Use of an Alpha-Galactosidase Gene as a Food-Grade Selection Marker for *Streptococcus thermophilus*

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ABSTRACT

The α–galactosidase gene (*aga*) of *Lactococcus raffinolactis* ATCC 43920 was previously shown to be an efficient food-grade selection marker in *Lactococcus lactis* and *Pediococcus acidilactici* but not in *Streptococcus thermophilus*. In this study, we demonstrated that the α-galactosidase of *L. raffinolactis* is thermolabile and inoperative at 42˚C, the optimal growth temperature of *S. thermophilus*. An *in vitro* assay indicated that the activity of this α-galactosidase at 42˚C was only 3% of that at 30˚C, while the enzyme retained 23% of is activity at 37˚C. Transformation of *S. thermophilus* RD733 with the shuttle-vector pNZ123 bearing the *aga* gene of *L. raffinolactis* (pRAF301) generated transformants that were stable and able to grow on melibiose and raffinose at 37˚C or below. The transformed cells possessed six-fold more α-galactosidase activity after growth on melibiose than cells grown on lactose. Slot blot analyses of *aga* mRNA indicated that repression by lactose occurred at the transcription level. The presence of pRAF301 did not interfere with the lactic acid production when the transformed cells of *S. thermophilus* were grown at the optimal temperature in milk. Using the recombinant plasmid pRAF301, which carries a chloramphenicol resistance gene in addition to *aga*, we showed that both markers were equally efficient at differentiating transformed from non-transformed cells. Taken altogether, the *aga* gene of *L. raffinolactis* can be used as a highly efficient selection marker in *S. thermophilus*.

Keywords: *Lactococcus raffinolactis*, vector, melibiose, raffinose, lactic acid bacteria

Abbreviation key: **GRAS** = generally recognized as safe, **LAB** = lactic acid bacteria,

INTRODUCTION

Streptococcus thermophilus is a lactic acid bacterium (**LAB**) used to manufacture yogurt and a number of cheeses. The extensive commercial use of this bacterium led to several fundamental and applied studies aimed at increasing our understanding of this LAB, in order to select better strains or to improve them through genetic modification (Turgeon et al., 2004; Vadeboncoeur and Moineau, 2004). Very few molecular tools are available for the genetic engineering of *S. thermophilus*. Such tools should be food-grade and applicable under laboratory and industrial conditions (Hansen, 2002). Food-grade systems are defined as an association of DNA that originates exclusively from "generally recognized as safe" (**GRAS**) organisms, including the selection marker (Johansen, 1999; Hansen, 2002; Kondo and Johansen, 2002). The criteria used for the classification as a food-grade marker include the safety of the genetic material transferred in the host, food compatibility, the absence of antibiotic resistance markers, the non-use of harmful compounds, and the applicability on an industrial scale or in food products (de Vos, 1999; Hansen, 2002). For a recent review on food-grade vectors, the readers are referred to Shareck et al. (2004).

Two general strategies are normally used to introduce genetic material into a relevant LAB: [1] integration into the host genome by homologous recombination (Biswas et al., 1993; Gosalbes et al., 2000; Henrich et al., 2002; Sasaki et al., 2004) or phage-mediated integration (MacCormick et al., 1995; Lillehaug et al., 1997; Martin et al., 2000); [2] introduction of autonomously replicating plasmids (Kok et al., 1984; Boucher et al., 2002; El Demerdash et al., 2003; Wong et al., 2003). Plasmids are often preferred because many copies of the target gene may be needed to obtain the

desired phenotype, notably for phage-resistance systems (O'Sullivan et al., 1995; Bouchard et al., 2002; Émond et al., 1997, 1998).

Few food grade markers are available for *S. thermophilus*. The cadmium resistance is a dominant selection marker, encoded by the *cadA* and *cadC* genes of *L. lactis,* and was successfully used in *S. thermophilus* (Liu et al., 1997; Wong et al., 2003). A β-galactosidase gene was proposed as a complementation marker for lactose-negative *S. thermophilus* strains (Herman and McKay, 1986). A *shsp* gene encoding a putative small heat shock protein conferring heat and acid resistance was also used as a food-grade selection in *S. thermophilus* (El Demerdash et al., 2003). Finally, thymidilate synthase genes from *S. thermophilus* or *Lactobacillus delbrueckii* subsp. *bulgaricus*, were shown to be suitable a selection marker in thymidine-requiring mutants of *S. thermophilus* (Sasaki et al., 2004).

The limitation of dominant selection markers is linked to the natural occurrence of the phenotype in *S. thermophilus* while complementation markers may be inconvenient to use as they require the prior isolation of appropriate mutants (Boucher et al., 2002). A dominant selection marker that takes advantage of the inability of a number of LAB strains to ferment melibiose was recently developed (Boucher et al., 2002). The dominant marker is based on the *aga* gene of *Lactococcus raffinolactis*, which is expressed using its own constitutive promoter (Boucher et al., 2002, 2003). This gene codes for an α-galactosidase that catalyzes the hydrolysis of the α 1–4 link of melibiose and the release of the two monosaccharides, glucose and galactose. This gene is able to convert the mesophilic species *Lactococcus lactis* and *Pediococcus acidilactici* from a melibiose-negative to a melibiose-positive phenotype. This marker was not functional in *S. thermophilus*

(Boucher et al., 2002). This result was unexpected because the LacS transporter of *S. thermophilus*, which is partially constitutive (Vaughan et al., 2001), is able to transport melibiose as well as lactose (Poolman et al., 1992; Veenhoff and Poolman, 1999). The presence of a functional α-galactosidase in *S. thermophilus* should allow its grow on melibiose by the metabolism of the glucose residue via the glycolytic Embden-Meyerhof-Parnas pathway. In this work, we show that the *aga* gene of *L. raffinolactis* can indeed be used as a dominant food-grade selection marker in *S. thermophilus* when the cells are grown at 37˚C or below.

MATERIALS AND METHODS

Bacterial strain, bacteriophage, plasmids, and media

The bacterial strain, plasmids, and phage used in this study are listed in Table 1. Unless mentioned otherwise, *S. thermophilus* was grown on M17 medium (Quélab, Montréal, Québec, Canada) at 42˚C. The carbohydrate fermentation assay was carried out in the Bromocresol Purple medium (BCP medium) (2% tryptone, 0.5% yeast extract, 0.4% NaCl, 0.15% Na-acetate, 40 mg/liter Bromcresol Purple) (Boucher et al., 2002; McKay et al., 1972). Cultures used for transcriptional analyses were grown in the same medium but without the Bromocresol Purple. Sugars were filter-sterilized (0.22 μm) and added at a final concentration of 0.5% in BCP or M17. The plasmid pRAF301 used in this study is a 2.5 kb PCR product composed of the *aga* gene of *L. raffinolactis* ATCC 43920 (accession number AY164273) cloned into the shuttle vector pNZ123 (Boucher et al., 2002). Plasmid pNZ123 (de Vos, 1987) carries a chloramphenicol acetyl transferase (*cat*) gene used for clone selection in both *E. coli* and *S. thermophilus* hosts. When needed, chloramphenicol was added to the growth medium at a concentration of 5 μg/ml. The virulent bacteriophage DT1, which is capable of propagating in *S. thermophilus* RD733, was used in a spot assay to confirm the identity of the transformants (Tremblay and Moineau, 1999). Sugar fermentation patterns were determined using API 50 CH strips with API 50 CHL medium (BioMérieux, St-Laurent, Québec, Canada) incubated at either 37˚C or 42˚C.

Plasmid isolation and electrotransformation

S. thermophilus plasmid DNA was isolated using silica-based method as described previously (Émond et al., 2001), with the following modifications: the lysozyme concentration

was increased to 60 mg/ml and the corresponding incubation was extended to 30 min at 37˚C. The protocol used for the electrotransformation of *S. thermophilus* was modified from the glycine-shock protocol reported elsewhere (Buckley et al., 1999). *S. thermophilus* cells were first grown at 42˚C in 100 ml of M17 medium containing of 0.5% lactose. When the culture reached an OD_{600nm} of 0.5, 100 ml of M17 containing 0.8 M sorbitol, 20% glycine, and 0.5% lactose was added to the medium. The culture was incubated at 42˚C for an additional 60 min. The cells were collected by centrifugation and washed three times in cold electroporation buffer (0.4 M sorbitol, 10% glycerol) and resuspended in a final volume of 1 ml. The cells were either used immediately or quickly frozen in an isopropanol (80%) bath maintained at –80˚C. For electroporation, the cells were thawed on ice, and 40 µl of cells were combined with 1 µg of plasmid DNA and transferred to a prechilled 0.2 cm electroporation cuvette (Bio-Rad, Mississauga, Ontario, Canada). The electroporation was performed using a Bio-Rad Gene Pulser apparatus with a Bio-Rad pulse controller set at 2.5 kV, 200 ohms, and 25 µF. The cells were then immediately recovered in 1 ml of cold recuperation medium (M17 with 0.4 M sorbitol, 20 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.5% lactose). After resting on ice for 5 min, the cells were incubated at 42˚C for 2 h. Aliquots were then plated on appropriate selective media.

Alpha-galactosidase activity

The α-galactosidase activity was measured as previously described (Boucher et al., 2002, 2003) with the following modifications: strains were grown in medium with 0.5% of the appropriate sugar but without Bromocresol Purple until the OD_{600nm} reached 0.3. Pellets from 10 ml cultures were washed twice with 1 ml of sodium phosphate buffer (50 mM, pH 7.0) and lysed with glass beads. The cell lysates (0.5 ml) were cleared by centrifugation and dialyzed

(6000–8000 kDa pores) against 250 ml of sodium phosphate buffer for 15 min as described previously (Boucher et al., 2002). The protein concentrations of the cell extracts were determined using the Bio-Rad DC protein assay. The α-galactosidase activity was assayed at 30˚C, 37˚C, and 42˚C at pH 7.0 using *p*-nitrophenyl-α-D-galactopyranoside (Sigma-Aldrich, Oakville, Ontario, Canada) as the substrate.

Transcriptional analysis

Total RNA was isolated from *S. thermophilus* as reported elsewhere (Boucher et al., 2002), with the following modifications: 1 ml of a cell culture (OD_{600nm} of 0.3) was mixed with 100 µl of rifampicin (2.5 mg/ml in methanol) and pelleted. The cells were gently resuspended in 100 µl of a lysozyme solution (60 mg/ml) diluted in 20% sucrose and incubated at 37˚C for 15 min. Total RNA was extracted using the RNeasy kit (Qiagen, Chatsworth, CA). Following the RNAse-free DNAse treatment, a slot blot analysis was performed on a positively charged Nylon membrane (Roche Diagnostic, Laval, Québec, Canada) using a Bio-Dot SF apparatus (Bio-Rad). One microgram of total RNA from each sample was denatured and applied to the slot blot apparatus (Sambrook and Russell, 2001). After fixing by UV exposure, the RNA was detected by a probe generated using a PCR DIG-labeling kit and corresponding to an 882 bp internal fragment of the *L. raffinolactis* ATCC 43920 *aga* gene (Primers: Raf66 5'-GCC CGC ATT TGC GCT GTA AT and Raf69 5'-GGG ATG GCA CCA GTT GTC AT).

Growth properties and plasmid stability

The growth of *S. thermophilus* strains in M17 medium supplemented with 0.2% lactose and chloramphenicol (5 µg/ml), when applicable, was monitored by following the OD_{660nm} at

42˚C. Generation times were calculated for cultures in exponential growth phase by plotting the logarithm of the OD_{660nm} against time. The values represent the means of three separate experiments. For growth in milk, *S. thermophilus* strains were inoculated at 5% (vol/vol) from an overnight culture (LM17) in 10 ml pasteurized milk (Natrel) and an aliquot was immediately taken for pH reading at time zero. The tubes were then incubated at 42˚C for seven hours. A second pH reading was taken and the ΔpH was obtained by substracting both values. The milk acidification assay was also performed in triplicates. To demonstrate the stability of the plasmid carrying the *aga* gene from *L. raffinolactis, S. thermophilus* RD733 carrying pNZ123 and *S. thermophilus* RD733 harboring pRAF301 were repeatedly cultivated in LM17 at 42˚C for 7 days without chloramphenicol. One hundred colonies of each strain were picked at random and tested for their resistance to chloramphenicol and, when applicable, growth on melibiose (at 37˚C).

RESULTS AND DISCUSSION

The *aga* gene of *L. raffinolactis* ATCC 43920 can confer a melibiose fermentation phenotype on *S. thermophilus*

We previously reported that *S. thermophilus* transformants containing pRAF301, a plasmid carrying the *L. raffinolactis aga* gene and its endogeneous promoter are unable to grow on melibiose (Boucher et al., 2002). However, the ability of these transformants to grown on melibiose was only tested at 42˚C, the optimal growth temperature for *S. thermophilus* but a non-permissive growth temperature for *L. raffinolactis*. When the incubation temperature for *S. thermophilus* was decreased to 37˚C, the industrial *S. thermophilus* RD733 strain carrying

pRAF301 was able to grow on a BCP medium containing melibiose. To confirm that melibiose fermentation by the *S. thermophilus* transformant was temperature-dependent, API strips were inoculated either with *S. thermophilus* RD733 containing the shuttle-vector pNZ123 or with *S. thermophilus* RD733 containing pRAF301 and incubated at 37˚C or 42˚C. Unlike the *S. thermophilus* strain carrying pNZ123, which was unable to ferment melibiose at either temperature, the strain carrying pRAF301 was able to ferment melibiose and raffinose at 37˚C but not at 42˚C. These results suggest that the α-galactosidase of *L. raffinolactis* is thermolabile or that the *aga* gene is not transcribed at 42˚C, or both.

Temperature sensitivity of the *L. raffinolactis* α-galactosidase

S. thermophilus RD733 carrying pRAF301 was grown at 30˚C in a melibiose-containing medium and a cell extract was obtained as described in Materials and Methods. The α-galactosidase activity of the cell extract was determined at 30˚C, the optimal growth temperature of *L. raffinolactis*, as well as at 37˚C and 42˚C (Table 2). When the temperature of the enzymatic assay was set at 42˚C, only 3% of the α-galactosidase activity found at 30˚C was detected in the cell extract. Likewise, only 24% of the α-galactosidase activity was retained at 37˚C.

Effect of lactose and temperature on *aga* expression

To determine the effect of lactose, the principal sugar found in milk, on the expression of α-galactosidase, *S. thermophilus* RD733 cells carrying pRAF301 were grown on lactose at 30˚C and α-galactosidase activity was measured in cell extracts at 37˚C. The α-galactosidase activity was six-fold lower in lactose- than in melibiose-grown cells (Table 2). To determine whether the

lactose repression occurred at the transcription level, total RNA was isolated during the exponential growth of *S. thermophilus* RD733 containing pRAF301 or pNZ123. An internal portion of the *aga* gene was used as a probe to detect specific mRNA (Figure 1). The amount of *aga* mRNA in *S. thermophilus* harboring pRAF301 was much lower in lactose- than in melibiose-grown cells, which is consistent with the levels of α-galactosidase activities. As expected, no *aga* mRNA was detected in *S. thermophilus* RD733 harboring the cloning vector pNZ123. The decrease in *aga* mRNA levels and α-galactosidase activity caused by growth on lactose suggested that the expression of *aga* is subject to repression by lactose. In *S. thermophilus*, lactose caused partial repression of the lactose operon by a mechanism involving the transcriptional regulator CcpA (van den Bogaard et al., 2000). This regulatory protein recognizes a specific DNA sequence called *cre* (catabolite responsive element) in the promoter region of target operons (Hueck et al., 1994; Miwa et al., 2000). A *cre* sequence encompasses the –35 region of the *aga* promoter (Boucher et al., 2002), suggesting a possible regulatory role for CcpA during growth on lactose.

To determine whether the growth temperature had an effect on the transcription of *aga*, we compared the amounts of *aga* mRNA in cells grown on lactose at 37˚C and 42˚C. The transcription of *aga* was similar at both temperatures (Fig. 1, lanes 2 and 5), indicating that the inability of the transformants to grow on melibiose at 42^{o}C most likely resulted from the heat sensitivity of the *L. raffinolactis* α-galactosidase rather than inefficient transcription of *aga*.

The *aga* gene may be used as a dominant selection marker on BCP medium

The efficacy of the *aga* gene as a dominant selection marker was determined by electroporating pRAF301 into the industrial *S. thermophilus* strains RD733. BCP medium was used to select melibiose-positive transformants on solid media because, unlike M17, it does not support residual growth without an external carbon source (data not shown). The ability to recover transformants on melibiose-containing medium using *aga* as a selection marker was also compared to the chloramphenicol resistance marker (Cm), which is also present on pRAF301. Following electroporation, *S. thermophilus* RD733 cells were plated on BCP-Mel, BCP-Mel-Cm, and BCP-Lac-Cm (Table 3). Similar electroporation efficiencies were obtained with each medium, showing that selection using melibiose is equivalent to selection using chloramphenicol. Fifty colonies were randomly picked from lactose plates containing chloramphenicol and streaked on melibiose medium. Forty-nine clones were able to readily grow on melibiose, confirming the presence and stability of the *aga* gene.

Plasmid stability and growth properties

Plasmid stability assays showed that pNZ123 and pRAF301 were highly stable in *S. thermophilus* RD733. Indeed, 98% to 99% of the colonies tested retained their ability to grow on chloramphenicol and melibiose (for pRAF301) even after repeated culturing without selective pressure (Table 4). The generation times (at 42˚C in LM17 medium) of the wild-type strain *S. thermophilus* RD733 as well as *S. thermophilus* RD733 carrying pNZ123 were identical at 29 min (Table 4). Under the same conditions, *S. thermophilus* harboring pRAF301 had a slightly longer generation time of 35 min in LM17 medium. The three strains were also grown in pasteurized milk and the milk acidification was monitored by measuring the difference in pH following a seven-hour incubation period at 42˚C. The milk assay showed that all three strains

decreased the pH at the same level (Table 4). This data suggest that the presence of pRAF301 has no effect on metabolic activities associated with milk acidification.

CONCLUSION

The *aga* gene was isolated from a GRAS organism and a plasmid containing *aga* was readily introduced into one industrial *S. thermophilus* strain. The α-galactosidase activity is inefficient at 42˚C, the optimum growth temperature of *S. thermophilus*, and its expression is down-regulated when the cells are cultivated on lactose, the main sugar in milk. This marker can thus be used in the laboratory to select recombinant *S. thermophilus* strains at 37˚C on a melibiose-containing medium. The marker did not interfere with the acidification of milk during fermentation. We are currently investigating the replication machinery of several natural *S. thermophilus* plasmids (Turgeon and Moineau, 2001, Turgeon et al., 2004) to identify several replicons that could be used in combination with the *aga* gene of *L. raffinolactis* to construct novel food-grade vectors.

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Table 1. Bacteria, bacteriophage, and plasmids used in this study.

Bacterial strain and plasmids	Relevant Characteristics [a]	Source
Streptococcus thermophilus RD733	Industrial strain, Glu^+, Lac^+, Mel^-, Raf^-, Suc^+	Danisco
pNZ123	*E. coli*/*S. thermophilus* shuttle cloning vector, Cm^r	de Vos, 1987
pRAF301	2.5 kb PCR amplicon containing the *aga* gene of *Lactococcus raffinolactis* ATCC 43920 cloned into pNZ123, Cm^r	Boucher *et al.,* 2002
Phage DT1	*cos*-type phage capable of infecting strains RD733	Tremblay and Moineau, 1999

[a] Cm^r, Chloramphenicol resistance; Glu, Glucose; Lac, Lactose; Mel, Melibiose; Raf, Raffinose; Suc, Sucrose.

Table 2. Effect of temperature on α-galactosidase activity in *S. thermophilus* RD733 containing pRAF301.

Temperature	Activity [a] for cells grown on	
	Melibiose	**Lactose**
30°C	2531 ± 295	ND [b]
37°C	611 ± 102	107 ± 30
42°C	83 ± 39	ND

[a] Values are the means ± standard error of 18 measurements from two cell extract quantities from three independent experiments. Activities are expressed as nanomoles of *p*-nitrophenol formed per milligram of total protein per minute. No activity was detected with the RD733 strain carrying pNZ123 (Mel^-) when grown on lactose. Cells were grown at 30°C.

[b] ND, Not determined.

435 **Table 3.** Electroporation efficiencies of pRAF301 and pNZ123 into *S. thermophilus* RD733.

Selective Media	Electroporation Efficiency [a]	
	pRAF301	**pNZ123**
Melibiose	$5.1 \pm 1.1 \times 10^4$	-
Melibiose-Cm	$5.3 \pm 1.0 \times 10^4$	-
Lactose-Cm	$8.3 \pm 2.7 \times 10^4$	$1.0 \pm 0.75 \times 10^6$

436 [a] Values are the means ± standard error of three independent experiments expressed as numbers
437 of transformants per μg of DNA per ml of electrocompetent cells.

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441 **Table 4.** Plasmid stability, generation time and milk acidification assays.

S. thermophilus **strains**	**Plasmid stability in LM17** [a]	**Generation time in LM17**[b]	**Milk acidification** [b,c]
RD733	-	29 ± 2 min	2.30 ± 0.08
RD733 + pNZ123	98	29 ± 1 min	2.24 ± 0.06
RD733 + pRAF301	99	35 ± 2 min	2.38 ± 0.06

442 [a] One hundred colonies were tested for the presence of plasmids after repeated culturing in LM17
443 without chloramphenicol. The values indicate the number of colonies that were able to grow in
444 the presence of melibiose and/or chloramphenicol after reapeated culturing.

445 [b] Values are the means ± standard error of three independent experiments.

446 [c] The values indicate the ΔpH obtained after seven hours of milk fermentation at 42˚C.

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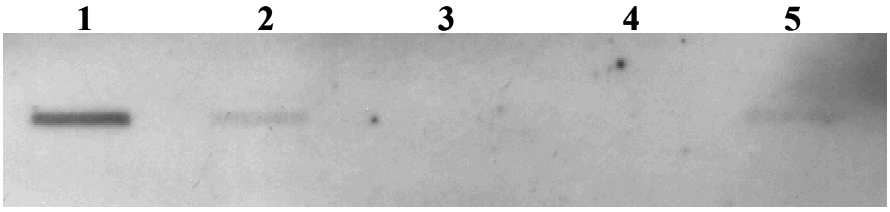

Figure 1. Slot blot hybridization using an 882 bp PCR-DIG-labeled internal section of the *L. raffinolactis aga* gene. Total RNA was isolated from *S. thermophilus* RD733 containing pRAF301 grown in the presence of melibiose at 37°C (lane 1), lactose at 42°C (lane 2), and lactose at 37°C (lane 5). Total RNA was isolated from *S. thermophilus* RD733 carrying pNZ123 grown in the presence of lactose at 37°C (lane 3) and lactose at 42°C (lane 4) and used as negative controls. *S. thermophilus* containing pRAF301 does not grow at 42°C in the presence of melibiose.