Short communication: Genome-wide identification of new reference genes for reverse-transcription quantitative PCR in *Streptococcus thermophilus* based on RNA-sequencing analysis

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ABSTRACT

Streptococcus thermophilus, one of the most important industrial lactic acid bacteria, is widely used for the production of fermented dairy products such as yogurt and cheese. The accuracy of gene expression-based analyses (e.g., reverse-transcription quantitative real-time PCR) relies heavily on the selection of reliable reference genes (RG), which provides the basis for correctly interpreting expression data. However, many traditional RG are not stably expressed in different systems. Here we used RNA-sequencing to systematically investigate gene expression variation at the genome scale and identify more stable RG in *S. thermophilus*. In total, 21 putative candidate RG were identified with variation coefficient values <10.0 based on the expression of all 1,911 genes under 4 different experimental conditions. We selected and validated 12 RG chosen from transcriptomes by using reverse-transcription quantitative real-time PCR, and ranked their expression stability by statistical algorithms geNorm and NormFinder. Compared with traditional RG *16S rRNA*, genes encoding glycine-tRNA ligase subunit β GlyS and fatty acid-binding protein DegV were more stable under all 4 treatments, which have never been used as RG in *S. thermophilus*. Our finding provides the foundation for more precise analysis of gene expression in *S. thermophilus* and other lactic acid bacteria species.

Key words: *Streptococcus thermophilus*, RNA sequencing, reverse-transcription quantitative real-time PCR, reference genes

Short Communication

Streptococcus thermophilus is one of the generally recognized as safe lactic acid bacteria (LAB) with

nonpathogenic, fermentative, and facultative anaerobic characteristics (Xiong et al., 2019a). As an important homo-fermentative streptococcal species of LAB, *S. thermophilus* is widely used for dairy industry such as yogurt and cheese production (Sørensen et al., 2016). The traits of *S. thermophilus* relevant to dairy products include lactose utilization, rapid acidification, improvement of the texture and flavor properties, as well as bacteriocin and exopolysaccharide synthesis (Xiong et al., 2019b). *Streptococcus thermophilus* prefers lactose over glucose as a carbon and energy source in dairy fermentation (Anbukkarasi et al., 2014), which can assist people with lactose intolerance to fully absorb milk nutrients. In addition, *S. thermophilus* has various probiotic functions (e.g., modulation of intestinal microbiota; Cui et al., 2016).

Reverse-transcription quantitative real-time PCR (RT-qPCR) is currently the most frequently used technique to quantify gene expression in diverse biological systems (Pombo et al., 2017). Selection of reliable reference genes (RG) is critical to affect the accuracy and interpretation of RT-qPCR results. Normalization of target gene expression by using invalidate RG will result in the questionable or even misleading results. Ideal RG are internal controls that should show minimal or no variation of their expression levels in various experimental conditions (Wu et al., 2017). Traditionally, RG are the most commonly used housekeeping genes that are presumed to have relatively stable expression in biological systems under different environments (Xia et al., 2014). However, recent studies have suggested that the transcript levels of these traditional RG are not as stable as we thought under different experimental conditions (Xia et al., 2014; Pombo et al., 2017; Wang et al., 2017). To date, most studies of gene expression by RT-qPCR in bacteria have still arbitrarily selected certain conventional RG without an evaluation of stability or even without an explanation (Krzyżanowska et al., 2019). Also, no in-depth analysis is available of stable RG in *S. thermophilus*, which limits accurately quantitative analysis of gene expression.

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With the rapid development of high-throughput sequencing, RNA-sequencing (**RNA-seq**) has recently been employed to uncover more reliable RG for RT-qPCR studies in various species under different experimental conditions (Xia et al., 2014; Pombo et al., 2017; Wang et al., 2017; Nong et al., 2019). Hence, to identify new RG with lower variation for RT-qPCR, we used RNA-seq to systematically assess gene expression variation of *S. thermophilus* under different treatments. Wild-type *S. thermophilus* S-3 (galactose-positive) and AR333 (galactose-negative) in this study were isolated from traditional Chinese dairy products (Xiong et al., 2019b); they were cultivated in both GM17 and LM17 liquid medium at 37°C without shaking for 24 h. Accordingly, the 4 experimental conditions were (1) strain S-3 grown in GM17 medium, (2) strain S-3 grown in LM17 medium, (3) strain AR333 grown in GM17 medium, and (4) strain AR333 grown in LM17 medium. According to our previous report (Kong et al., 2019), total RNA of *S. thermophilus* samples was extracted using Trizol reagent (Thermo Fisher Scientific, Waltham, MA) and ribosomal RNA was removed by a Ribo-Zero Magnetic kit (Epicenter, Madison, WI). The cDNA libraries were prepared using the TruSeq RNA Library Preparation kit (Illumina, San Diego, CA) and sequenced on the Illumina HiSeq 4000 platform by Shanghai Majorbio Bio-pharm Technology Co. Ltd. (Shanghai, China). The strength of gene expression could be reflected by fragments per kilobase of gene model per million mapped reads (**FPKM**), which was calculated using the FPKM function from edgeR v.3.4.0.3 (Kong et al., 2019). Based on the reference genome data of *S. thermophilus* LMD-9 (NC_008532), the transcriptional profiling of all genes in strains S-3 and AR333 was sorted by FPKM under the 2 target culturing conditions (GM17 and LM17 media). To rank the transcript level stability of the predicted 1,911 genes, we calculated the average reads per kilobase of transcript per million mapped reads (RPKM) value, standard deviations, and variation coefficient (**VC**, the ratio between SD and the average RPKM of each gene expression) using all the RPKM obtained from the above 4 treatments. We screened 21 genes that satisfied with $VC < 0.1$ and ordered the gene list according to the lowest VC value (Table 1). For this set of genes, VC ranged from 4.10% to 9.88%. The lower VC indicated more stable gene expression across all the conditions. Interestingly, none of 21 selected genes had been used previously as RG in *S. thermophilus*. Primarily house-keeping genes such as *16S rRNA* (16S ribosomal RNA), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), and *recA* (DNA recombination/repair protein) have been widely used as traditional RG in bacteria. However, higher VC values of *16S rRNA* (19.9%), *GAPDH*

(30.8%), and *recA* (43.4%) across all the treatments suggested the possibility of low gene expression stability of traditional RG (Table 1). The *16S rRNA* gene had a low VC value in comparison with the other 2 RG. Moreover, the *16S rRNA* gene is the most commonly used RG in *S. thermophilus* (Anbukkarasi et al., 2014). Hence, we selected *16S rRNA* as the control for further validation by RT-qPCR.

To assess the possibility of 21 candidate genes as RG, we extracted the nucleotide sequence of each gene from the genome data of LMD-9 (Supplemental File S1; <https://doi.org/10.3168/jds.2020-18672>) and designed primers (Supplemental Table S1; <https://doi.org/10.3168/jds.2020-18672>) using Vector NTI Advance software (ver. 11.5, Invitrogen, Carlsbad, CA). The method of RNA extraction and RT-qPCR was previously described by Xiong et al. (2020). The specificity of the amplification for all the pair of primers was evaluated by melting curves and agarose gel electrophoresis. If there was not a single peak in the melting curve or no single amplification product in the 2% agarose gel, we discarded that pair of primers (Supplemental Table S1). We used cDNA dilutions (1, 1:10, 1:100, 1:1,000, and 1:10,000) for RT-qPCR. Amplification efficiency (**E**) was measured as $10^{-1/\text{slope}}$ and expressed in percentage (Supplemental Table S1). Except for abnormally high *E* values ($>150\%$) of the primers of *STER_RS01160*, *STER_RS06475*, *STER_RS01675*, *STER_RS06355*, and *STER_RS08540*, other primers showed good amplification with *E* values ranging from 80.7% to 129.9%. We also evaluated the performance of these candidate genes under the above 4 treatments by cycle amplification values (**Cq**, Figure 1). Average Cq values ranging from 18.7 (*STER_RS10000*) to 31.7 (*STER_RS05195*) suggested that most of the genes (except for *STER_RS05195*) have suitable Cq values within the recommended values (15–30) for RT-qPCR RG (Pombo et al., 2017). According to *E* and Cq values, we abandoned the genes *STER_RS01160*, *STER_RS06475*, *STER_RS01675*, *STER_RS06355*, *STER_RS08540*, and *STER_RS05195* as candidate RG.

To evaluate the expression stability of our selected genes, we used 2 different tools to analyze the RT-qPCR data across all 4 conditions. The geNorm software (Vandesompele et al., 2002) first determined the average expression stability value *M* (the average pairwise variation of a particular gene with all other control genes) under various experimental conditions. A gene with a lower *M* value is more stable. All the analyzed genes showed lower *M* values than that of the common RG *16S rRNA* ($M = 3.83$, Figure 2A and Supplemental Table S2; <https://doi.org/10.3168/jds.2020-18672>). The most stable genes were *STER_RS10085*,

Table 1. Expression (reads per kilobase of transcript per million mapped reads, RPKM) and variation coefficient (VC) of the selected genes for the stability analysis by RNA-sequencing¹

Gene ID	Gene	Protein ID	Function	AR333-GM17	AR333-LM17	S-3-GM17	S-3-LM17	Average RPKM	SD	VC (%)
<i>STER_RS10085</i>	<i>degV</i>	WP_082306385.1	Fatty acid-binding protein DegV	199.55	184.92	183.10	194.35	190.48	7.80	4.10
<i>STER_RS01160</i>	<i>ptsG</i>	WP_014607909.1	PTS glucose transporter subunit IIA	12.41	13.46	14.31	14.01	13.55	0.84	6.18
<i>STER_RS09650</i>	—	WP_002952260.1	Hypothetical protein	147.59	154.84	132.97	148.17	145.89	9.22	6.32
<i>STER_RS07945</i>	—	WP_011681553.1	Amino acid ABC transporter substrate-binding protein	170.02	186.50	184.64	199.04	185.05	11.89	6.42
<i>STER_RS02845</i>	—	WP_011680871.1	Multidrug ABC transporter ATP-binding protein	3.04	3.38	3.26	2.94	3.15	0.20	6.44
<i>STER_RS02880</i>	—	WP_004197224.1	ABC transporter ATP-binding protein	24.47	26.40	25.57	22.58	24.75	1.65	6.67
<i>STER_RS10000</i>	<i>rpmG</i>	WP_002946904.1	50S ribosomal protein L33	2,020.3	2,008.2	1,965.6	1,739.3	1,933.38	131.43	6.80
<i>STER_RS06475</i>	<i>pbuX</i>	WP_014608487.1	Xanthine permease	98.41	104.19	115.79	103.74	105.53	7.33	6.94
<i>STER_RS02660</i>	<i>glyS</i>	WP_011680845.1	Glycine-tRNA ligase subunit β	335.40	370.72	339.30	389.63	358.76	25.95	7.23
<i>STER_RS03165</i>	<i>murN</i>	WP_011680915.1	Peptidoglycan branched peptide synthesis protein	224.86	199.30	241.88	220.07	221.53	17.53	7.91
<i>STER_RS05350</i>	<i>deoB</i>	WP_011681180.1	Phosphopentomutase	1,509.9	1,802.1	1,782.26	1,800.6	1,723.72	142.79	8.28
<i>STER_RS06365</i>	<i>desK</i>	WP_011681331.1	Histidine kinase	15.94	15.09	13.07	14.32	14.60	1.22	8.35
<i>STER_RS06935</i>	—	WP_011681418.1	ISL3 family transposase	6.93	8.09	6.83	6.81	7.16	0.62	8.62
<i>STER_RS05195</i>	<i>ubiB</i>	WP_011681158.1	Transporter	480.77	454.63	498.11	403.70	459.30	41.15	8.96
<i>STER_RS01675</i>	<i>ssrT</i>	WP_011680729.1	Serine/threonine transporter SsrT	54.68	55.99	65.68	54.93	57.82	5.27	9.12
<i>STER_RS06880</i>	<i>ftsY</i>	WP_011681410.1	Signal recognition particle-docking protein FtsY	115.32	96.73	96.08	110.71	104.71	9.78	9.34
<i>STER_RS06355</i>	—	—	Sugar ABC transporter ATP-binding protein	21.54	21.45	19.35	24.38	21.68	2.06	9.51
<i>STER_RS06240</i>	—	WP_011681309.1	DUF5067 domain-containing protein	38.21	38.83	46.91	42.15	41.53	3.99	9.60
<i>STER_RS08540</i>	—	WP_002947111.1	Hypothetical protein	186.33	150.17	179.33	161.37	169.30	16.53	9.76
<i>STER_RS07550</i>	<i>murF</i>	WP_011681505.1	UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-alanine ligase	68.39	78.45	86.85	81.59	78.82	7.77	9.86
<i>STER_RS00395</i>	<i>arsC</i>	WP_002949058.1	Arsenate reductase	308.03	275.95	279.83	340.10	300.98	29.74	9.88
<i>STER_RS06855</i>	<i>16S rRNA</i>	WP_014608533.1	16S rRNA [cytosine(967)-C(5)]-methyltransferase	272.37	248.07	354.26	229.98	276.17	54.88	19.9
<i>STER_RS06125</i>	<i>GAPDH</i>	WP_011681296.1	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	2,651.0	1,442.6	1,565.5	1,617.7	1,819.2	559.37	30.8
<i>STER_RS00390</i>	<i>recA</i>	WP_011680606.1	DNA recombination/repair protein RecA	201.12	339.09	559.69	297.30	349.30	151.69	43.4

¹AR333-GM17 = *Streptococcus thermophilus* AR333 cultivated in GM17 medium; AR333-LM17 = *S. thermophilus* AR333 cultivated in LM17 medium; S-3-GM17 = *S. thermophilus* S-3 cultivated in GM17 medium; S-3-LM17 = *S. thermophilus* S-3 cultivated in LM17 medium.

Figure 1. Cycle quantification (Cq) values of 21 candidate genes and *16S rRNA*. Box and whisker plot graph showing Cq values of each selected gene in all the experimental conditions. Upper and lower edges of boxes indicate the 25th and 75th percentiles. The lines across the boxes depict the median. Whisker caps represent the minimum and maximum values.

STER_RS02660, and *STER_RS03165* with *M* values of 1.43, 1.44, and 1.48, respectively, by the geNorm algorithm. We further investigated expression stability of these selected genes with NormFinder software (Andersen et al., 2004), which can also calculate *M* index with taking into account the intragroup and intergroup variations. Like geNorm, NormFinder analysis presented the similar result that all candidate genes have lower *M* values than *16S rRNA* ($M = 2.55$, Figure 2B and Supplemental Table S2). The most reliable genes were also *STER_RS02660*, *STER_RS10085*, and *STER_RS03165* with *M* values of 0.13, 0.18, and 0.28, respectively. Although the results of gene expression stability obtained from geNorm and NormFinder were largely consistent, a few discrepancies of ranking orders were observed by these 2 statistical programs based on different algorithms. Pombo et al. (2017) recommended considering the complementarity between these methods and globally analyzing the results. Thus, the arithmetical mean of the ranking value by different algorithms was calculated for each candidate gene. As expected, *STER_RS02660* (*glyS*) and *STER_RS10085* (*degV*) were sorted as the most stable genes with a mean value of 1.5 (Supplemental Table S2). Thus, we recommend *glyS* and *degV* as the better choices for universal RG in *S. thermophilus*.

The gold standard of gene expression quantification is RT-qPCR (DeLorenzo and Moon, 2018), which highly

depends on RG for the accuracy and reliability of the data. Unfortunately, no in-depth analysis of stable RG has been performed in *S. thermophilus* and other LAB species. In this work, we used RNA-seq as a new approach for genome-wide identification of new stable RG in *S. thermophilus*. To our knowledge, this is the first time of identifying RG in *S. thermophilus*. A set of new candidate RG with low VC values was selected from the expression of all 1,911 genes in the genome and further validated by RT-qPCR. Based on the analysis of expression stability by geNorm and NormFinder algorithms, all candidate genes were more stable than traditional RG *16S rRNA* under 4 experimental conditions. Our results strongly supported the use of high-throughput transcriptomic data for mining the highly stable RG in RT-qPCR experiments (Xia et al., 2014; Pombo et al., 2017; Wang et al., 2017), which provides a valuable starting point for more precise results of gene expression in *S. thermophilus* and other LAB.

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Figure 2. Expression stability of selected genes in *Streptococcus thermophilus* using geNorm (A; Vandesompele et al., 2002) and NormFinder (B; Andersen et al., 2004) analysis. The analysis was performed using expression data from all biological replicates and treatments. The M value represents the average pairwise variation of a particular gene in comparison with all other control genes.

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