, and transformation procedures

Thermus brockianus ITI360 and *T. thermophilus* TH125 were grown at 65°C under strong aeration in the nutrient medium 162 of Degryse et al. (1978), with 0.25% trypton and 0.25% yeast extract at pH 7.5. Growth of *T. brockianus* ITI360 on single carbon sources was tested on agar plates with a minimal medium 162 containing 0.05% NH_4Cl, biotin ($50\,\mu g\,l^{-1}$), and thiamin ($1\,mg\,l^{-1}$). Growth of *T. thermophilus* TH125 on single carbon sources was tested on the same medium with a slight modification. Instead of titriplex I, EGTA (ethylene glycol-*O*, *O*′-bis(2-aminoethyl)-*N*,*N*,*N*′*N*′-tetraacetic acid), was used as a chelating agent ($15\,mg\,l^{-1}$). The medium was also supplied with tryptophan ($50\,\mu g\,ml^{-1}$). The method of Koyama et al. (1986) was used with a slight modification for the *Thermus* transformation as previously described (Fridjonsson et al. 1999b). Transformants were selected at 60°C on 162 agar plates containing $20\,\mu g\,ml^{-1}$ kanamycin. The *E. coli* strains were grown in Luria Bertani medium (LB) at relevant temperatures. When necessary, selective antibiotics were added ($100\,\mu g\,ml^{-1}$ ampicillin, $25\,\mu g\,ml^{-1}$ kanamycin). *E. coli* transformation was performed according to the TSS (transformation and storage solution) method (Chung et al. 1989).

Enzyme assays

Cells of 10-ml *Thermus* cultures were harvested by centrifugation, washed, and resuspended in 0.1 M potassium phosphate buffer, pH 6.5. Crude extracts were prepared by sonication and debris was removed by centrifugation. The protein concentration of crude extracts was estimated by the method of Bradford (Bradford 1976) using bovine serum albumin as a standard. α-Galactosidase activity was determined by measuring the hydrolyzing rate of para-nitrophenyl-α-D-galactoside (pNP-α-galactoside), 13.3 mM ($4\,mg\,ml^{-1}$) in 0.1 M potassium buffer, pH 6.5, as previously described (Ganter et al. 1988). One unit (U) of activity is defined as the amount of enzyme that liberates 1 μmol *p*-nitrophenol per minute under the given assay conditions. β-Galactosidase activity was determined by using para-nitrophenyl-β-D-galactoside (pNP-β-galactoside), 13.3 mM ($4\,mg\,ml^{-1}$), under the same conditions as described for pNP-α-galactoside. Activity tests were usually carried out at 70°C. For the determination of temperature optimum of activity, the tests were performed at the temperature range from 25° to 100°C.

Cloning of *T. thermophilus* TH125 α-galactosidase gene (*agaT*)

Recombinant DNA techniques were performed by conventional protocols (Sambrook et al. 1989). DNA was extracted from the thermophilic bacteria, and a size-fractional partial *Xho*II digest was used to construct a genomic phage λ-RESIII library. The library was amplified in the *E. coli* strain TAP90. Plaque hybridization was performed by using a DIG-labeled (Boehringer Mannheim) *agaT* gene fragment from *T. brockianus* ITI360 as a probe (Fridjonsson et al. 1999b); the hybridization solution contained 50% formamide. The λ-RESIII vector allows the excision of cloned fragments by site-specific recombination from the λ DNA and conversion into autonomously replicating plasmids, achieved by infecting the *E. coli* strain HB101 harboring the transposon Tn*1739tnpR* on a F' plasmid (Altenbuchner 1993). The presence of an α-galactosidase gene on a plasmid, designated pOF455, was confirmed by measuring α-galactosidase activity at 70°C in crude extracts of the plasmid harboring the *E. coli* strain, using pNP-α-galactoside as a substrate.

Cloning of a β-glycosidase gene (*bglT*) from *T. thermophilus* TH125

The *E. coli* strain HB101 F'*lac*::Tn*1739tnpR* was infected with about 2000 recombinant phages from a *T. thermophilus* genomic library. Following infection and plasmid conversion, the cells were plated on LB agar containing kanamycin ($25\,\mu g\,ml^{-1}$) and incubated at 37°C overnight. Single colonies were immobilized on nylon membranes (Qiagen). For inactivation of the host's own β-galactosidases, the membranes were placed on filter papers saturated with potassium phosphate buffer in petri dishes and

incubated for 30 min at 70°C in a water bath. The membranes were again placed on filter papers in petri dishes, saturated with phosphate buffer containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactospyranoside, 100 μg ml^{-1}), and incubated in a water bath for 6 h at 70°C. Colonies matching immobilized colonies that turned blue were picked, cultured, and the harboring plasmids subjected to restriction analysis. One plasmid, designated pOF151, was subjected to DNA sequencing.

DNA sequencing

Restriction fragments of plasmids pOF455 and pOF151 were subcloned into pUC18/19 for sequencing. To generate templates for sequencing the entire insert of plasmid pOF932, ~8000 base pairs (bp) containing the α-galactosidase gene cluster of *T. brockianus* ITI360 (Fridjonsson et al. 1999b), a transposon mutagenesis method with Tn*5491* was employed (Fischer et al. 1996). Sequencing reactions of double-stranded DNA were carried out according to the dideoxy chain termination method with universal and internal primers (Sanger et al. 1977). DNA was analyzed with an automated laser fluorescent A.L.F. sequencer (Pharmacia). The nucleotide sequence was analyzed on a Sun workstation with programs from the University of Wisconsin Genetics Computer Group package, version 8.01 (Devereux et al. 1984). All database searches were run with the program BLASTX on a server from the National Center for Biotechnology Information, Bethesda, MD, USA (Altschul et al. 1990).

Inverse PCR

An inverse PCR was performed to isolate DNA sequences upstream of the insert of pOF932. *T. brockianus* ITI360 chromosomal DNA was digested with various restriction enzymes, blotted, and hybridized, using a 500-bp fragment from one end of the pOF932 insert as a probe (region upstream of *agaT*). *Bgl*II restriction fragments corresponding to a band detected by the Southern hybridization (4.4 kb) were eluted from an agarose gel and ligated with T4 ligase for circularization. Primers directing divergent DNA synthesis from sites internal to the insert of pOF932 were used to amplify DNA from such circular molecules. A DNA product of the expected size was cloned into pUC18 to produce pOF1072 and sequenced. A new inverse PCR was performed, now using circularized *Sac*I digest (4.2-kb fragment) and primers directing divergent DNA synthesis from sites internal to the insert of pOF1072 and near the *Sac*I restriction site in the 5′-sequence region of the pOF932 insert. A DNA product of the expected size was cloned into pUC18. The resulting plasmid, pOF1810, was used for sequence analysis.

Subcloning of *bgaT* and *bglT* into pBTac1

bgaT and *bglT* from *T. brockianus* ITI360 and *T. thermophilus* TH125, respectively, were amplified by PCR, cloned into pBTacI, and expressed in *E. coli* JM109. For the amplification of the *bgaT* gene, the forward primer S1937, CGG AAT TCT TAT GCT AGG TGT TTG CTA C, and the reverse primer S1230, GGA ATT CCA TAT GCC CTT AAC CCT CCT CCC AAA C, with *Eco*RI linkers, were used. For the *bglT* amplification, the forward primer S1669, CCG GAA TTC TTA TGA CCG AGA ACG CCG AA, and the reverse primer S1670, CCG GAA TTC TTA GGT CTG GGC CCG CG, with *Eco*RI linkers, were used.

Insertial inactivation of *agaT* in *T. thermophilus* TH125

For the chromosomal inactivation of *agaT* in *T. thermophilus* TH125 the following integration cassette was constructed. A *Bam*HI–*Bgl*II fragment from pOF455, encoding the C-terminal end of *T. thermophilus agaT*, was cloned into pUC18 to produce pOF552. A 1030-bp sequence upstream of the *agaT* gene in *T. thermophilus* TH125 (5′ flanking sequence) was amplified by PCR, using the forward primer S939, CGG GAA TTC GCC GCC ATG GGA ATT, with a *Eco*RI linker and the reverse primer S940, GAA TTC CAT ATG CCT CCC TCC TTG CCG C, with a *Nde*I linker. The gene coding for the thermostable kanamycin resistance protein from pYK134 (*kan*) (Hoshino et al. 1993), kindly supplied by T. Hoshino, was amplified by PCR using the forward primers S941, GAA TTC CAT ATG AAT GGA CCA ATA ATA ATG, with a *Nde*I linker and the reverse primer S718, CGG GAT CCG TCA TCG TTC AAA ATG G, with a *Bam*HI linker. Subsequently, the amplified 5′ flanking sequence was cut with *Eco*RI and *Nde*I and the amplified *kan* fragment was cut with *Nde*I and *Bam*HI. The two fragments were inserted between the *Eco*RI and *Bam*HI site of pOF552 in a three-fragment ligation. The resulting plasmid (pOF1051) was brought into *E. coli* JM109 by transformation. Further, *T. thermophilus* TH125 was transformed with pOF1051 prepared from *E. coli*. The resulting transformants were analyzed for α-galactosidase activity and for growth on minimal medium agar plates containing single carbohydrate sources. Chromosomal DNA of the wild-type strain TH125 and a deletion strain, named OF1051, was digested with *Sac*I/*Bgl*II, and Southern hybridizations were carried out with DIG-labeled (Boehringer Mannheim) *agaT*- and *kan* fragments as probes. The same DNA was digested with *Bam*HI/*Bgl*II, and Southern hybridizations were performed with the 5′ flanking sequence and the *galT* fragment (3′-sequence) as probes.

Inactivation of *bglT* in *T. thermophilus* TH125

For the inactivation of *bglT* in *T. thermophilus* TH125, a 790-bp fragment downstream of the *bglT* gene in pOF151 was amplified via PCR, using the forward primer S1598, CGG GAT CCC CGC CAG AGG GGC GG, with a *Bam*HI linker and a reverse primer S1600, CCC AAG CTT CTC CGA CGG CCA GTC C, with a *Hin*dIII linker. The fragment was cut with *Bam*HI and *Hin*dIII and inserted between the *Bam*HI and *Hin*dIII of pUC18 to produce

pOF10723. A 390-bp fragment upstream from the *bglT* gene in pOF151 was amplified via PCR with the forward primer S1596, CCG GAA TTC CTC AGG GGC TTC TTC G, with a *Eco*RI linker and the reverse primer S1597, GAA TTC CAT ATG AGG CGT TTC TCT CCA A, with a *Nde*I linker. The amplified fragment cleaved with *Eco*RI and *Nde*I was ligated along with the amplified *kan* fragment described earlier, cleaved with *Nde*I and *Bam*HI, into pOF10723 following *Eco*RI and *Bam*HI digestion. The resulting plasmid, pOF10725, was brought into JM109 by transformation. Further, *T. thermophilus* TH125 was transformed with pOF10725. The transformants were analyzed for β-galactosidase activity. Chromosomal DNA of the wild-type strain TH125 and a deletion strain, named OF10725, was digested with *Sac*I, and Southern hybridizations were performed with DIG-labeled (Boehringer Mannheim) *T. thermophilus bglT* gene fragment, *kan* gene fragment, and 5′-and 3′-flanking sequence fragments as probes.

Results

α- and β-Galactosidase activities in crude extracts of *T. brockianus* ITI360 and *T. thermophilus* TH125

A prerequisite for a "thermoadaptation" of a metabolizing enzyme in a thermophile is the capability of the host to import and metabolize the relevant substrates. The galactoside utilization of the *Thermus* strains was studied by investigating the corresponding enzyme activities and the growth on minimal medium containing galactosides as a sole carbohydrate source.

The effect of various sugars on the α- and β-galactosidase activity in strains ITI360 and TH125 was examined by adding them to the culture medium. In *Thermus* ITI360, the α-galactosidase activity increased about tenfold and the β-galacosidase activity about fivefold after the addition of galactose. Less effect was observed by addition of melibiose or lactose, that is, twofold increase of the α- and β-galactosidase activity, likely because of the release of galactose by the hydrolysis of melibiose and lactose in the cell. In contrast, neither galactose nor the disaccharides in culture medium had any effect on the α- and β-galactosidase activity in *T. thermophilus* TH125 (Table 1).

Growth on minimal agar medium containing a single carbohydrate source

The two *Thermus* strains were tested for growth on minimal agar medium. The glucose content was 0.1% and the galactose and galactoside content was 0.4%. Growth was generally achieved by incubating the minimal agar plates 2–5 days at 65°C, depending on carbon source. *T. brockianus* ITI360 was able to use all sugars tested as a single carbohydrate source. Growth on medium supplemented with galactose, melibiose, raffinose, and lactose was achieved after incubation for 2–3 days. Growth on glucose was poor but could be adapted, which was observed by the appearance of single colonies (after 3–4 days). *T. thermophilus* TH125 grew well on glucose (incubation for 1–2 days). However, in spite of the observed constitutive α- and β-galactoside hydrolyzing activities, the strain exhibited poor growth on raffinose and lactose (>5 days). Only a slow growth on minimal medium with melibiose and galactose was achieved (4–5 days).

The sequence analysis of the flanking regions of *agaT* from *T. brockianus* ITI360

Expression of heterologous galactosidase genes in a thermophile and subsequent growth selection of a thermostable enzyme variant require the establishment of a suitable host strain. Thereby, the corresponding host genes must be inactivated. Sequence data of the genes and the flanking regions facilitate construction of knockout mutants and simplify the development of such in vivo selection systems.

The nucleotide sequence and the expression of an α-galactosidase gene (*agaT*) from *T. brockianus* ITI360 are described elsewhere (Fridjonsson et al. 1999b). The gene was isolated on a 8.0-kb chromosomal fragment from the strain ITI360 and cloned into *E. coli.* To achieve sequence data of the flanking regions of *agaT* and to examine whether the gene is a part of an operon, the upstream and downstream regions of *agaT* were sequenced. Furthermore, fragments upstream and overlapping with the 8.0-kb fragment, obtained by inverse PCR, were sequenced. The locations, sizes, and order of detected genes are represented diagrammatically in Fig. 1.

In a region of 4572 bp upstream of *agaT*, four open reading frames (*orfs*) were identified. The predicted amino acid

Table 1. α-Galactosidase and β-galactosidase activity ($mU\,mg^{-1}$) in crude extracts of *Thermus brockianus* ITI360 and *T. thermophilus* TH125

Strain	Enzyme activity	Without sugar	Melibiose	Lactose	Galactose
T. brockianus ITI360	α-galactosidase	16	41	37	147
	β-galactosidase	38	76	70	210
T. thermophilus TH125	α-galactosidase	72	68	59	63
	β-galactosidase	195	195	178	191

The strains were grown for 15 h in 162 nutrient medium with 0.4% galactose, lactose, or melibiose, or without sugar. Activity tests (in triplicates) were performed at 70°C using pNP-α- and pNP-β-galactopyranoside as substrates. The maximum variation from the mean values (shown) was less than 5%

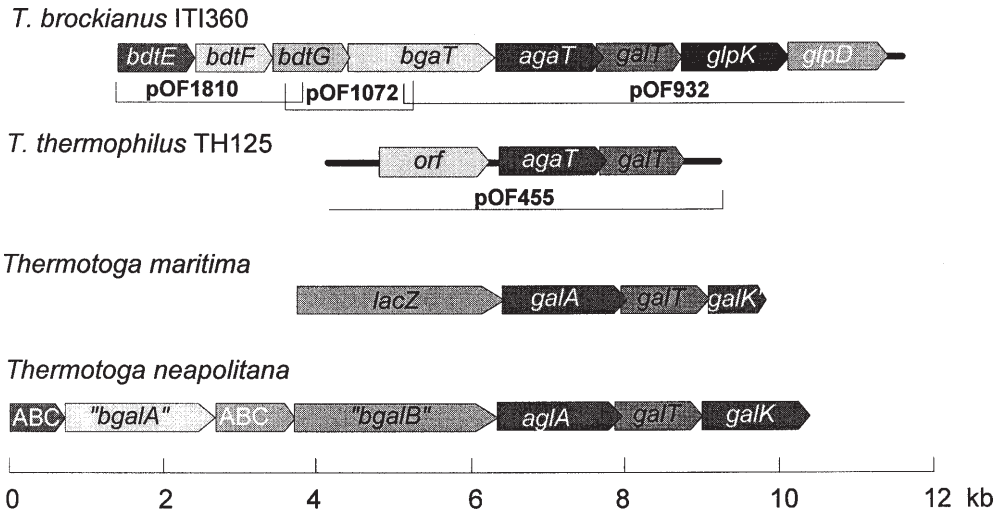

Fig. 1. Schematic representation of galactoside utilization gene clusters in *Thermus brockianus* ITI360 (10-kb sequence region), *Thermus thermophilus* TH125 (5.3-kb sequence region), *Thermotoga maritima* (6.2-kb region shown), and *Thermotoga neapolitana* (10.3-kb region shown). The designation, position, and orientations of the *orfs* (represented as *pointed boxes*) are shown. The *Thermus* sequence regions corresponding to inserts of gene library plasmids and plasmids containing fragments obtained by inverse PCR are shown below the relevant maps. *bdtE*, *bdtF*, and *bdtG*, binding protein-dependent transport system genes; *ABC*, putative ABC transporter genes; *bgaT*, *lacZ*, "*bgalA*," "*bgalB*," β-galactosidases; *agaT*, *galA*, *aglA*, α-galactosidases; *galT*, galactose-1-phosphate uridylyl-transferase homologous proteins; *glpK*, glycerol kinase; *glpD*, glycerol dehydrogenase; *galK*, galactokinase. The *orf* upstream of *agaT* in *T. thermophilus* displays similarity to *aidB* of *E. coli*. Only a partial sequence of *galK* in *Thermotoga maritima* is available. A scale (in kb) is shown below the diagrams. The GeneBank accession numbers are AF135398 and 135399 for the gene cluster sequences of ITI360 and TH125, respectively, and AJ001072 and AF055482 for the sequences of *T. maritima* and *T. neapolitana*, respectively

Table 2. Summary of *Thermus brockianus* ITI360 and *Thermus thermophilus* TH125 genes presented in this study

Strain	Gene	% GC content	Protein/function	Homologous protein, accession number, % aa sequence identity	No. of amino acids	M_r (Da)	Calculated pI
Thermus brockianus ITI360	*bdtF*	57	Putative integral membrane protein, BdtF	LacF of *Agrobacterium radiobacter* (X66596), 45%; Putative LacF of *Thermus* A4 (D85027), 88%	281	31518	9.98
	bdtG	56	Putative integral membrane protein, BdtG	LacG of *A. radiobacter* (X66596), 34%; Putative LacG of *Thermus* A4 (D85027), 85%	275	30286	9.60
	bgaT	62	β-Galactosidase, BgaT	BgaA of *Thermus* T2 (g2765752), 80%	645	73420	6.07
	agaT	62	α-Galactosidase, AgaT	GalA of *Thermotoga maritima* (AJ001072), 29%	476	53810	5.62
	galT	68	Putative galactose-1-phosphate uridylyl-transferase, GalT	GalT of *Streptomyces lividans* (P13212), 40%; Putative GalT of *T. maritima*; (AJ001072), 43%	348	39877	5.95
	glpK	71	Putative glycerol kinase, GlpK	GlpK of *Thermus flavus* (AB004569), 54%	495	53414	5.51
	glpD	72	Putative glycerol dehydrogenase, GlpD	GlpD of *Bacillus subtilis* (121415), 33%	511	56271	9.08
	*orf*1	71	Putative adaptive response gene product	AidB of *E. coli* (543789), 29%	447	50148	9.58
Thermus thermophilus TH125	*agaT*	71	α-Galactosidase, AgaT	AgaT of *T. brockianus* (AF135398), 75%	476	53645	5.33
	galT	69	Putative galactose-1-phosphate uridylyl-transferase, GalT	Putative GalT of *T. brockianus* ITI360 (AF135398), 83%	349	39451	5.84
	bglT	70	β-Glycosidase, BglT	*Streptomyces* sp. (S45675), 54%	379	42100	6.39

(aa) sequences encoded by the first three *orfs* revealed similarity to different components of the binding proteindependent lactose transport system (*lac*E, F, G) in *Agrobacterium radiobacter* (Williams et al. 1992). Also, a high similarity was observed to a sequenced region from *Thermus* strain A4 containing genes of a potential binding protein-dependent lactose transport system, i.e., the putative *lac*E, *lac*F, and *lac*G (Ohtsu et al. 1998). The corresponding genes in *T. brockianus* ITI30 were designated *bdtE*, *bdtF*, and *bdtG*, respectively (for *b*inding protein-*d*ependent *t*ransport) (Table 2). The putative BdtF and BdtG of strain ITI360 exhibit the characteristic properties

of integral membrane proteins from various bacterial binding protein-dependent transport systems (Higgins et al. 1990), i.e., regions of particularly high hydrophobicity that are probably capable of spanning the membrane. The calculated average hydropathic index was 0.79 and 0.95 fro BdtF and BdtG, respectively (Kyte and Doolittle 1982).

The fourth *orf* was identified as a β-galactosidase gene, designated *bgaT*. The biological activity was verified by the cloning of the gene into pBTacl and its expression in *E. coli* JM109. The recombinant enzyme hydrolyzed pNP-β-galactoside with a optimum initial hydrolyzing rate measured at 90°C (results not shown). The enzyme displayes similarity to β-galactosidases of *Thermus* strains T2 (Vian et al. 1998) and A4 (Ohtsu et al. 1998).

An *orf* was identified downstream of and overlapping with the *agaT* gene in *T. brockianus* ITI360. The deduced protein displays aa sequence similarity to galactose-1-phosphate uridylyl transferases, GalT, of various bacterial sources (Table 2). The start codon is 9 nucleotides (nt) downstream of a sequence that resembles a stable ribosome site and 11 nt upstream of the TAA stop codon of the *agaT* gene. A similar gene arrangement was observed in *Thermotoga maritima* and *Thermotoga neapolitana* (Liebl et al. 1998; King et al. 1998), in which galactose utilization genes (*galT* and *galK*) are located downstream of galactoside utilization genes (see Fig. 1).

The stop codon of the putative *galT* in *T. brockianus* ITI360 overlaps with a start codon of a 1487-nt-long *orf*. The deduced protein displays only a low-level sequence similarity to galactokinases, e.g., 22% identity with the GalK of *S. lividans* (accession number P13227) and 17.8% identity to the putative GalK of *T. neapolitana*. On the other hand, 54% aa sequence identity to a glycerol kinase, GlpK, of *Thermus flavus* is observed (see Table 2). The predicted aa sequence of an *orf* immediately downstream from the putative glycerolkinase gene (*glpK*) in ITI360 displays similarity to aa sequences of FAD-dependent glycerol-3-phosphate dehydrogenases, e.g., of *Bacillus subtilis* (*glpD*) (Table 2). The biological function of those genes will be a subject of another study.

Sequences resembling typical ribosome-binding sites were generally observed 4–9 bp upstream of the start codons of the predicted *orf*s. The exceptions were the first *orf* (*bdtE*), which appears to be truncated at the N-terminus by the cloning procedure and the second *orf* (*bdt*F). The overall GC content of the gene cluster averages 63.8%. The genes encoding the putative integral membrane proteins of the protein-dependent transport system, with relatively high content of hydrophobic aa, exhibit the lowest GC content, 56.9% on average. *glpK* and *glpD* exhibit the highest GC content, 71.7% on average (Table 2). As expected for organisms with a relatively high G + C content, Arg, Pro, Ala, and Gly codons occur with a higher frequency than GC-poor codons, e.g., Asn, Lys, Tyr, Phe, and Ile codons.

The GeneBank accession number for the nucleotide sequence containing the galactosidase gene cluster of *T. brockianus* ITI360 is AF135398.

Cloning and sequence analysis of the α-galactosidase gene and flanking sequences of *T. thermophilus* TH125

To achieve structural information about the α-galactosidase gene locus in *T. thermophilus* TH125, the α-galactosidase gene and flanking sequences were cloned into *E. coli*. Several clones were isolated by plaque hybridization of the *T. thermophilus* TH125 gene library using the ITI360 *agaT* gene as a probe. Following plasmid formation, restriction analysis, and southern blot, the α-galactosidase gene was localized on a mutual 1.8-kb *Sac*I fragment of overlapping gene library clones. One gene library clone, pOF455, was selected for sequence analysis. A continuous sequence about 5300 nt long was shown to contain three *orf*s (see Fig. 1, Table 2). The central *orf* is similar to the *agaT* gene of strain ITI360. As in *Thermus* ITI360, an *orf* is located downstream of the α-galactosidase gene, *agaT*, with a overlapping coding region of 14 nt excluding the *agaT* stop codon. The deduced protein is a counterpart of the putative GalT of *Thermus* ITI360 (Table 2).

A 290-nt sequence downstream of *galT* in *T. thermophilus* TH125 was analyzed. BLASTX search did not reveal any significant similarity to known aa sequences. A 2560-nt sequence upstream of the α-galactosidase gene was analyzed. One *orf* was found with a preceding ribosome-binding site resembling a sequence 7 nt upstream of a GTG start codon. The deduced protein displays aa sequence similarity to the translation product of the *E. coli aidB* gene, which is involved in the repair of MNNG-specific DNA lesions or in a MNNG detoxification pathway and exhibits acyl coenzyme A dehydrogenase activity (Landini et al. 1994). No potential promoter −35/−10 consensus sequences (Maseda and Hoshino 1995) were recognized in the sequenced region upstream of *agaT* in *T. thermophilus* TH125.

The GeneBank accession number for the nucleotide sequence containing the α-galactosidase gene and flanking sequences of *T. thermophilus* TH125 is AF135399.

Inactivation of *agaT* in *T. thermophilus* TH125

The inactivation of *agaT* in *Thermus brockianus* ITI360 has been described (Fridjonsson et al. 1999b). To use *T. thermophilus* TH125 for expression of heterologous α-galactosidases, the *agaT* gene was inactivated. An integration cassette was constructed in which the α-galactosidase gene was insertionally inactivated in vitro with a gene (*kan*) encoding a thermostable kanamycin resistance protein (Matsumura et al. 1984). By the construction, the 3′-region of *agaT* and the 5′-region of *galT* were left intact because of the overlapping coding regions. The *T. thermophilus* TH125 was transformed with the corresponding plasmid, pOF1051, and transformants were selected on kanamycin plates incubated at 60°C. Because pOF1051 cannot replicate in *Thermus*, resistant colonies should be those in which the integration cassette has inserted into the chromosome by homologous recombination between the *agaT* flanking sequences in the plasmid and the chromosome. To verify

the integration of the *kan* gene into the α-galactosidase gene locus, chromosomal DNA from a transformant strain (OF1051) and the wild type, digested with *Sac*I/*Bgl*II, was analyzed by Southern hybridization.

Figure 2A shows a Southern blot following hybridization with an *agaT* fragment probe. A 1.8-kb fragment, containing the complete *agaT* gene, is seen by the wild-type strain (lane 2). This fragment is absent in the mutant strain; instead, a 0.98-kb band appears (land 3), corresponding to the 3′-region of *agaT* between the *Bgl*II restriction site in the *kan* gene and a *Sac*I restriction site downstream of *agaT*. Figure 2B shows a Southern blot following hybridization with a *kan* fragment probe. The probe did not hybridize to the chromosomal DNA of the wild-type strain (lane 2). In the mutant strain, the expected double band of 971 and 978 bp is seen (lane 3). To confirm intact flanking sequences in the mutant strain, chromosomal DNA of TH125 and OF1051 was digested with *Bam*HI/*Bgl*II and Southern hybridization was performed using fragments of flanking sequences as probes. The results are shown in Fig. 2C,D for hybridization with a 1 kb probe covering the 5′-flanking region of *agaT* and a *galT* probe, respectively. Expected fragments are detected according to the restriction map represented in Fig. 2E. The molecular analysis revealed the insertion of *kan* into the *agaT* locus by homologous recombination, via double crossover, creating the mutant strain OF1051 (Δ*agaT*::*kan*).

Phenotypic analysis of integration mutants, OF1051 and OF642

Thermus thermophilus OF1051 and *T. brockianus* OF642 (Δ*agaT*::*kan*) were tested for growth on glucose, melibiose, and galactose. Both mutant strains were unable to use melibiose as well as galactose as a single carbohydrate source. pNP-α-galactoside and pNP-β-galactoside hydrolyzing activities were measured in crude extracts of the mutant strains (Table 3). As expected, the α-galactosidase activity of both strains was abolished. In *T. thermophilus* OF1051 the pNP-β-galactoside hydrolyzing activity was unaffected and remained the same as in TH125. In strain OF642, the pNP-β-galactoside hydrolyzing activity, without addition of galactose to the culture medium, was similar to that in strain ITI360 following induction with galactose.

Isolation of a TH125 gene library clone displaying pNP-β-galactoside hydrolyzing activity

According to the sequence analysis of the α-galactosidase gene locus in TH125, the gene (genes) responsible for the pNP-β-galactoside hydrolyzing activity in the strain TH125 is separated from *agaT*. To isolate the corresponding β-galactosidase gene, a Southern hybridization of the *T. thermophilus* chromosomal DNA was carried out using the *bgaT* gene fragment from strain ITI360 as probe. An ITI360 *agaT* gene fragment probe served as a control. No hybridization signals were detected (except with the *agaT* probe),

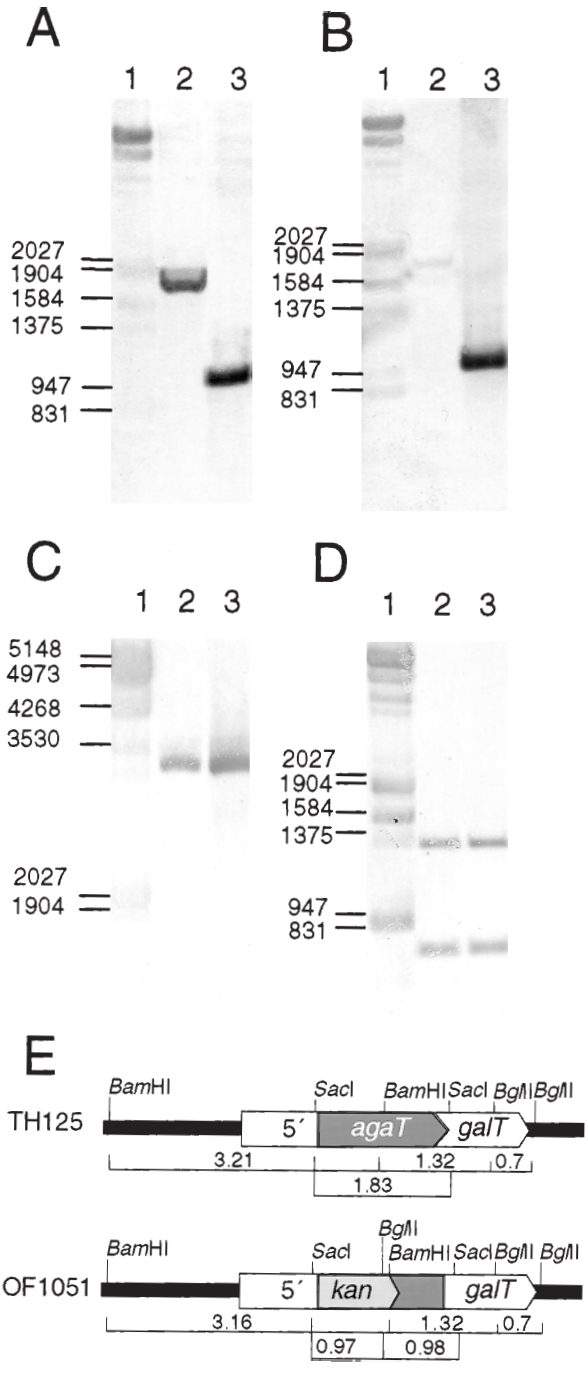

Fig. 2A–E. Southern blot analysis of *Thermus thermophilus* TH125 chromosomal DNA. DNA was digested with *Sac*I/*Bgl*II (**A** and **B**) or *Bam*HI/*Bgl*II (**C** and **D**), electrophoresed on 1% agarose gel, transferred to a nylon membrane, and hybridized. Hybridization solution contained DIG-labeled (Boehringer Manheim) λ DNA for the hybridization of the marker. **A** Southern hybridization with the *T. thermophilus agaT* gene fragment as a probe. **B** Hybridization with the *kan* fragment as a probe. **C** Hybridization with 1-kb 5′-flanking sequence of *agaT* as a probe. **D** Hybridization with the *galT* gene (3′-flanking sequence) as a probe. In each part: *lane 1*, λ *Eco*RI/*Hin*dIII marker; *lane 2*, TH125 (wild type); *lane 3*, OF1051 (Δ*agaT*::*kan*). Sizes of the marker bands are indicated. **E** Restriction map of the strains. Sizes (in kb) of restriction fragments detected by the Southern hybridization are indicated. The 5′-flanking sequence of *agaT* corresponding to the sequence used for the construction of the integration cassette in pOF1051 and as a hybridization probe is represented as a *box*

Table 3. α-galactosidase and β-galactosidase activity (mU mg^{-1}) in *T. brockianus* ITI360 and *T. thermophilus* TH125 and their mutant strains *T. brockianus* OF642 (Δ*agaT*::*kan*), *T. thermophilus* OF1051 (Δ*agaT*::*kan*), and *T. thermophilus* OF10725 (Δ*bglT*::*kan*)

Enzyme activity	ITI360 +Gal	OF642 −Gal	OF642 +Gal	TH125 −Gal	OF1051 −Gal	OF1051 +Gal	OF10725 −Gal
α-Galactosidase	147	<1	<1	72	<1	<1	75
β-Galactosidase	210	235	250	195	212	215	87

The strains were grown 15 h in 162 nutrient medium with 0.2% galactose or without sugar. Activity tests were performed in triplicates at 70°C using pNP-α- and pNP-β-galactopyranoside as substrates. The maximum variation from the mean values (shown) was less than 5%

although low stringency conditions were used. Also, *bdtE*, *bdtF*, and *bdtG* fragment probes failed to hybridize (data not shown). Therefore, an attempt was made to isolate a gene encoding a pNP-β-galactoside hydrolyzing enzyme by using the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as described in Materials and methods. Three positive clones were shown to contain identical inserts. One clone was subjected to sequence analysis. An *orf* of 1289 nt was identified. The deduced amino acid sequence shows similarity to β-glucosidases of various bacterial sources (family 1 of glycosyl hydrolases) (see Table 2). Only low-level similarity to *bgaT* of ITI360 is observed (22% sequence identity). The gene, designated *bglT*, was amplified, cloned in pBTac1, and expressed in *E. coli* JM109. The recombinant enzyme was able to hydrolyze pNP-β-glucopyranoside as well as pNP-β-galactopyranoside, which is a common property of β-glucosidases of family 1 glycosyl hydrolases (Gabelsberger et al. 1993; Breves et al. 1997; Takase and Horikoshi 1988).

The GeneBank accession number for the nucleotide sequence containing the β-glycosidase gene and flanking sequences of *T. thermophilus* TH125 is AF135400.

Inactivation of *bglT* in *T. thermophilus* TH125

An *bglT* gene inactivation was carried out to examine if *T. thermophilus* TH125 possessed β-galactoside hydrolyzing enzymes other than BglT and for a potential application of the resulting mutant strain as a host for heterologous β-galactosidase genes. *T. thermophilus* TH125 was transformed with plasmid pOF10725, which contains the thermostable kanamycin resistance marker (*kan*) between the flanking sequences of *bglT*. Two transformants were analyzed by Southern hybridization after *Sac*I digestion of their chromosomal DNA. Figure 3A shows a Southern blot after hybridization with a *bglT* probe. The *bglT* gene appears in the wild type and strain OF1051 (Δ*agaT*::*kan*) (lanes 1 and 2, respectively), on a 1.0-kb and 1.2-kb fragment. The gene is not detectable in the *bglT* deletion mutant strains (lanes 3 and 4). No signal is detected in the wild-type strain with the *kan* gene fragment probe (Fig. 3B, lane 1), whereas in the mutant strains a 1.7-kb fragment is detected (Fig. 3B, lanes 3 and 4). Also, expected fragments are detected after hybridization with 5′- and 3′-flanking sequence probes (Fig. 3C, D), according to the restriction map represented in Fig. 3E. This molecular analysis revealed the integration of the *kan* gene into the *bglT* gene locus of TH125 by homologous recombination, creating the mutant strain OF10725 (Δ*bglT*::*kan*).

The pNP-β-galactoside hydrolyzing activity at 70°C in crude extract of the mutant strains was determined. A specific activity of 87 mU mg^{-1} was observed, whereas in crude extract of TH125 a specific activity of 195 mU mg^{-1} was measured (see Table 3). Thus, the inactivation of the *bglT* gene led to 55% reduction of the pNP-β-galactoside hydrolyzing activity in the mutant strain in comparison to the wild type.

Discussion

The aim of this work was to characterize metabolic activities and genomic structures in two *Thermus* strains for a subsequent engineering of a host strain for an "in vivo selection" of galactosidases.

Several *Thermus* isolates have been described for the production of galactosidases (Berger et al. 1995). In the *Thermus* strain T2, an α-galactosidase activity was detected along with a β-galactosidase activity, following induction with galactose (Ulrich et al. 1972). Also, it was demonstrated that the α- and β-galactosidase activities in strain T2 were of two distinct enzymes, probably of a common gene regulation system (Koyama et al. 1990).

The molecular analysis described here revealed a different organization of α-/β-galactosidase genes in two different species of the genus *Thermus*. In *T. brockianus* ITI360, the α-galactosidase gene is located downstream of a β-galactosidase gene as described for *Thermus* strain T2 (Koyama et al. 1990), in a cluster of closely linked galactoside utilization genes. Lactose is usually transported into gram-negative bacteria via proton symport or phosphotransferase systems. An exception is the lactose transport in *Agrobacterium radiobacter*, where the uptake of lactose is via a binding protein-dependent uptake system (Greenwood et al. 1990). The presence of genes upstream of the *bgaT* in strain ITI360, encoding proteins similar to the lactose transport proteins of *A. radiobacter*, indicate that some *Thermus* bacteria also seem to use a binding protein-dependent transport system for the uptake of galactosides.

Location of a gene presumably encoding an enzyme of the galactose metabolism of *T. brockianus* (*galT*) immediately downstream of the β-and α-galactosidase genes might explain the role of galactose as an inducer of these genes. In bacteria such as *E. coli*, *Streptococcus mutans*, and *Streptomyces lividans*, *galT* constitutes an operon along with genes encoding enzymes of the Leloir pathway for galactose metabolism, i.e., *galK*, the galactokinase-encoding gene and *galE*, the UDP glucose-4-epimerase-encoding gene (Adhya 1987; Ajdic et al. 1996; Adams et al. 1988). Hence it was not surprising to find a gene downstream from *galT* in *Thermotoga maritima* and *Thermotoga neapolitana* encoding a enzyme displaying aa sequence similarity to galactokinases (accession numbers AJ001072 and AF055482, respectively). Surprisingly, the genes downstream of *galT* in *Thermus brockianus* ITI360 resemble genes required for the biosynthesis of triacylglycerols.

According to the sequence analysis of the α-galactosidase gene region in *T. thermophilus* TH125, *agaT* is closely linked to a putative *galT* gene as in *T. brockianus* ITI360. On the other hand neither a β-galactosidase gene nor genes of a protein-dependent transport system were identified in a sequence region upstream *agaT*. In fact, our data indicate the lack of *bdtE*, *bdtF*, and *bdtG* and *bgaT* homologous genes in *T. thermophilus* TH125. This result is in agreement with that of Tabata and Hoshino (1996), who could not locate *lac* genes on the physical map of *T. thermophilus* HB27 chromosome. This lack may be the reason for the poor growth of *T. thermophilus* TH125 on lactose or raffinose. A broad-specificity β-glycosidase (BglT) can partially account for the pNP-β-galactoside hydrolyzing activity in TH125. The remaining activity, following insertional inactivation of *bglT*, indicates the existence of another β-galactoside hydrolyzing enzyme and gene in *T. thermophilus* TH125. The cloning and inactivation of this gene(s) will be required for a potential thermoadaptation of β-galactosidases in *T. thermophilus*.

In addition to the dissimilar growth capacities of the *Thermus* strains on minimal medium, the different gene organization may explain the unlike behavior with respect to pNP-α-galactoside and pNP-β-galactoside hydrolyzing activities and the effect of galactose. In strain ITI360, the galactoside utilization genes (and *galT*) are closely linked and seem to be regulated together. The α-galactosidase gene in *T. thermophilus* TH125 is constitutively expressed and not under a common transcriptional control of a mutual promoter, along with a β-galactosidase gene.

Although this article does not include data on the cotranscription of the genes described, several observations suggest that the α-galactosidase genes of both strains are a part of a polycistronic message. (i) The α-galactosidase and β-galactosidase activity increase concurrently following addition of galactose in *T. brockianus* ITI360. (ii) The α-galactosidase gene locus in *T. brockianus* ITI360 contains a cluster of genes that either overlap or are closely linked. (iii) *agaT* genes of both strains overlap with a putative *galT* gene. (iv) The capability to metabolize galactose is restricted by the *agaT* deletion strains. The results can be interpreted as a polar transcriptional effect on the down-

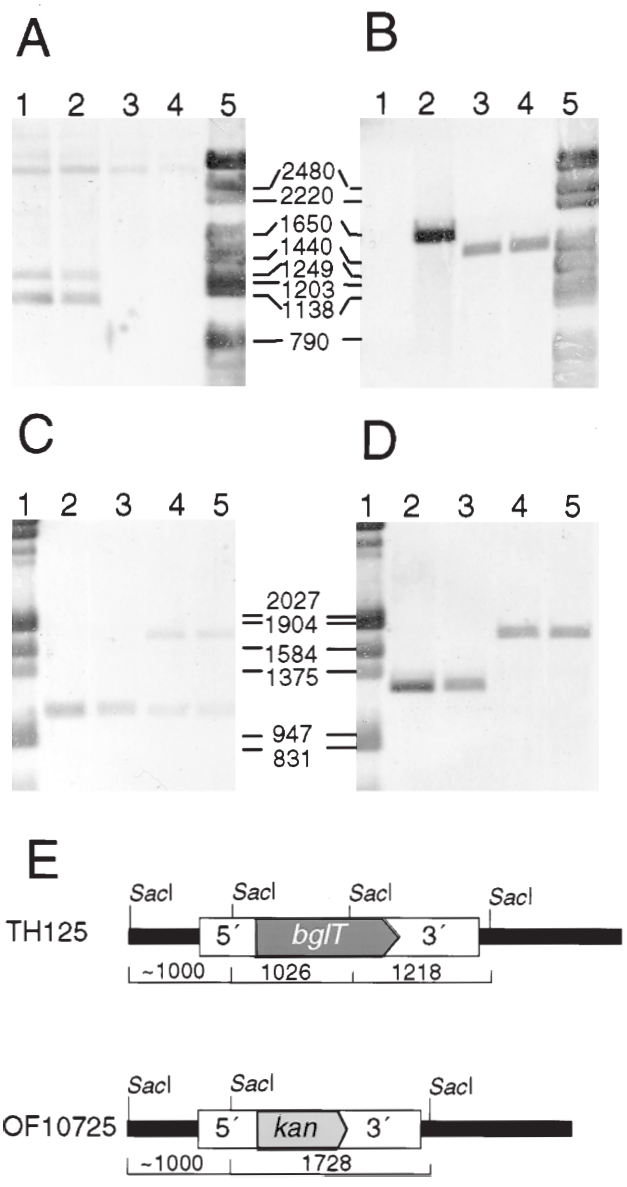

Fig. 3A–E. Southern blot analysis of *T. thermophilus* TH125 chromosomal DNA. DNA was digested with *Sac*I electrophoresed on 1% agarose gel, transferred to a nylon membrane, and hybridized. Hybridization solution contained DIG-labeled (Boehringer Manheim) λ DNA for the hybridization of the marker. Southern blots after (**A**) hybridization with the *T. thermophilus bglT* gene fragment as a probe, (**B**) hybridization with the *kan* gene as a probe, (**C**) hybridization with the 0.4-kb 5′-flanking sequence of *bglT* as a probe, and (**D**) hybridization with the 0.8-kb 3′-flanking sequence as a probe. In **A** and **B**: *lane 1*, TH125; *lane 2*, OF1051; *lanes 3* and *4*, OF10725; *lane 5*, λ-marker *Bgl*I digested. In **C** and **D**: *lane 1*, λ marker *Eco*RI/*Hin*dIII digested; *lane 2*, TH125 (wile type); *lane 3* OF1051 (Δ*agaT*::*kan*); *lanes 4* and *5*, OF10725. **E** Restriction map of the strains. Sizes (bp) of restriction fragments detected by the Southern hybridization are indicated. The 5′-flanking and 3′-flanking sequences of *bglT* corresponding to the flanking sequences used for the construction of the integration cassette in pOF10725 and as hybridization probes are represented as *boxes*. Position of the chromosomal *Sac*I site at the left end of the restriction maps is approximated from the size of the band seen in **C**

stream *galT* genes. A similar effect was reported for the inactivation of the *S. mutans aga* gene, which led to the loss of the ability to ferment not only melibiose but also isomaltose. The dextran glucosidase-encoding gene, *dex*, is located downstream of *aga* in *S. mutans* in operon-like arrangement (Aduse-Opoku et al. 1991). (v) Integration of a kanamycin resistance gene into the *agaT* locus of *T. brockianus* ITI360 and selection with kanamycin may have involved a selection of constitutive mutants to enable growth on T162 kanamycin medium, which is reflected by the concomitant constitutive activity of the β-galactosidase. That fact would be a further indication of a mutual transcriptional control of the galactoside utilization genes in *T. brockianus* ITI360. However, other β-galactosidases may also be responsible for the detected constitutive activity.

Because of the complex regulation and structure of the galactosidase genes in *T. brockianus*, in addition to low transformation efficiency (Fridjonsson et al. 1999b), *T. thermophilus* is more likely to suit as a host strain for in vivo protein engineering of galactoside hydrolyzing enzymes. The observation that an insertional inactivation of the α-galactosidase genes restricts the capability of the mutant strain to metabolize galactose may be an obstacle to our purposes, however. This problem might be solved by complementing the mutant with a *galT* gene as well as with galactosidase genes. Also, a different constellation of integration modules for the inactivation of *agaT* might circumvent the predicted polarity problem.

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