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Bacterial reference genes for gene expression studies by RT-qPCR: survey and analysis

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Abstract The appropriate choice of reference genes is essential for accurate normalization of gene expression data obtained by the method of reverse transcription quantitative real-time PCR (RT-qPCR). In 2009, a guideline called the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) highlighted the importance of the selection and validation of more than one suitable reference gene for obtaining reliable RT-qPCR results. Herein, we searched the recent literature in order to identify the bacterial reference genes that have been most commonly validated in gene expression studies by RT-qPCR (in the first 5 years following publication of the MIQE guidelines). Through a combination of different search parameters with the text mining tool MedlineRanker, we identified 145 unique bacterial genes that were recently tested as candidate reference genes. Of these, 45 genes were experimentally validated and, in most of the cases, their expression stabilities were verified using the software tools geNorm and NormFinder. It is noteworthy that only 10 of these reference genes had been validated in two or more of the studies evaluated. An enrichment analysis using Gene Ontology classifications demonstrated that genes belonging to the functional categories of DNA Replication (GO: 0006260) and Transcription (GO: 0006351) rendered a proportionally higher number of validated reference genes. Three genes in the former functional class were also among the top five most stable genes identified through an analysis of gene expression data obtained from the Pathosystems Resource Integration Center. These results may provide a guideline for the initial selection of candidate reference genes for RT-qPCR studies in several different bacterial species.

Keywords RT-qPCR · Reference genes · Bacteria · Normalization · Gene expression · MIQE guidelines

Introduction

Relative quantification of mRNA expression by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) has become a method of choice for gene expression studies in various organisms (Thellin et al. 2009). For accurate quantification of gene expression using RT-qPCR, a series of experimental parameters must be carefully observed, including: (i) the determination of an appropriate number of biological replicates; (ii) the strict quality control in the steps of RNA sample extraction and reverse transcription; and (iii) the

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suitable selection and validation of reference genes that will be used as controls to normalize the data in relative quantification analysis (Taylor et al. 2010; Taylor and Mrkusich 2014).

In order to standardize the design of qPCR and RT-qPCR assays and to reduce the number of inconsistent results due to inappropriate experimental design, guidelines were proposed in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) in 2009 (Bustin et al. 2009). The current state of implementation of the MIQE standards has been recently reviewed (Bustin et al. 2013; Taylor and Mrkusich 2014). Specifically, the guidelines point out that the use of a single, unvalidated reference gene for normalization of RT-qPCR results is generally not acceptable (Kozera and Rapacz 2013). The most accurate study design would require between three and five good reference genes (Taylor et al. 2010). For this, it is recommended to test the stability of approximately 6–10 candidate reference genes in each of the experimental conditions evaluated, using appropriate software such as geNorm and NormFinder (Taylor and Mrkusich 2014).

In the present study, we searched the recent literature in order to identify the bacterial genes that have been most commonly selected and validated as reference genes in gene expression studies by RT-qPCR, during the first 5 years following publication of the MIQE guidelines. We were able to compile a list of genes that might serve as a guide for the initial reference gene selection to be used in gene expression studies in a variety of bacterial species. Importantly, appropriate validation of a set of candidate reference genes chosen in every new experimental condition tested is necessary to obtain consistent RT-qPCR results.

Materials and methods

Literature search and survey of validated bacterial reference genes

A literature search was performed in the NCBI's PubMed database for studies specifically aimed at selection and validation of bacterial reference genes for use in gene expression analyses by the RT-qPCR methodology, during the first 5 years after publication of the MIQE guidelines (2010–2014). Initially, a combination of various search terms using the Boolean operator 'AND' rendered 52 different search parameters that were then used in the 'Advanced Search' functionality of PubMed (Table S1). The search terms used were: (i) reference genes; (ii) internal controls; (iii) normalization; (iv) normalizers; (v) qPCR; (vi) realtime PCR; (vii) real-time PCR; (viii) RT-qPCR; (ix) bacteria; and (x) bacterium.

All the publications retrieved in the initial searches were manually inspected for specificity (*i.e.,* studies dedicated to validate bacterial reference genes) and different combinations of the most specific abstracts were used to compose a training set for the text mining tool MedlineRanker (Fontaine et al. 2009). This tool allows for ranking of the literature available in Medline according to the relevance related to a given topic (Fontaine et al. 2009). The publication period analyzed ranged from 2009 to 2014. Citation records for each of the identified articles were retrieved from Google Scholar (scholar.google.com).

The most relevant results obtained from the two search strategies were combined, and a list of validated bacterial reference genes was then obtained from the final set of articles selected (Table S2). The following information was retrieved for each one of the identified genes: (i) gene name, description and identifier; (ii) bacterial species; and (iii) experimental strategy and software used for reference gene validation.

Functional annotation and enrichment analysis using Gene Ontology (GO)

The GO Enrichment Analysis tool (The Gene Ontology Consortium 2015) was used to retrieve functional annotation and enrichment analysis for all the candidate bacterial reference genes identified previously in the text mining approach. A list containing all the identified gene names and/or identifiers was used as query to obtain functional classifications of the PANTHER (protein annotation through evolutionary relationship) classification system (Mi et al. 2013), using *Escherichia coli* as the reference organism. GO biological process terms for each gene, as well as the frequency of each functional annotation in the input list, were obtained by this analysis.

Analysis of gene expression profiles of the identified bacterial reference genes

The expression profiles of several bacterial genes commonly selected as candidate reference genes in the

RT-qPCR studies were evaluated across nearly 1000 different experiments, with eight different bacterial species, using the Gene Page Transcriptomics functionality of the Pathosystems Resource Integration Center (PATRIC) (www.patricbrc.org) (Wattam et al. 2014). The PATRIC database contains transcriptomic datasets for more than 65 bacterial genera, obtained from high-throughput studies of gene expression using either microarrays or RNA-seq. The expression profile of a given gene, over various experimental comparisons, was normalized and Log2-transformed allowing for comparison between experiments (Wattam et al. 2014). Expression analysis of the 18 selected genes was done for representatives of seven different bacterial phyla: Actinobacteria; Betaproteobacteria; Gammaproteobacteria; Epsilonproteobacteria; Chlamydiae; and Firmicutes. An alteration in gene expression for a specific gene was considered significant for any variation higher than 2-fold. The percentage of experiments in which the expression of the candidate reference genes did not vary significantly was calculated. All experiments that involved mutant strains were excluded from this analysis. Correlation analysis was performed using GraphPad Prism (GraphPad Software, Inc.).

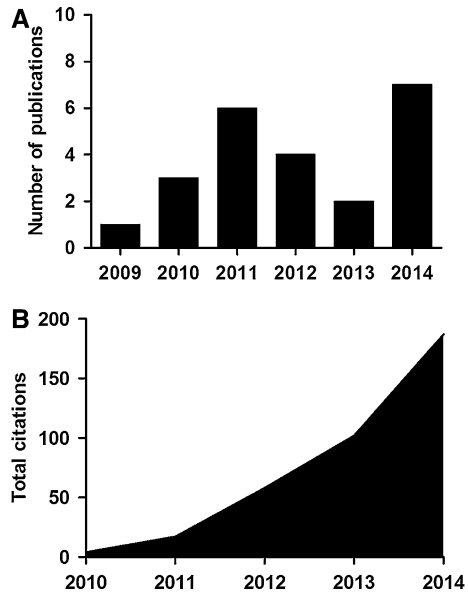

Fig. 1 Distribution of studies aimed at selecting and validating bacterial reference genes for normalization of RT-qPCR results, published during the 5 years of the MIQE guidelines. The most specific studies were identified through a combined search strategy, using different search terms and the text mining tool MedLineRanker. **a** Distribution of studies identified per year. A single study was identified in 2009 when the MIQE standards were first published. **b** Cumulative citations to the 23 specific studies identified by Google Scholar searches

Results and discussion

Publication distribution and validation strategies

We used a combined strategy to search the PubMed database for recent literature on the selection and validation of suitable reference genes for gene expression studies in bacteria. Firstly, the 52 different search parameters presented in supplementary Table S1 were employed in the advanced search extension of PubMed. Then, a collection of the most specific abstracts identified in these searches was used as query in the text mining tool MedlineRanker. Following manual inspection to remove unspecific results, it was possible to retrieve 23 publications that were specifically dedicated to identification of appropriate bacterial reference genes to be used as controls to normalize the data in RT-qPCR studies (in the first five-years after publication of the MIQE guidelines) (Table S2) (Fig. 1a). A single study was identified in 2009, the year of publication of the MIQE standards, and was therefore included in the analysis (Fig. 1a; Table S2).

We observed an increasing number of citations to these studies over the recent years (Fig. 1b), which might be indicative of a growing interest of the scientific community working with bacterial gene expression analysis by RT-qPCR in using validated reference genes, as suggested by the MIQE criteria. Specifically, the guidelines recommend that normalization of RT-qPCR results should always be performed with more than one validated reference gene (Bustin et al. 2009; Kozera and Rapacz 2013). For suitable selection and validation of these genes intended to be used as normalizers, it is appropriate to identify between six and ten candidate reference genes. Their stabilities should be then evaluated in RT-qPCR reactions using dedicated software tools such as geNorm, NormFinder and BestKeeper (Taylor et al. 2010; Kozera and Rapacz 2013; Taylor and Mrkusich 2014).

Figure 2 shows that most of the evaluated studies comply with these recommendations. The great majority of the studies tested a minimum of six candidate reference genes (median = 10; range: 5–22

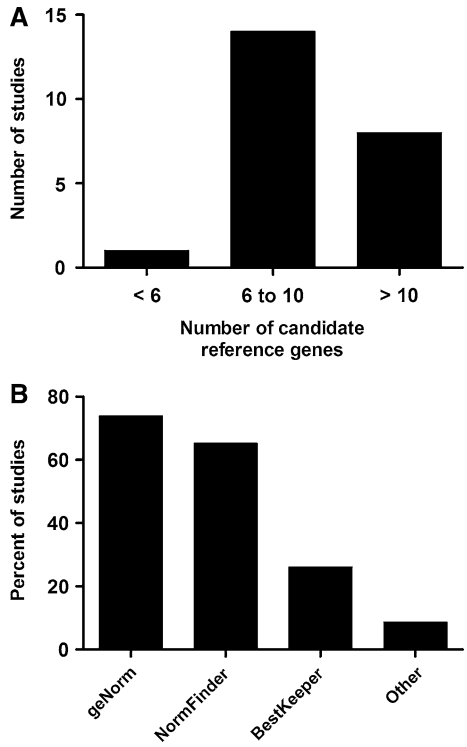

Fig. 2 Compliance of the identified studies with the MIQE guidelines recommendations for the selection and validation of reference genes. **a** Numbers of candidate bacterial reference genes tested in each study. **b** Statistical strategies used in the various studies for analyzing the stabilities of expression of the candidate reference genes

genes) (Fig. 2a). Eighteen out of the twenty-three studies analyzed used a combination of algorithms for evaluating gene expression stability, with nine studies using a combination of the geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) software (Fig. 2b). These same programs were used in the remaining studies in combination with BestKeeper (Pfaffl et al. 2004) or using another specific statistical approach. Overall, nearly 74 % of the studies made use of geNorm for determining gene expression stability and an approximate 65 % used NormFinder (Fig. 2b).

List of validated bacterial reference genes and functional annotations

In total, 145 unique bacterial genes, belonging to diverse functional classes, have been tested as candidate reference genes in the studies evaluated (Table S3). Of these, 45 genes have been experimentally validated (these genes were analyzed by RT-qPCR reactions in the various studies and were demonstrated to be the most stably expressed in all the conditions tested) (Table S3). For a good review on primer validation and reference gene validation, please refer to Taylor and Mrkusich (2014). Noteworthy, only 18 candidate reference genes have been evaluated in more than three studies and, among these, only 10 have been validated in two or more studies (Table 1).

The *16S rRNA* gene, encoding the 16S ribosomal RNA (rRNA), was by far the most tested candidate reference gene in the studies analyzed. However, the proportion of studies in which it was experimentally validated as a suitable reference gene for normalization of RT-qPCR was rather low (only 4 out of 17 studies) (Table 1). The reasons why this gene was not validated in the majority of the studies include: (i) its low stability in several of the experimental conditions evaluated (Stenico et al. 2014; Carvalho et al. 2014); and (ii) the very high expression rate, what makes this gene unreliable for normalization of the expression levels of very low abundant transcripts (Kozera and Rapacz 2013).

An enrichment analysis using Gene Ontology biological process classifications showed that, among the 145 candidate bacterial reference genes identified, the most commonly found functional annotations were RNA Metabolic Process (GO: 0016700) and Translation (GO: 0006412) (Fig. 3). When only the genes that have been experimentally validated by the studies evaluated are analyzed, these same functional classes persist (Fig. 3). Nevertheless, if we now consider the proportion of genes within each functional class that had been validated, then the classes DNA Replication (GO: 0006260) and Transcription (GO: 0006351) will stand out (Fig. 3). This might be due to a consistently higher stability of expression of the genes belonging to these functional categories. However, this may also reflect the higher number of studies that have tested these genes as candidate reference genes (Table 1).

Expression profiles of bacterial reference genes

We selected a list of bacterial reference genes belonging to eight different functional classes

Table 1 The most commonly tested and validated bacterial reference genes identified by this study

Gene name[a]	Gene description[b]	Experimental validation by RT-qPCR		References (PMID)[c]
		No. studies (tested)	No. studies (validated)	
16S rRNA	16S ribosomal RNA	17	4	21047531; 22634000; 20599622; 23651807; 19555508; 21513543; 21104423; 22496882; 22003014; 23452832; 24389585; 24657392; 21318633; 20619305; 24841066; 24893820; 25115691
gyrA	DNA gyrase A	11	6	22634000; 20599622; 23651807; 19555508; 23053071; 23452832; 25017489; 24389585; 24657392; 21318633; 24841066
recA	Recombinase A	10	3	22814372; 22634000; 23651807; 19555508; 23053071; 21104423; 24657392; 24037409; 24841066; 24893820
rpoB	DNA-directed RNA polymerase, beta subunit	9	2	21047531; 22634000; 23053071; 23452832; 25017489; 21318633; 24037409; 24893820; 24657392
rpoA	DNA-directed RNA polymerase, alpha subunit	7	2	22814372; 21047531; 20599622; 23651807; 23053071; 22496882; 24037409
gyrB	DNA gyrase B	6	3	22814372; 23053071; 21104423; 25017489; 21318633; 24893820
gap	Glyceraldehyde-3-phosphate dehydrogenase	5	4	24657392; 23053071; 24841066; 21104423; 24037409
rho	Rho termination factor	5	3	22814372; 21047531; 20599622; 23651807; 24893820
ftsZ	Cell division protein FtsZ	3	2	23053071; 23452832; 24893820
secA	Protein translocase subunit SecA	2	2	22496882; 25115691
rpoC	DNA-directed RNA polymerase, beta prime subunit	5	1	19555508; 25017489; 24389585; 21318633; 22814372
gmk	Guanylate kinase	4	1	19555508; 23053071; 22814372; 21047531
adk	Adenylate kinase	3	1	21620905; 20599622; 24389585
rpoD	RNA polymerase sigma factor	3	1	24037409; 24841066; 22814372
dnaG	DNA primase	3	1	25017489; 22814372; 21047531
glnA	Glutamine synthetase	3	0	24037409; 22634000; 24657392
ldhD	D-Lactate dehydrogenase	3	0	22814372; 23053071; 21104423
recF	DNA replication and repair protein	3	0	24841066; 22814372; 21047531

All the genes that have been validated in two or more studies are marked bold

[a] Gene symbols according to NCBI's Gene Database

[b] According to UniProt Database

[c] PubMed IDs: 22814372 (Galisa et al. 2012); 21047531 (Thomas et al. 2011); 21620905 (Reiter et al. 2011); 22634000 (Florindo et al. 2012); 20599622 (Metcalf et al. 2010); 23651807 (Liu et al. 2013); 19555508 (Nieto et al. 2009); 21513543 (Zhou et al. 2011); 23053071 (Sumby et al. 2012); 21104423 (Zhao et al. 2011); 21637857 (Hommais et al. 2011); 22496882 (Pinto et al. 2012); 22003014 (Turroni et al. 2011); 23452832 (Brudal et al. 2013); 25017489 (Carvalho et al. 2014); 24389585 (Kirk et al. 2014); 24657392 (Stenico et al. 2014); 21318633 (Jacob et al. 2011); 20619305 (Borges et al. 2010); 24037409 (Løvdal and Saha 2014); 24841066 (McMillan and Pereg 2014); 24893820 (Sihto et al. 2014); 25115691 (Szekeres et al. 2014)

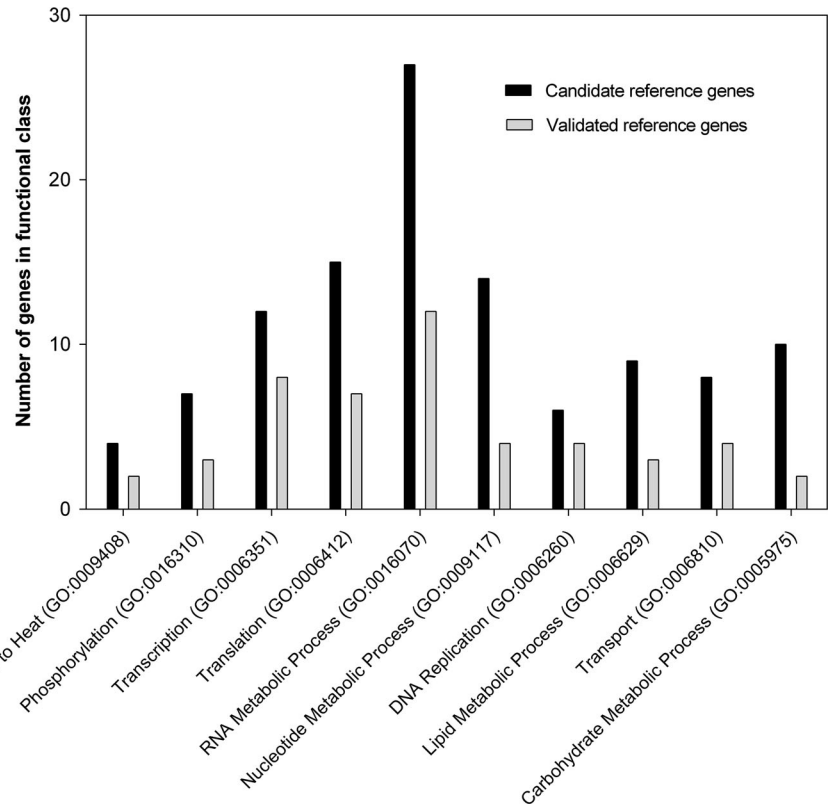

Fig. 3 Functional enrichment analysis of the identified bacterial reference genes using Gene Ontology biological process classifications. All of the candidate bacterial reference genes that were tested and validated in the studies evaluated were subjected to functional enrichment analysis using the GO Enrichment Analysis tool (http://geneontology.org/page/go-enrichment-analysis)

(primarily the categories that showed the highest proportions of validated reference genes). Next, we conducted an analysis of their expression levels over hundreds of different experimental conditions by using the PATRIC platform (please refer to item 2.3). Basically, normalized expression data was retrieved for all the genes listed in Table 2 in various experimental conditions with eight different bacterial species (*Bacillus subtilis*, *Burkholderia pseudomallei*, *Campylobacter jejuni*, *Chlamydophila pneumoniae*, *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Salmonella enterica* var. Typhimurium and *Staphylococcus aureus*). Then, we calculated for each species the proportion of experiments in which a given gene did not exhibit a significantly altered expression level, (*i.e.,* the expression variation was below a 2-fold cut-off in every experimental comparison that included this gene). The results were expressed as the percentage of experiments in which the specific gene did not show a significant alteration in expression (Table 2).

The bacterial genes *gyrA* and *gyrB*, coding for subunits of the enzyme DNA gyrase, showed an invariable expression pattern in the highest proportion of the studies evaluated (Table 2). On the other hand, the gene encoding the 60 kDa heat shock protein (*hsp60*) showed the most variable expression among the genes evaluated, even though it had been selected in some studies as a candidate bacterial reference gene (Table 2).

We observed a moderate positive correlation between the expression stability data retrieved from the analyses using PATRIC and the proportion of studies that experimentally validated a given bacterial reference gene (Fig. 4a). Nonetheless, there was no significant correlation between the observed stability of a specific gene and the number of studies that selected this gene as a candidate reference gene through a literature search-based approach (Fig. 4b). These results indicate that selection of the candidate reference genes based on their perceived stabilities in previous studies (as shown in Table 2) might be a useful strategy for increasing the chances of validating a specific reference gene for a given bacterial species.

Table 2 Meta-analysis of gene expression stability using the PATRIC database

Gene name	Functional category[a]	Expression stability analysis in the PATRIC database	
		No. experiments evaluated	Proportions of studies with invariable expression (weighted average)
gyrB	DNA replication/DNA metabolic process	1009	95.83
gyrA	DNA replication/DNA metabolic process	1010	94.14
era	Translation	968	93.25
secA	Transport	1006	93.14
dnaG	DNA replication/DNA metabolic process	1000	92.50
adk	Phosphorylation/Nucleotide metabolism	1012	90.41
gmk	Phosphorylation/Nucleotide metabolism	1000	90.30
ftsZ	Cell division	970	88.96
recA	DNA replication/DNA metabolic process	1013	86.27
rpoB	Transcription	1000	86.09
gap	Energy metabolism	970	85.56
rpoC	Transcription	886	84.76
rpoD	Transcription	1051	84.29
rho	Transcription	1037	82.59
tuf	Translation	1004	81.77
fusA	Translation	969	81.01
rpoA	Transcription	971	79.09
hsp60	Response to stress	1006	68.09

[a] This analysis included bacterial genes in the most commonly tested and/or validated functional categories

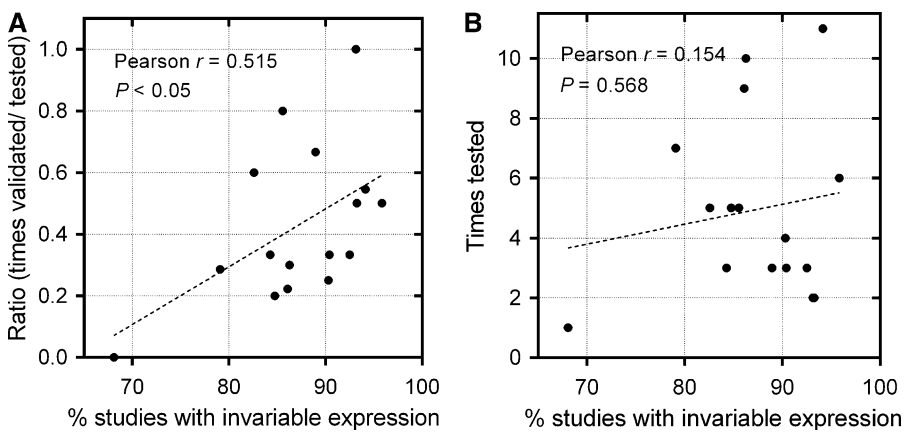

Fig. 4 Correlation analysis between the gene expression stabilities retrieved from PATRIC and the success rate of validation of specific bacterial reference genes. The expression profiles of several candidate bacterial reference genes throughout many different experiments were retrieved from PATRIC. The proportion of experiments in which a specific gene did not show an altered expression pattern was calculated for each bacterial species analyzed and then a weighted average was obtained, taking into account the different numbers of experiments identified for each species. **a** Correlation analysis between the proportion of studies with invariable expression and the success rate of validation (ratio: times validated/times tested) of each bacterial reference gene. **b** Correlation analysis between the proportion of studies with invariable expression and the number of studies that tested a specific candidate bacterial reference gene. Genes that were tested in a single study were excluded from these analyses

Conclusions

We identified many different bacterial genes that have been tested as candidate reference genes for normalization of RT-qPCR studies, in the first 5 years following publication of the MIQE guidelines. This demonstrates an increased interest of the community working with gene expression studies in various bacterial species in the adoption of MIQE criteria for selection and validation of reference genes. However, the literature-driven selection of candidate housekeeping genes has led to a high proportion of the genes not being experimentally validated, mostly due to unstable expression. Herein, we observed that genes belonging to some specific functional categories, such as DNA replication, have been validated in a higher proportion of studies than the most commonly used reference gene, which encodes the 16S ribosomal RNA. We anticipate that the list of genes evaluated in this study may provide a guideline for the initial selection of candidate reference genes for RT-qPCR studies in several different bacterial species. It is noteworthy that the stability of expression of each newly selected gene must be evaluated in all novel experimental conditions tested, according to the recommendations of the MIQE standards.

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