Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR

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Abstract

Listeria monocytogenes is an important food-borne pathogen that can tolerate a wide range of stress conditions. However, its stress adaptation processes are still poorly understood. Real-time-based quantitative RT-PCR (qRT-PCR) provides a tool to probe gene expression changes underlying stress adaptation. But, a limitation to study mRNA levels by real-time qRT-PCR is that validated reference genes are required for normalization. Such genes are currently lacking for experimental models that may be applied to evaluate stress-related gene expression changes in *L. monocytogenes*. Therefore, five housekeeping genes (HKG) were studied as potential reference genes. Their expression stability was evaluated across 16 *L. monocytogenes* strains. Three experimental models designed to assess gene expression changes induced by cold, acid and high NaCl concentration stress adaptation were applied. The 16S rRNA gene was consistently the most stably expressed HKG across the different *L. monocytogenes* strains under all the experimental conditions. While the expressions of β-glucosidase (*bglA*), Glyceraldehyde-3P-dehydrogenase (*gap*), RNA polymerase beta subunit (*rpoB*) and Ribosomal protein L4 (*rplD*) was stable amongst the different *L. monocytogenes* strains, they were prone to significant variations under the different stress adaptation models.

Introduction

The food-borne pathogen *Listeria monocytogenes* continues to be involved in both sporadic and epidemic food-borne illnesses around the world. These organisms must frequently adapt and overcome various forms of hostile conditions during their transmission in foods and related environments. Some of the frequent forms of stress found along the food supply chain include cold stress due to low temperatures, low pHs associated with organic acids and osmotic stress associated with higher NaCl concentrations. The versatile adaptation capacity of *L. monocytogenes* to these stress challenges stems from a well-coordinated appropriate induction of multiple molecular response mechanisms by stressed cells (Kazmierczak *et al.*, 2006; Tasara & Stephan, 2006). Although our knowledge about the different stress response mechanisms involved remains obscure, some information on this phenomenon has emerged over the past few years (Liu *et al.*, 2002; Kazmierczak *et al.*, 2003; Wemekamp-Kamphuis *et al.*, 2004b). Examples also include numerous studies linking the stress-responsive alternative sigma factor σ^B to the regulation of various environmental stress response mechanisms in this organism (Becker *et al.*, 1998, 2000; Ferreira *et al.*, 2001; Fraser *et al.*, 2003; Kazmierczak *et al.*, 2003). Thus, an improved understanding of the gene expression patterns in this organism might help reflect some of the molecular underpinnings behind its adaptation to different stress conditions along the food chain, as well as during the infection of human hosts. The application of quantitative gene expression analysis techniques such as real-time RT-PCR (qRT-PCR) contributes immensely to the understanding of the complex biological processes behind stress adaptation. As a result, these molecular techniques are now increasingly being applied for rapid and sensitive quantification of the desired mRNA targets in cells (Bustin, 2000; Bustin *et al.*, 2005). However, various challenges remain due to variations associated with RNA isolation, enzymatic efficiencies and standardization of the quantification results (Bustin, 2000; Vandesompele *et al.*, 2002; Bustin *et al.*, 2005). The most frequently used standardization methods involve the normalization of quantification results to an internal reference gene. This approach therefore allows controlling all the different variables associated with qRT-PCR process. Consequently, conclusions

drawn based on relative qRT-PCR methods are also dependent on the choice of reference gene. So far, it has been shown that there is no universal internal control reference gene in eukaryotic or prokaryotic cells (Thellin *et al.*, 1999; Vandecasteele *et al.*, 2001; Vandesompele *et al.*, 2002; Gilsbach *et al.*, 2006). Thus, frequently used internal control reference genes are based on housekeeping genes or rRNA genes. These type of genes, although originally assumed to be constitutively and uniformly expressed, have also been found to be regulated and vary with experimental conditions (Thellin *et al.*, 1999; Vandecasteele *et al.*, 2001; Vandesompele *et al.*, 2002). Therefore, the validity of reference gene choices in specific experimental conditions must be determined before application for quantitative mRNA expression studies. Some previous studies using either semi or relative qRT-PCR approaches to study stress related gene expression changes in *L. monocytogenes* have been described (Sleator *et al.*, 2003b; Sue *et al.*, 2004; Wemekamp-Kamphuis *et al.*, 2004a; Kazmierczak *et al.*, 2006). The real-time relative qRT-PCR methods described so far have applied *rpoB* or *gap* gene for normalization (Sue *et al.*, 2004; Schwab *et al.*, 2005; Kazmierczak *et al.*, 2006). There is, however, little validation of reference genes for stress response-related gene transcript quantification using relative qRT-PCR in view of the different stress adaptation models. The present study was therefore conducted to evaluate the suitability of five housekeeping genes (HKG) as potential reference genes in three food-related stress adaptation experimental models. The aim was to identify internal control reference genes suitable for normalization in experimental models exploring gene expression changes associated with adaptation to cold stress, acid stress and osmotic stress due to elevated NaCl salt concentrations.

Materials and methods

Bacterial strains

The different *L. monocytogenes* strains used in this study with their sources, serotypes and genetic lineages are shown in Table 1. Serotyping of the strains was performed at the Swiss National Centre for Listeriosis (Lausanne, Switzerland), and genetic lineages were determined based on the ASO-PCR multiplex system targeting the *prfA* virulence gene cluster as described previously (Ward *et al.*, 2004).

Bacterial growth conditions and stress adaptation

The different *L. monocytogenes* strains were available as frozen ($-70\,^{\circ}C$) stock cultures. They were revived by plating out onto columbia agar plates supplemented with 5% sheep blood (Becton and Dickenson), and single colonies were obtained after incubation for 18–24 h at 37 °C. Single colonies from each strain were used to inoculate 10 mL brain-heart infusion (BHI). The cultures were grown for 18 h with shaking (250 r.p.m.) at 37 °C to reach the stationary phase. This was confirmed by following the growth of each strain by absorption measurements at 600 nm. To assess target HKG expression in the cold stress model, such stationary-phase cultures were pelleted by centrifugation (4000 ***g*** for 5 min). The supernatants were discarded and the pellets were once again resuspended in 10 mL of fresh BHI broth. Each culture was further subdivided into two aliquots of 5 mL each, followed by a 6-h incubation at 4 and 37 °C, respectively. Thereafter, the samples were processed for total RNA isolation. The influence of acid stress on target HKG expression stability was assessed in an organic acid-based stress model using BHI acidified with lactic acid. Briefly, 10 mL stationary-phase cultures of each strain were prepared in BHI broth as described. These were subsequently divided into two 5 mL aliquots for each strain and centrifuged (4000 ***g*** for 5 min). The supernatants were discarded and the pellets were retained. From each strain, one of the pellets was resuspended in 5 mL normal BHI and the other in 5 mL of BHI broth acidified to pH 5.5 using lactic acid. The samples were incubated at 37 °C for 2 h and then processed for total RNA template isolation. The influence of NaCl salt stress adaptation on target HKG expression was evaluated in stationary phase cells adapted to defined medium (DM) and DMS (DM plus 3% NaCl; Sleator *et al.*, 2003a). Briefly,

Table 1. Serotypes, genetic lineages and sources of the *Listeria monocytogenes* strains

Strain designation	Serotype	Lineage	Source
Lm 22/3A	1/2a	II	Human asymptomatic
Lm 60/2006	1/2a	II	Human clinical case
Lm 69/2006	1/2a	II	Human clinical case
Lm 56/2A	1/2a	II	Food (meat sample)
Lm 217	1/2a	II	Food environment (meat processing line)
Lm 288	1/2a	II	Food environment (meat processing line)
Lm 9	1/2b	I	Food (meat sample)
Lm 19/2005	1/2b	I	Human clinical case
Lm 45/2005	1/2b	I	Human clinical case
Lm 22/2005	1/2c	II	Human clinical case
Lm 28/2005	1/2c	II	Human clinical case
Lm 760	1/2c	II	Food environment (meat processing line)
Lm 8	4b	I	Food (meat sample)
Lm 49	4b	II	Food (meat sample)
Lm 58/2006	4b	I	Human clinical case
Lm 72/2006	4b	I	Human clinical case

10 mL stationary-phase BHI cultures of each strain were prepared as described above and subdivided into two 5 mL aliquots. These were pelleted by centrifugation (4000 ***g*** for 5 min). At this point, one pellet from each strain was resuspended in 5 mL DM (Premaratne *et al*., 1991), and the other in a similar volume of DMS. The samples were incubated for a 2 h at 37 °C, followed by total RNA isolation. The various total RNA templates prepared from the different samples were subsequently applied for the analysis of target HKG expression in gene-specific real-time RT-PCR assays.

Total RNA isolation

Total RNA was isolated from each sample using the RNeasy plus mini kit (Qiagen), and following the guidelines of the kit protocol. *Listeria monocytogenes* cells prepared under the different experimental conditions of the stress adaptation models described above were harvested by centrifugation of 1 mL cultures (8000 ***g*** for 5 min at 4 °C). The pellets were resuspended in 500 μL of kit lysis buffer and transferred onto a lysing bead matrix in MagNA lyser tubes. The bacterial cells were mechanically disrupted in the MagNA Lyser instrument (Roche Molecular Diagnostics). Two times disruption at 6500 r.p.m. for 60 s was performed as outlined in the instrument protocol. Samples were cooled at 4 °C for 1 min in between the two mechanical disruption steps. Thereafter, total RNA was isolated from the bacterial cell lysates following the RNeasy plus mini kit protocol. This included two steps to remove or minimize genomic DNA contamination. The lysates were first passed over a DNA-binding column provided in the kit, followed by an on-column DNAse I digestion of the samples bound to the RNA-binding column. The RNA templates were eluted in 30 μL of RNAse free water. The RNA concentration was measured by absorbance at 260 nm, while purity was monitored by inspection of the 260/280 nm and 230/260 nm ratios to assess protein and organic substances contamination, respectively, using the Nanodrop instrument (Nanodrop Technologies, Delaware). The quality and integrity of the total RNA templates were assessed by electrophoresis of aliquots of selected samples from each run on a 2% (w/v) denaturing formaldehyde agarose gel electrophoresis.

Reverse transcription

The reverse transcription step was performed using the Quantitect Reverse Transcription Kit (Qiagen AG, Hombrechtikon, Switzerland). This procedure incorporated an optimized blend of oligo-dT and random hexamers as primers for cDNA synthesis. Three-hundred nanograms of the RNA template from each sample was converted into cDNA in 20 μL. As controls, similar amounts of total RNA of each sample were also subjected to the cDNA synthesis reaction without the reverse transcriptase enzyme. These provided the minus RT control samples used in the gene-specific real-time PCR assays to assess the potential residual DNA contamination of each sample. The reverse transcription reactions therefore generated 300 ng cDNA template pool from each sample, which was subsequently used to assess the various HKG transcript levels in gene-specific real-time PCR assays.

Real-time PCR

The gene expression stability under the different experiments of the stress adaptation models focused on five HKGs. These target genes and their primer sets are shown in Table 2. The real-time PCR reactions were performed in the Light Cycler 480 instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland) in a total reaction volume of 20 μL. This reaction contained 5 ng cDNA template (5 μL of 1 : 15 dilution of the original cDNA pool), 0.5 μM of each primer and 1 × LightCycler^{R} 480 SYBR Green I master mix (Roche Molecular Diagnostics, Penzburg, Germany). The real-time PCR run protocol consisted of (1) preincubation (4 min at 95 °C); (2) amplification (10 s at 95 °C; 20 s at 56 °C; 20 s at 72 °C; 5 s at 80 °C with a single fluorescent measurement); (3) melting curve (65–97 °C at 2.2 °Cs^{-1} and a continuous fluorescent measurement); and (4) cooling. Amplification was monitored in the appropriate LC 480 channel and specific amplification confirmed by single peaks in melting curve analysis. Genomic DNA-based standard curves were used to determine the efficiencies of the HKG target amplification by real-time PCR. The reactions for each target gene were performed in duplicate per sample and the results here reflect the means. Data on the expression level of the HKGs were obtained in the form of crossing points (CP) values based on the 'second derivative maximum' method as

Table 2. Details of primers and amplicons for each of the five evaluated candidate genes of *Listeria monocytogenes*

Gene	Forward primer	Reverse primer	Amplicon size	E (%)	Reference
16S rRNA	TTAGCTAGTTGGTAGGGT	AATCCGGACAACGCTTGC	550	92.5	Fraser *et al*. (2003)
rpoB	AATCGGGGACAATGACT	GTGTGCGGAAACCTAC	362	95.5	This study
rplD	GTATTCGGCCCAACAC	AGCACCTCCTCTACTT	353	100	This study
gap	ACCAGTGTAAGCGTGAA	TCACAGCGCAAGACAAA	248	93.5	This study
bglA	GCCTACTTTTTATGGGGTGGAG	CGATTAAATACGGTGCGGACATA	417	97.5	Salcedo *et al*. (2003)

computed by the LIGHT CYCLER 480 software (Roche Molecular Diagnostics). Further data analysis was performed with the CP raw data using the Excel based application BESTKEEPER tool programme (Pfaffl *et al.*, 2004).

Results and discussion

To identify HKG candidates suitable for normalization in qRT-PCR-based gene expression analysis in food-related stress adaptation models, the expression stability of five HKG targets in 16 *L. monocytogenes* strains was investigated. The genes selected consisted of some genes previously used as references in relative qRT-PCR studies (*rpoB* and *gap*), as well as some genes with a presumed HKG function (*rplD* and *bglA*) in this organism (Salcedo *et al.*, 2003; Sue *et al.*, 2004; Wemekamp-Kamphuis *et al.*, 2004a; Schwab *et al.*, 2005; Kazmierczak *et al.*, 2006). Initially, the RNA template isolation protocol and real-time RT-PCR assays were optimized. A total RNA isolation protocol that combined rapid mechanical lysis of *L. monocytogenes* cells and column-based purification yielding nondegraded DNA free RNA templates was developed. The optimization of real-time PCR assays included determination of amplification specificity, appropriate primer concentration and amplification conditions. Single product bands in agarose gels and single peaks in melting curve analysis confirmed the amplification specificity of the selected primer sets. The PCR efficiencies and linearity of the HKG-specific real-time PCR assays were defined using standard curves based on *L. monocytogenes* genomic DNA samples. The HKG target gene amplifications had PCR efficiencies ranging from 92.5% to 100% (see Table 2). The expression stability of the HKGs was first analyzed across the 16 different strains of *L. monocytogenes*. These strains were from different origins grouping into

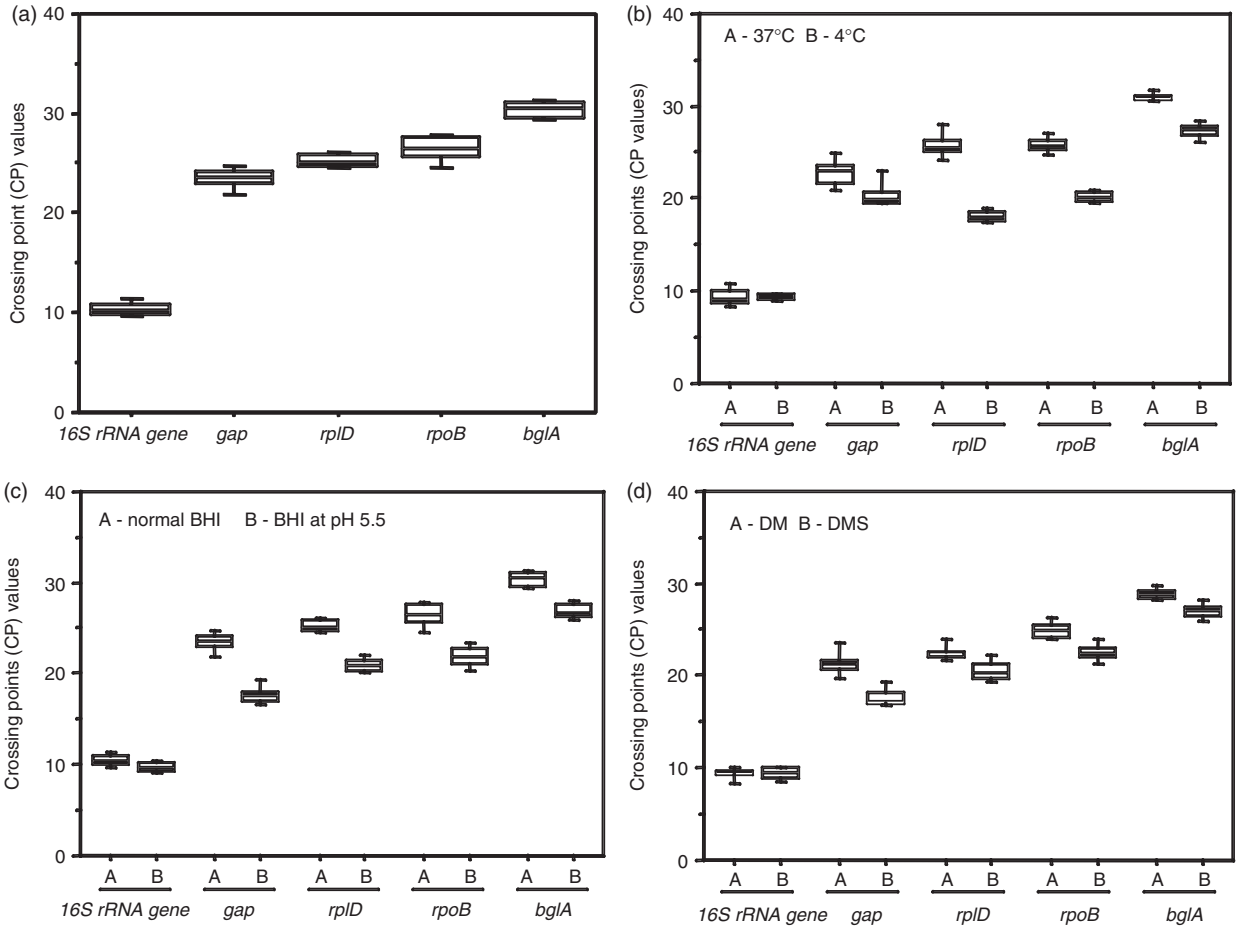

Fig. 1. Box plot overview of CP values from stationary phase cells of 16 *Listeria monocytogenes* strains (a) grown at 37 °C; (b) adapted to temperature conditions of 37 and 4 °C respectively; (c) adapted to normal BHI and acidified BHI (pH 5.5) conditions; d) adapted to DM and DMS (5 DM plus 3% NaCl) conditions. Three hundred nanograms total RNA from cells of each strain adapted to appropriate experimental conditions were converted into a cDNA pool by reverse transcription. Five nanograms aliquots from these cDNA pools were applied as templates in gene specific real-time PCRs targeting the *16S rRNA*, *rpoB*, *bglA*, *rplD* and *gap* genes to determine their CP values. The box plots highlight the median, 25th and 75th percentiles and expression range across the strain collection.

genetic lineages I and II, and into serotypes 1/2a, 1/2b, 1/2c and 4b. An overview of the individual HKG expression levels across the 16 strains based on the CP is shown as box plots in Fig. 1a. The medians, 25th and 75th percentiles, as well as ranges in HKGs expression across the strains are shown. The five HKGs displayed a relatively wide range in their expression levels. As can be expected, the highly expressed transcripts were encoded by the 16S rRNA gene. The lowest expressed transcripts were encoded by the *bglA* gene. The CP data obtained were further analyzed using BESTKEEPER tool programme. This is an excel-based software that determines 'optimal' housekeeping reference genes. Firstly, the software computes the overall candidate gene expression stability by calculation of CP data variation based on calculated means, CP data ranges, SD and coefficient of variation (CV). Table 3A presents an overview of expression variation of the five candidate genes derived from the CP data obtained across the 16 strains. The evaluation of these data shows that the highest interstrain expression stability (SD $< \pm 1$ CP and CV $< 2.5\%$ CP) is for the expressions of *bglA* and *rplD* genes. More importantly, however, these data shows that there are generally low levels of interstrain variation associated with the expression of all the five candidate genes supported by the low SD (0.54 CP $<$ SD < 1.02 CP) and CV (2.3% CP $<$ CV $< 5.19\%$) values. Therefore, none of the tested HKGs can be considered as displaying inconsistent expression among the different *L. monocytogenes* strains. Secondly, the BESTKEEPER software performs comparative analysis based on numerous pair-wise correlation analyses of all the candidate gene expression data against each other. The highly correlated candidate genes are then computed into the *BestKeeper* index. The relationship between the *BestKeeper* index and the contributing candidate HKGs is further defined by pair-wise correlation analysis. A comparative evaluation of the five candidate genes by pair-wise correlation to the *BestKeeper* index revealed strong correlations ($0.778 < r < 0.859$) between the five candidate genes and the index (Table 3B). The 16S rRNA gene was the gene displaying the lowest correlation coefficient to the *BestKeeper* index as a consequence of its relatively high expression levels compared with the other four targets. Therefore, based on the observations on the expression data of the five HKGs presented so far, it was determined that all the five tested genes displayed relatively stable interstain expression and therefore deemed to qualify as potential reference gene candidates. An additional important reference gene criterion is that its expression must display consistency or minimal variation under the different experimental conditions where quantitative gene expression analysis is to be performed. Therefore, this phenomenon was investigated in the next step by examining the HKGs expression stability after exposure to the experimental conditions of three models of food-related stress adaptation. In the first instance, the HKG expression stability was monitored in the context of a cold stress adaptation model. This experimental model is designed to investigate gene expression changes associated with *L. monocytogenes* cold stress adaptation. A comparative quantitative analysis of gene expression between cells adapted to optimal (37 °C) and cold stress (4 °C) temperature conditions would be performed using the qRT-PCR approaches. Therefore, the expression of the five HKGs was monitored in stationary-phase cells of the 16 strains preadapted to 37 and 4 °C, respectively. An overview of individual HKG expressions across the 16 strains adapted to these temperature conditions is shown in Fig. 1b. In general, the expression of all the

Table 3A. Results from BESTKEEPER descriptive statistical analysis showing variation in the CP values*

Factor	*16S rRNA gene*	*gap*	*rplD*	*rpoB*	*bglA*
N	16	16	16	16	16
GM [CP]	10.36	23.59	25.27	26.31	30.43
AM [CP]	10.38	23.63	25.27	26.34	30.44
Min [CP]	9.16	21.13	23.94	24.00	28.43
Max [CP]	11.41	26.96	26.40	28.09	31.45
SD [$\pm$CP]	0.54	0.91	0.59	1.02	0.70
CV [%CP]	5.19	3.86	2.34	3.86	2.31

*HKG expression assessed on total RNA templates isolated from stationary-phase cultures of 16 *Listeria monocytogenes* strains grown at 37 °C.
N, number of samples; GM [CP], the geometric mean of CP; AM [CP], the arithmetic mean of CP; Min [CP] and max [CP], the extreme values of CP; SD [$\pm$CP], the SD of the CP; CV [%CP], the coefficient of variance expressed as a percentage on the CP level.

Table 3B. Results from *BestKeeper* correlation analysis*

BestKeeper vs.	*16S rRNA gene*	*gap*	*rplD*	*rpoB*	*bglA*
Coefficient of corr. [r]:	0.859	0.778	0.794	0.923	0.791
P-value	0.001	0.001	0.001	0.001	0.001

*Measures of correlation between each candidate gene expression and the *BestKeeper* index computed from the best candidate genes.

Table 4. Results from BESTKEEPER descriptive statistical analysis showing variation in the CP values obtained across the cold stress adaptation model*

Factor	*16S rRNA gene*	*gap*	*rplD*	*rpoB*	*bglA*
N	32	32	32	32	32
GM [CP]	9.35	21.37	21.53	22.81	29.12
AM [CP]	9.39	21.45	21.88	22.99	29.19
Min [CP]	8.20	17.89	17.25	19.11	25.89
Max [CP]	10.91	26.15	28.31	27.21	31.71
SD [± CP]	0.53	1.58	3.81	2.78	1.83
CV [%CP]	5.61	7.35	17.41	12.10	6.26

*HKG expressions were assessed on total RNA templates isolated from stationary-phase cultures of 16 *Listeria monocytogenes* strains adapted to temperatures of 37 and 4 °C.
N, number of samples; GM [CP], the geometric mean of CP; AM [CP], the arithmetic mean of CP; Min [CP] and max [CP], the extreme values of CP; SD [± CP], the SD of the CP; CV [%CP], the coefficient of variance expressed as a percentage on the CP level.

Table 5. Results from BESTKEEPER descriptive statistical analysis showing variation in the CP values across the acid stress adaptation model*

Factor	*16S rRNA gene*	*gap*	*rplD*	*rpoB*	*bglA*
N	32	32	32	32	32
GM [CP]	10.04	20.41	23.00	23.91	28.62
AM [CP]	10.06	20.66	23.11	24.05	28.69
Min [CP]	8.71	16.28	19.87	20.16	25.78
Max [CP]	11.41	26.96	26.40	28.09	31.45
SD [± CP]	0.55	2.97	2.17	2.291.02	1.77
CV [%CP]	5.48	14.36	9.38	9.54	6.17

*HKG expressions were assessed on total RNA templates isolated from stationary-phase cultures of 16 *Listeria monocytogenes* strains adapted to normal BHI and acidified BHI (pH 5.5).
N, number of samples; GM [CP]: the geometric mean of CP; AM [CP], the arithmetic mean of CP; Min [CP] and max [CP], the extreme values of CP; SD [± CP], the SD of the CP; CV [%CP], the coefficient of variance expressed as a percentage on the CP level.

Table 6. Results from BESTKEEPER descriptive statistical analysis showing variation in the CP values across the NaCl stress adaptation model*

Factor	*16S rRNA gene*	*gap*	*rplD*	*rpoB*	*bglA*
N	32	32	32	32	32
GM [CP]	9.38	19.41	21.38	23.76	27.95
AM [CP]	9.40	19.52	21.42	23.82	27.98
Min [CP]	8.10	16.61	19.35	20.67	25.71
Max [CP]	11.33	23.87	24.13	26.60	30.29
SD [± CP]	0.48	1.89	1.10	1.35	1.01
CV [%CP]	5.11	9.66	5.11	5.66	3.59

*HKG expressions were assessed on total RNA templates isolated from stationary-phase cultures of 16 *Listeria monocytogenes* strains adapted to DM and DMS (DM plus 3% NaCl) conditions.
N, number of samples; GM [CP], the geometric mean of CP; AM [CP], the arithmetic mean of CP; Min [CP] and max [CP], the extreme values of CP; SD [± CP], the SD of the CP; CV [%CP], the coefficient of variance expressed as a percentage on the CP level.

HKGs tested seems to be influenced to varying extents by the temperature conditions applied. However, there are considerably strong increases in *bglA*, *rplD* and *rpoB* expression at 4 °C compared with those at 37 °C. On the other hand, only modest increases seem to be induced in *gap* and 16S rRNA gene expressions under these conditions. These observations are also in agreement with HKG expression stability analysis based on inspection of the CP data variations based on all the CP values derived from the strain collection under the two temperature conditions using the BESTKEEPER program (Table 4). In fact, 16S rRNA gene expression appears to be the most stable with the lowest SD (± 0.51) and CV (5.6%) values. Thus, based on these assessments of HKG gene expression variation, the candidate stabilities are ranked in the order: *16S rRNA* > *gap* > *bglA* > *rpoB* > *rplD* in this cold stress adaptation model. Next, the expression stability of this HKG set was examined in an experimental model designed to investigate gene expression changes related to acid stress adaptation. The experimental conditions applied in this model are designed for the quantitative comparison of gene expression changes in *L. monocytogenes* cells adapted to acid stress in BHI at pH 5.5 and those adapted to regular

BHI medium conditions. Therefore, HKG expression was evaluated in stationary-phase cells of the 16 strains pre-adapted to normal BHI or BHI adjusted to pH 5.5 using lactic acid. The box plots in Fig. 1c highlight the variation in individual HKGs expressions, across the 16 strains under these two experimental conditions. Once again, the adaptation of *L. monocytogenes* cells to acid stress conditions also variably induces changes in the expression levels of *rpoB*, *rplD*, *gap* and *bglA*. Meanwhile, the influence of acid stress on 16S rRNA gene expression seems to be minimal (Fig. 1c). This was also confirmed upon inspection of HKG expression stability based on the variation computed from the CP raw data from of this experimental setup (Table 5). Once again, 16S rRNA gene transcripts display maximum expression stability in this setup [SD ($\pm$0.55) and CV (5.48%)], whilst *gap* expression is the least stable [SD ($\pm$2.97) and CV (14.36%)]. The expression stability analysis in this experimental model therefore ranks the HKGs in the order: *16S rRNA* > *bglA* > *rplD* > *rpoB* > *gap*. Finally, the HKG expression stability in the NaCl salt stress adaptation model was investigated. In this case, variation in HKGs transcript levels was assessed in stationary-phase cells adapted to DM in comparison with those adapted to NaCl stress in DM plus 3% NaCl (DMS; Fig. 1d). This experimental model also shows that the expression of all the HKGs is more stable but still slightly influenced during adaptation of *L. monocytogenes* cells to NaCl salt stress. Calculated variations of the CP raw data from this experimental setup are shown in Table 6. This shows that 16S rRNA gene [SD ($\pm$0.48) and CV (5.11%)], followed by *bglA* [SD ($\pm$1.01) and CV (5.11%)] are the most stably expressed genes, while once more the *gap* [SD ($\pm$1.89) and CV (9.66%)] transcripts are also the least stably expressed in this experimental setup. The overall HKG expression stability based on this analysis therefore ranks the HKGs in the order: *16S rRNA* > *bglA* > *rplD* > *rpoB* > *gap* in this stress adaptation model.

Conclusions

This study was performed to select optimal internal control reference genes for qRT-PCR assays designed to quantitatively assess gene expression changes in stress adaptation of *L. monocytogenes*. The data showed a high degree of homogeneity and expression stability of 16S rRNA gene transcripts in all the three experimental stress models tested. The optimal reference gene function of 16S rRNA gene can be attributed to its high levels of expression (low CP values). Therefore, quantitative differences in expression profiles tend to be leveled off by the high target copy numbers available for PCR amplification. The next most stable HKG is the *bglA* gene. It displays low interstrain variation and is the second gene that shows minimal experimental conditions-dependent fluctuation in the three stress models. Its rather low expression levels, however, mean that it might be more prone to variation arising from experiment to experiment fluctuation. Meanwhile, there were variable degrees of stability in *gap*, *rplD* and *rpoB* gene expressions in the stress adaptation models. Their use in such stress adaptation models should therefore be carefully evaluated as their own variation in expression might be high enough to mask or distort the sought gene expression changes. Therefore, based on the evaluations presented here, 16S rRNA gene is recommended as the optimal reference gene for relative qRT-PCR analysis of gene expression in the three stress adaptation models described.

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