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RESEARCH LETTER

Validation of reference genes for normalization of qPCR mRNA expression levels in *Staphylococcus aureus* exposed to osmotic and lactic acid stress conditions encountered during food production and preservation

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Abstract

Staphylococcus aureus represents the most prevalent cause of food-borne intoxications worldwide. While being repressed by competing bacteria in most matrices, this pathogen exhibits crucial competitive advantages during growth at high salt concentrations or low pH, conditions frequently encountered in food production and preservation. We aimed to identify reference genes that could be used to normalize qPCR mRNA expression levels during growth of *S. aureus* in food-related osmotic (NaCl) and acidic (lactic acid) stress adaptation models. Expression stability of nine housekeeping genes was evaluated in full (LB) and nutrient-deficient (CYGP w/o glucose) medium under conditions of osmotic (4.5% NaCl) and acidic stress (lactic acid, pH 6.0) after 2-h exposure. Among the set of candidate reference genes investigated, *rplD*, *rpoB*, *gyrB*, and *rho* were most stably expressed in LB and thus represent the most suitable reference genes for normalization of qPCR data in osmotic or lactic acid stress models in a rich medium. Under nutrient-deficient conditions, expression of *rho* and *rpoB* was highly stable across all tested conditions. The presented comprehensive data on changes in expression of various *S. aureus* housekeeping genes under conditions of osmotic and lactic acid stress facilitate selection of reference genes for qPCR-based stress response models.

Introduction

Staphylococcal food poisoning is the most prevalent cause of food-borne intoxications worldwide. Consumption of staphylococcal enterotoxins preformed in food causes acute gastroenteritis and can be fatal in sensitive populations such as children and the elderly. *Staphylococcus aureus* represents the most osmotolerant food-borne pathogen and is also highly resistant to acidic stress (Shebuski *et al.*, 2000). Although growth of this organism is repressed by competing bacteria in most matrices, it possesses a crucial competitive advantage under osmotic or acidic stress, conditions that are frequently encountered in food production and preservation (Scott, 1953; Minor & Marth, 1972; Hurst, 1973).

Numerous publications investigated the production of enterotoxins under stress conditions using immunological methods (Genigeorgis & Sadler, 1966; McLean *et al.*, 1968; Troller, 1971; Domenech *et al.*, 1992). However, these data are of limited value, as it was later shown that loss of serological recognition does not equal loss of biological/emetic activity (Bennett, 2005). Quantitative real-time PCR (qPCR) techniques have opened up new possibilities to investigate enterotoxin gene expression under stress conditions. Evaluation of enterotoxin gene expression on mRNA level was suggested to be a useful tool to determine the risk of *S. aureus* intoxication (Lee *et al.*, 2007). However, sound normalization of quantification data is crucial to yield reliable qPCR results. Normalization allows for comparison of mRNA concentrations among different samples, as it controls for variations regarding the yield of both RNA extraction and reverse transcription, as well as amplification efficiency (Bustin *et al.*, 2009). It is widely regarded to be the most appropriate strategy to normalize quantification results using expression levels of several stably expressed house-

keeping genes (HKGs) as an internal reference (Bustin *et al.*, 2009). While qPCR is increasingly used to determine the expression of different enterotoxin genes (Derzelle *et al.*, 2009; Duquenne *et al.*, 2010), there is no information on HKGs suitable for normalization of quantification data of enterotoxin gene expression under osmotic and acidic stress.

In this study, the suitability of nine *S. aureus* HKGs as reference genes for normalization of qPCR mRNA expression levels in food-related stress adaptation models was investigated. We aimed to identify reference genes that could be applied in experimental models investigating enterotoxin gene expression changes related to adaptation of *S. aureus* to NaCl and lactic acid stress encountered during food production and preservation.

Materials and methods

Bacterial strains

Staphylococcus aureus strains used in this study, including sources, clonal complexes, and *spa* types, are listed in Table 1. Strains from different clonal complexes and sources were selected to reflect the variability of *S. aureus* strains that can be detected in foodstuff.

Bacterial growth and collection of samples

Staphylococcus aureus strains were grown and subjected to control (media alone), as well as acid (0.08% lactic acid, pH 6.0) and osmotic (4.5% NaCl) stress conditions in both the nutrient-deficient glucose-free casamino acids yeast extract glycerophosphate broth (CYGP w/o glucose, Novick 1991) and the nutrient-rich medium LB (Bertani, 1951). Media ingredients were obtained from Difco laboratories (Detroit, MI), Oxoid (Cambridge, UK), Becton Dickinson (Allschwil, Switzerland), and Sigma (Buchs, Switzerland). Frozen stock cultures (−80 °C) of *S. aureus* strains were resuscitated by plating on 5% sheep blood agar and incubation at 37 °C over night. Two single colonies per strain were grown in parallel in 10 mL of LB broth cultures for 18 h (37 °C, 225 r.p.m. shaking) to reach the stationary phase. Three identical 5-mL cultures were generated by three times pooling 2.5-mL aliquots of both stationary phase cultures. The pooled cultures were centrifuged (4000 ***g*** for 5 min), and the recovered cells were resuspended in 5 mL of fresh medium adjusted to three different conditions: (1) LB or CYGP only (serving as a control), (2) LB or CYGP w/o glucose adjusted to 4.5% NaCl, (3) LB or CYGP w/o glucose adjusted to pH 6.0 using lactic acid. All samples were incubated in a shaking incubator at 37 °C and 225 r.p.m. for 2 h to allow for adaptation to control and stress conditions. Two samples (400 μL) were taken from each culture, and stress-adapted cells were harvested by centrifugation (8000 ***g***, 5 min, 4 °C). The cells were resuspended in 500 μL RNA protect bacteria reagent (Qiagen, Hombrechtikon, Switzerland), incubated at room temperature for 5 min, and harvested again by centrifugation (3100 ***g***, 5–10 min). Cell pellets were stored at −80 °C. This procedure was repeated to gain two independent samples of each strain grown adapted to these different growth conditions.

Selection of reference genes

Expression stability was analyzed for nine candidate reference genes that have previously been used as references in qPCR studies (Lee *et al.*, 2007; Theis *et al.*, 2007; Derzelle *et al.*, 2009; Duquenne *et al.*, 2010; Stutz *et al.*, 2011). For a detailed list of all selected reference genes and their function see Table 2.

Cell lysis and RNA extraction

Cell pellets were resuspended in 500 μL RLT plus buffer (Qiagen) and mechanically disrupted using a MagNA lyser (Roche, Rotkreuz, Switzerland) in two intervals of 6500 r.p.m. for 60 s with an intermediate cooling step (cells were put on ice for 1 min). Total RNA was isolated using the RNeasy plus mini kit (Qiagen) following the manufacturer's instructions. Two DNA contamination

Table 1. Clonal complexes, *spa* types, and sources of the *Staphylococcus aureus* strains used in this study

Strain ID	Clonal complex	*spa* type	Enterotoxins	Source
KLT_6	CC12	t160	*seb*	Food-borne outbreak
RKI2	CC8	t008	*sea, sed, sej, ser*	Food-borne outbreak
RKI3	CC30	t018	*sea, egc cluster*	Food-borne outbreak
RKI4	CC9	t733	*seb, egc cluster*	Food-borne outbreak
SAI_06	CC97	t276	–	Human bronchial secretion
SAI_23	CC22	t8019	*egc cluster*	Human bronchial secretion
SAK_09	CC5	t8456	*seb, seg, sei*	Rabbit carcass
SAR_1	CC151 (CC705)	t529	*egc cluster*	Bovine mastitis milk

Table 2. Primer pairs, including amplicon sizes, primer concentrations, and *E*-values for the nine HKG in this study

Gene	Function	Primer pair (5′-3′)	Amplicon size (bp)	*E* (%)	Primer c (nM)	Reference
16S rRNA	16S ribosomal RNA subunit	TGT CGT GAG ATG TTG GG CGA TTC CAG CTT CAT GT	270	95	500 500	Stutz *et al.* (2011)
ftsZ	Cell division protein	TAT TAC TGG TGG CGA GTC A AGT ATT TAC GCT TGT TCG GA	223	93	250 250	This study
gyrB	DNA gyrase (subunit B)	GTC GAA GGG GAC TCT G GCT CCA TCC ACA TCG G	242	95	250 250	This study
proC	Pyrroline-5-carboxylate reductase	GGC AGG TAT TCC GAT TG CTT CCG GTG ATA GCT GTT A	231	97	800 1000	This study
pyk	Pyruvate kinase	GCT AGT GAC GTT GCC A ATA GTA CGT GCC GTT G	284	95	500 500	This study
recA	Recombinase A	AAG TAC GTC GTG CAG A TGA CCC ATT CGT TCG C	229	93	250 250	This study
rho	Transcription termination factor	GAA GCT GCT GAA GTC G CGT CCA TAC GTG AAC CC	319	95	250 250	This study
rplD	Ribosomal protein L4	TTC GGA CCA ACT CCA AGA CGA GCA CCT CCT CAA C	352	91	250 250	This study
rpoB	RNA polymerase (beta chain)	CTA AGC ACA GAG GTC GT ACG GCA TCC TCA TAG T	298	98	250 250	This study

removal steps were included by passing the cell lysates through a genomic DNA-binding column and performing an on-column DNase I digestion of the samples bound to the RNA spin column. The RNA templates were eluted in 50 μL RNase-free water. The quantity and quality of RNA were assessed using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and Bioanalyzer (Agilent Technologies, Waldbronn, Germany) instruments, respectively.

Reverse transcription

Reverse transcription was performed using the Quantitect Reverse Transcription Kit (Qiagen). cDNA synthesis was primed using an optimized blend of oligo-dT and random hexamer primers provided in this kit. We converted 480 ng of total RNA of each sample to cDNA in a total volume of 20 μL. We created RT minus controls of each sample by performing the same reaction without reverse transcriptase to be able to subsequently screen each sample for residual DNA contamination in the gene-specific qPCR assay.

Real-time PCR

Primers used are listed in Table 2. Real-time PCR experiments were performed using Light Cycler 480 (Roche). Reactions were performed in a total volume of 10 μL, including 4.8 ng cDNA template (except for 16S rRNA gene primers, for which 0.48 ng cDNA was used), optimized primer concentrations (Table 2), and the LIGHTCYCLER 480 SYBR Green I master mix (Roche). Water (no template) and RT minus samples served as controls. PCR cycling conditions included 8 min of hot start at 95 °C, 45 amplification cycles (95 °C for 10 s, 57 °C for 15 s, 72 °C for 20 s, 78 °C for 1 s with a single fluorescence measurement), a melting curve (60–95 °C at 2.2 °C s^{-1}, and a continuous fluorescence measurement), and a final cooling step. Single peaks in the melting curve analysis confirmed specificity of amplification. Standard curves based on genomic DNA were generated to determine the efficiency of HKG target amplification by real-time PCR. All reactions were performed in two separate sets of experiments using triplicates for each sample, and results are presented as mean values. Expression levels of HKGs were compared using crossing points (CP) based on the 'second derivative maximum' computed by the LIGHTCYCLER 480 software.

Determination of HKG expression stability using BESTKEEPER and GENORM

BESTKEEPER (Pfaffl *et al.*, 2004) and GENORM (Vandesompele *et al.*, 2002) programs were used to compare expression stability of the candidate reference genes under the tested stress conditions. BESTKEEPER uses raw CP values to compare expression stability of HKGs based on a multitude of pair-wise correlation analyses and determines the BESTKEEPER index. The software compares each gene to this index, thus calculating the Pearson correlation coefficient (r) and the correlation probability (P) between the index and the contributing candidate HKG. It also calculates standard deviations (SD) of the CP values, as well as a coefficient of variance (CV) that is expressed as a percentage

on the CP level. The genes with the highest coefficient of correlation and SD ≤ 1.0 are considered to be most stably expressed (Pfaffl *et al.*, 2004). The GENORM module integrated into qbase^{plus} (Biogazesse, Zulte, Belgium) allows calculation of the gene expression stability measure *M*, based on the average pair-wise variation value (*V*) of a single candidate reference gene with all other control genes. Thus, the gene with the lowest *M* value is most stably expressed. The least stably expressed genes are stepwise excluded, and *M* is recalculated. The recommended number of reference genes is determined using *V* with a cutoff of 0.15 as threshold (Vandesompele *et al.*, 2002).

Results

RNA quality assessment using Bioanalyzer revealed high RNA integrity values (RIN scores of 7.3–9.8) for all tested samples. Real-time PCR assays were optimized with regard to primer concentrations (Table 2) and run conditions for each target to assure high amplification specificity and efficiency. Single peaks in melting curve analyses, as well as single product bands on agarose gels, confirmed target-specific amplifications for all primer pairs used. PCR efficiencies ranged from 91% to 98% (Table 2).

Interstrain HKG expression stability was initially assessed among *S. aureus* strains exposed to individual experimental conditions associated with the LB- and CYGP-based osmotic and organic acid stress adaptation models. This revealed that there was high interstrain variation in HKG expression stability under osmotic and acidic stress conditions in comparison with growth under control conditions in both LB and CYGP w/o glucose (Figs 1 and 2; Table 3). Next, we assessed changes in expression levels due to exposure to osmotic or acidic stress (Table 4). In the NaCl stress adaptation model, we found the most stably expressed HKGs were *rplD*, *rpoB*, and *rho* in nutrient-rich (LB) medium, and *rho*, *gyrB*, and *rpoB* in nutrient-deficient (CYGP w/o glucose) medium. Greater variability in HKG expression levels was detected under the combined experimental conditions of the lactic acid stress adaptation model. In the LB-based lactic acid stress adaptation model, *rplD*, *rpoB*, *gyrB*, and *rho* were ranked as the best four reference genes using BESTKEEPER and GENORM. When we assessed expression stability in the CYGP w/o glucose-based lactic acid stress adaptation model, *rho* represented the most stably expressed candidate reference gene.

Finally, the generated data also allowed us to determine which of the candidate reference genes exhibits the most stable expression across all three tested conditions (Table S3). When including all three conditions, *rplD* was most stably expressed in LB, whereas *rpoB* (BESTKEEPER) and *rho* (GENORM) exhibited the most stable expression in CYGP w/o glucose. In general, we observed less variation in the HKG expression across all conditions in LB than in CYGP w/o glucose.

Discussion

In this study, we investigated the suitability of nine HKGs as reference genes for normalization of qPCR mRNA expression levels in food-related stress adaptation models in *S. aureus*. Our objective was to identify reference genes that could be used as endogenous controls in the development of experimental models for investigation of enterotoxin gene expression changes associated with the exposure and adaptation of enterotoxigenic *S. aureus* strains to NaCl and lactic acid stress encountered during food production and preservation.

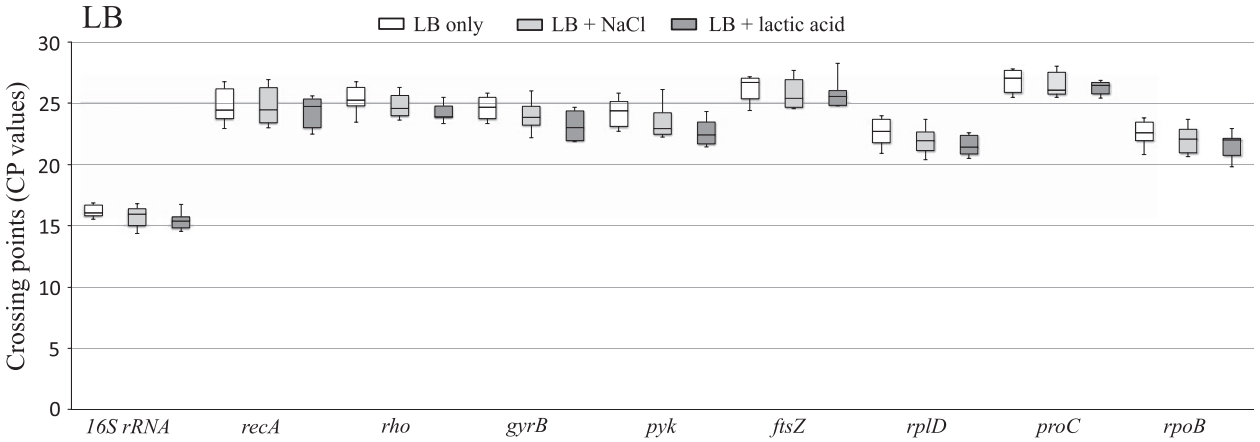

Fig. 1. Changes in CP values of different candidate reference genes when *Staphylococcus aureus* strains were exposed to LB only, LB with 4.5% NaCl, and LB adjusted to pH 6.0 using lactic acid. The graph depicts ranges of HKG expression across the tested strains as well as medians and 25th/75th percentiles.

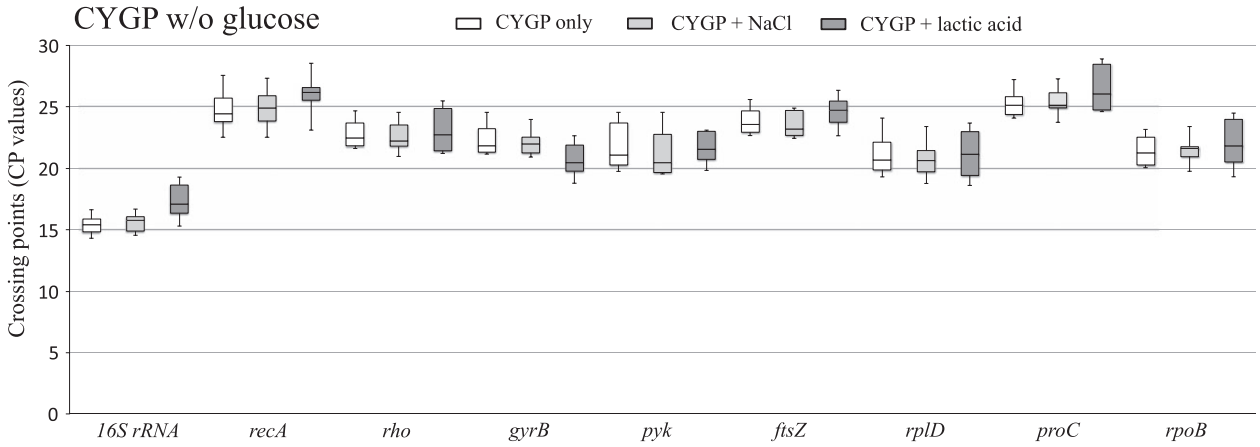

Fig. 2. Changes in CP values of different candidate reference genes when *Staphylococcus aureus* strains were exposed to CYGP only, CYGP with 4.5% NaCl, and CYGP adjusted to pH 6.0 using lactic acid. The graph depicts ranges of HKG expression across the tested strains as well as medians and 25th/75th percentiles.

Table 3. Ranking of reference genes suitable for normalization of experiments comparing expression across strains within a single experimental condition

		Ranking								
Condition	Software	1	2	3	4	5	6	7	8	9
LB	BESTKEEPER	*rplD*	*rpoB*	*gyrB*	*rho*	*pyk*	*ftsZ*	*proC*	*16S rRNA*	
	GENORM	*gyrB*	*rpoB*	*rplD*	*rho*	*pyk*	*proC*	*ftsZ*	*16S rRNA*	*recA*
CYGP	BESTKEEPER	*rplD*	*gyrB*	*rho*	*rpoB*	*recA*	*ftsZ*	*proC*	*16S rRNA*	
	GENORM	*rplD*	*16S rRNA*	*rho*	*proC*	*ftsZ*	*rpoB*			
LB-NaCl	BESTKEEPER	*rplD*	*rho*	*pyk*	*rpoB*	*ftsZ*	*proC*	*gyrB*	*16S rRNA*	
	GENORM	*rho*	*rplD*	*rpoB*	*proC*	*ftsZ*	*pyk*	*gyrB*	*recA*	
CYGP-NaCl	BESTKEEPER	*rho*	*rplD*	*gyrB*	*rpoB*	*proC*	*recA*	*ftsZ*	*16S rRNA*	
	GENORM	*rho*	*rpoB*	*gyrB*	*proC*	*ftsZ*	*rplD*	*recA*		
LB acid	BESTKEEPER	*rplD*	*rpoB*	*pyk*	*recA*	*gyrB*	*rho*	*ftsZ*	*16S rRNA*	*proC*
	GENORM	*rplD*	*pyk*	*rho*	*rpoB*	*gyrB*	*recA*	*ftsZ*	*proC*	*16S rRNA*
CYGP acid	BESTKEEPER	*16S rRNA*	*pyk*	*recA*	*ftsZ*					
	GENORM	*rho*	*rpoB*	*proC*	*ftsZ*					

Only HKGs that met the criteria of SD ≤ 1.0 (BESTKEEPER) and $M \leq 1.0$ (GENORM) were included. For more detailed results, see Table S1.

Table 4. Ranking of reference genes suitable for normalization of stress adaptation models comparing changes in expression levels due to exposure to osmotic stress (4.5% NaCl) or acidic stress (lactic acid, pH 6.0)

		Ranking								
Medium	Software	1	2	3	4	5	6	7	8	9
LB vs. LB + NaCl	BESTKEEPER	*rplD*	*rpoB*	*rho*	*gyrB*	*ftsZ*	*proC*	*16S rRNA*		
	GENORM	*rplD*	*rho*	*rpoB*	*pyk*	*gyrB*	*proC*	*ftsZ*	*recA*	*16S rRNA*
CYGP vs. CYGP + NaCl	BESTKEEPER	*rho*	*gyrB*	*rpoB*	*proC*	*ftsZ*	*16S rRNA*			
	GENORM	*rho*	*rpoB*	*gyrB*	*proC*	*ftsZ*	*rplD*	*recA*		
LB vs. LB + acid	BESTKEEPER	*rplD*	*rpoB*	*gyrB*	*rho*	*proC*	*ftsZ*	*16S rRNA*		
	GENORM	*rplD*	*rho*	*rpoB*	*gyrB*	*pyk*	*proC*	*ftsZ*	*16S rRNA*	*recA*
CYGP vs. CYGP + acid	BESTKEEPER*	*rho*	*proC*	*ftsZ*	*gyrB*					
	GENORM	*rho*	*rpoB*	*proC*	*ftsZ*					

Only HKGs that met the criteria of SD ≤ 1.0 (BESTKEEPER) and $M \leq 1.0$ (GENORM) were included. For more detailed results, see Table S2.
*When all HKGs were included, BESTKEEPER determined that only *ftsZ* would be suitable as a reference gene (SD = 0.96, $r = 0.880$, $P = 0.001$). Results listed depict the ranking of reference genes, when strain KLT_6 leading to the most variable results was excluded.

With this objective in mind, stress adaptation experiments were performed using both a nutrient-rich (LB) and a nutrient-deficient (CYGP w/o glucose) medium adjusted to 4.5% NaCl and pH 6.0 (lactic acid stress) to reflect stress conditions relevant to the food production. Both media do not contain glucose, which was reported to have a negative effect on enterotoxin expression (Regassa *et al.*, 1991). CYGP w/o glucose was selected as a nutrient-deficient medium, after assessing staphylococcal growth in various minimal media, including chemically defined medium (Hussain *et al.*, 1992), *S. aureus* synthetic medium (Gertz *et al.*, 1999), AAM (Rudin *et al.*, 1974), as well as broth containing 10 g L^{-1} hydrolyzed casamino acids and 122 μM tryptophan. However, CYGP w/o glucose represented the only nutrient-deficient medium enabling sufficient growth of all tested strains.

In this study, we evaluated expression stability using both BESTKEEPER and GENORM, applications designed to identify the best-suited reference genes out of a set of HKGs. Rank assignment of the candidate reference genes under the tested experimental conditions varied using these two methods. This is expected because the two applications rely on different assumptions and algorithms for reference gene ranking. Firstly, BESTKEEPER determines overall stability of candidate gene expression by comparison of CP variation based on mean values. Several HKGs exhibited low interstrain variability of expression levels across all growth conditions and tested media (SD ≤ 1 CP), including *16S rRNA* gene, *proC*, *rho*, *rpoB, ftsZ,* and *gyrB. 16S rRNA* gene exhibited the lowest interstrain variation in our study, supported by low SD ($0.41 \leq SD \leq 1.0$) and CV (2.51% $CP \leq CV \leq 6.14\%$) values. While this gene is commonly used in *S. aureus* for normalization of qPCR data due to its high target copy number (Eleaume & Jabbouri, 2004; Lee *et al.*, 2007; Stutz *et al.*, 2011), others have suggested that this gene may not be suitable as a reference gene in *S. aureus* because *16S rRNA* gene transcripts were shown to by far exceed most other transcripts in stability (McKillip *et al.*, 1998) and do not reflect overall mRNA in this organism (Theis *et al.*, 2007). Secondly, BESTKEEPER performs numerous pair-wise correlation analyses between the candidate reference genes and computes highly correlated genes into an index. Subsequently, the software compares each gene to this index, thus calculating the Pearson correlation coefficient (r) and the correlation probability (P) between the index and the contributing candidate HKG.

In contrast, the GENORM algorithm relies on the assumption that the expression ratio of the two most adequate reference genes should be highly similar among all samples irrespective of the tested conditions. The GENORM software also calculates an optimal number of reference genes. In our study, GENORM determined the use of two reference genes (*rplD* and *rho*) to be optimal for normalization in osmotic and acid stress adaptation models that determine expression response changes by comparing gene expression between strains in LB supplemented with NaCl (4.5%) or LB adjusted to pH 6.0 using lactic acid, relative to controls that are exposed to regular LB media. However, this number would not suffice to normalize expression data when similar stress adaptation models are investigated in the nutrient-deficient medium CYGP w/o glucose. For these conditions, the software suggested the use of three reference genes for the NaCl assay and five reference genes for the lactic acid assay.

We evaluated the suitability of nine HKGs as internal controls for normalization of qPCR mRNA expression levels in food-related stress adaptation models in *S. aureus*. Among the set of HKGs tested, *rplD*, *rpoB*, *gyrB*, and *rho* were determined to be most stably expressed in LB and were therefore considered to represent the most suitable reference genes for assays investigating *S. aureus* stress response to osmotic or acidic conditions in this medium. In CYGP w/o glucose, *rho* and *proC* were stably expressed across all tested conditions. We recommend the use of HKGs for normalization of qPCR mRNA expression levels that ranked in high positions in both BESTKEEPER and GENORM rankings under the specific experimental conditions employed. This is the first study presenting comprehensive data on changes in expression of various *S. aureus* HKGs under conditions of osmotic and lactic acid stress, enabling selection of reference genes for qPCR-based stress response models in *S. aureus*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Ranking of reference genes suitable for normalization of experiments comparing expression across strains within one experimental condition only.

Table S2. Ranking of reference genes suitable for normalization of stress adaptation models by comparison of expression levels between one stress condition (either NaCl or acidic stress) and the control condition without stress.

Table S3. Ranking of HKG that exhibited the highest stability over all three tested conditions (NaCl stress, lactic acid stress, control).