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Research paper

Identification and validation of reliable *Aeromonas salmonicida* subspecies *salmonicida* reference genes for differential gene expression analyses

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ABSTRACT

Aeromonas salmonicida subsp. *salmonicida* is a Gram-negative, facultative intracellular pathogen of a wide range of freshwater and marine fish species. *A. salmonicida* is the causative agent of furunculosis, an immunosuppressive disease that typically progresses to septicemia. Several aspects of *A. salmonicida* pathogenesis has already been described, but fundamental genetic aspects of the psychrophilic lifestyle of this bacterium remain unknown.

Reverse transcription quantitative real-time polymerase chain reaction (qPCR) is a precise molecular technique used to detect very slight changes in gene expression. The appropriate choice of reference genes is essential for accurate normalization of qPCR gene expression data. Despite the available abundance of validated reference genes for mesophilic pathogens, a broad list of validated reference genes for *A. salmonicida* is not available. Here, we evaluated seven *A. salmonicida* reference genes under different culture conditions, including different growth phases, iron-limited and iron-supplemented conditions, and thermal stress. We determined that *hfq* maintained the most stable expression, followed by *era, recA, rpoB, 16S, fabD*, and *gapA*. The results of this study provided with an expanded list of reliable reference genes for *A. salmonicida* gene expression studies using qPCR.

1. Introduction

Aeromonas salmonicida subsp. *salmonicida* (hereafter *A. salmonicida*), the causative agent of furunculosis, is one of the oldest known bacterial pathogens of fish (Cipriano and Bullock, 2001). *A. salmonicida* is a Gram-negative, psychrotrophic, non-motile, and facultative intracellular pathogen, which infects both marine and freshwater fish (Dallaire-Dufresne et al., 2014). *A. salmonicida* has a broad host range, affecting salmonid species (Austin and Austin, 2007), and a variety of non-salmonid fish, including sablefish (*Anoplopoma fimbria*), lumpfish (*Cyclopterus lumpus*), cunner (*Tautogolabrus adspersus*), turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*), Atlantic cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*), lamprey (*Petromyzon marinus*), carp (*Cyprinus carpio*), and European eel (*Anguilla anguilla*) (Beaz-Hidalgo and Figueras, 2013; Dallaire-Dufresne et al., 2014:, Lafferty et al., 2015; Powell et al., 2017). Several aspects of the pathogenesis of *A. salmonicida* has already been described (Dallaire-Dufresne et al., 2014), but fundamental transcriptional regulatory aspects of the psychrotrophic nature of this bacterium remain unknown.

Reverse transcription quantitative real-time polymerase chain reaction (qPCR) is a standard method utilized for gene expression evaluation (Thellin et al., 2009; Flores-Herrera et al., 2018). The selection of appropriate reference genes is an essential prerequisite to differentiate between basal levels of gene transcription and experimentally induced expression during qPCR experiments (Rocha et al., 2015). Reference genes are described as single copy genes whose expression remains unchanged under different physiological conditions (Rocha et al., 2015; Taylor and Mrkusich, 2014). According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, an accurate qPCR gene expression study requires two to five validated reference genes to ensure explicit reliability and reproducibility of experimental results (Bustin et al., 2009; Bustin et al., 2010).

The most commonly validated bacterial reference genes in mesophilic bacteria, including *gyrA*, *gyrB*, *gapA*, *recA*, *fabD*, *rpoA*, *rpoB*, and *16S* (Rocha et al., 2015), are not considered ideal universal reference genes (Savli et al., 2003; Nieto et al., 2009; Galisa et al., 2012; Sumby et al., 2012). *A. salmonicida gyrB*, *proC, rpoC*, *rpoD,* and *fabD* reference

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Table 1
Description of the selected reference genes.

Gene	Encoded protein	Function	Gene location	GenBank accession ID
gapA	Glyceraldehyde-3-phosphate dehydrogenase A	Glycolysis pathway	973,560–974,993	ABO89078.1
era	GTPase-binding protein	GTPase	3,777,317–3,778,216	ABO91453.1
16S	16S Ribosomal RNA	Ribosomal structure and function	4,537,410–4,538,946	CP000644.1
recA	Recombinase A	DNA repair	4,100,706–4,101,767	ABO91769.1
fabD	Malonyl CoA-acyl carrier protein transacylase	Fatty acid biosynthesis	2,183,301–2,184,230	ABO90122.1
rpoB	RNA polymerase sigma factor B	β-subunit of bacterial RNA polymerase	296,661–300,800	ABO88471.1
hfq	RNA-binding protein	sRNA chaperone	3,643,806–3,644,069	ABO91347.1

genes have been evaluated during a single *in vitro* growth condition (Rivera et al., 2015), and as mentioned previously, a broad list of validated reference genes for *A. salmonicida* is not available. Therefore, in this study, we evaluated *gapA*, *era*, *recA*, *rpoB*, *hfq*, *16S* (*rrn*), and *fabD* reference genes in *A. salmonicida* J223. *A. salmonicida* J223 was isolated from an outbreak in Atlantic salmon (*Salmo salar*) during 1999 and recently fully sequenced (Valderrama et al., 2017a). We have shown that *A. salmonicida* J223 can infect and kill rainbow trout (*Oncorhynchus mykiss*) and lumpfish (Valderrama et al., 2017a; Chakraborty et al., 2018). Determining stable reference genes for *A salmonicida* J223 under different conditions represents an important step to study fundamental transcriptional regulatory aspects of this psychrotrophic pathogen.

A. salmonicida exhibits optimal growth in complex media in aerobic conditions, between 15 and 18 °C, and reaches stationary growth in approximately 24 h (Cipriano and Bullock, 2001; Valderrama et al., 2017a). The late stationary growth phase of *A. salmonicida* is characterized by the production of an opaque brown pigment (Cipriano and Bullock, 2001). In this study, we evaluated the transcription of the selected candidate reference genes under mid-logarithmic, late-logarithmic, and stationary growth phases.

Elemental iron is an essential requirement for growth and cellular homeostasis in bacteria and eukaryotic organisms (Cassat and Skaar, 2013). Vertebrates sequester iron from invading pathogens as a means of nutritional immunity, using high-affinity iron-binding proteins to limit the levels of free-iron present in host biological fluids and tissues (Santander et al., 2012). In animal hosts, iron is typically present in transferrin or lactoferrin complexes, which are iron-binding glycoproteins that circulate through the bloodstream (Chart and Trust, 1983). Invading bacterial pathogens can sense this iron-depletion as a signal that they have successfully entered a host, and subsequently induce the expression of genes that facilitate iron uptake as a means to overcome host defenses (Chart and Trust, 1983; Ebanks et al., 2004; Guan et al., 2013; Hirst et al., 1991; Najimi et al., 2009; Santander, 2012). Therefore, the transcriptional stability of each reference genes was evaluated under iron-limiting and iron-rich conditions.

It has also been shown that *A. salmonicida* under temperatures over 24–26 °C induces the selection of sub-populations lacking virulence factors (Ishiguro et al., 1981; Daher et al., 2011). Thus, in this study, the expression of selected *A. salmonicida* reference genes was also evaluated under thermal stress conditions.

Here, we evaluated the transcriptional expression of *gapA*, *hfq*, *era*, *16S*, *recA*, *fabD*, and *rpoB,* in *A. salmonicida* under different growth conditions, including mid-logarithmic, late-logarithmic, and stationary growth phases, iron-limited and iron-supplemented conditions, and thermal stress. We determined that *hfq* was the most stable expressed gene across the different tested conditions, followed by *recA, gapA, era, 16S, fabD* and *rpoB* genes.

2. Material and methods

2.1. Bacterial strain, media, reagents, and culture conditions

A. salmonicida J223 (NZ_LSGV00000000) (Valderrama et al., 2017a) was utilized in this study. Bacteriological media and components were from Difco (Franklin Lakes, NJ). Tryptic Soy Broth (TSB) complex media was used for routine bacterial growth. The culture media was supplemented with 1.5% agar when required. *A. salmonicida* was incubated at 15 °C aerobically (180 rpm) until mid-logarithmic (O.D. 600 nm ~0.7), late-logarithmic (O.D. 600 nm ~1.5), and stationary growth phase (O.D. 600 nm ~2.5) (Leboffe and Pierce, 2015).

When required, *A. salmonicida* J223 was grown at 28 °C, under iron-limited conditions (TSB supplemented with 100 μM of 2,2′-dipyridyl (Sigma)), or iron-rich conditions (TSB supplemented with 100 μM of $FeCl_3$ (Sigma)) until mid-logarithmic phase. Bacterial growth was monitored spectrophotometrically and/or by plating (Valderrama et al., 2017a). Bacterial cell shape and Gram stain was observed through light microscopy (Olympus CX21, New York, USA), under X1000 magnification. Gram stain was performed according to standard procedures (Leboffe and Pierce, 2015).

2.2. Reference gene selection and primer design

The selection of candidates reference gene (Table 1), from the annotated *A. salmonicida* genome J223 (Valderrama et al., 2017a), was made according to the MIQE guidelines (Bustin et al., 2009; Bustin et al., 2010; Bustin and Huggett, 2017). All primers were designed using the Sci Tools at IDT (https://www.idtdna.com/pages/tools), considering forward and reverse primers with similar melting temperature (Tm; < 1 °C variation), oligo length of 20 nucleotides, and PCR products with a size of 100–150 bp (Table 2 and Table S1). The optimal Tm for each primer set was evaluated *in silico*, using AmplifX 1.7.0 (Nicolas Jullien; CNRS, Aix-Marseille Université - https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr) and verified through gradient PCR. Additionally, all the primers were

Table 2
A. salmonicida primers for qPCR analysis.

Gene	Forward primer	Tm	Reverse primer	Tm	Amplicon size (bp)	% efficiency
gapA	5′-TCGTTTCGTGTTCCGTCTG-3′	55.2	5′-TCGTACTTCAGCATGTAAGCC-3′	54.7	102	101.52
era	5′-GCCTTACTCTGTGACCGTG-3′	55.4	5′-TGTGCCTATGGTCTTGATCTTC-3′	54.5	148	102.34
16S	5′-TGGAAACGACTGCTAATACCG-3′	54.7	5′-TCATCCTCTCAGACCAGCTAG-3′	55.3	150	97.45
recA	5′-AAGGCGAGATGGGTGATTC-3′	54.8	5′-TGACACCAATCTTCATCCGG-3′	54.8	138	92.11
fabD	5′-GAGTTGAAGTTGACCGGAGAG-3′	55.2	5′-GGTTGAACTGCGTGGTTTG-3′	54.9	150	109.83
rpoB	5′-GCCTCCCGTATCGTTATCAAG-3′	55.1	5′-AAGGACGCTGGTTGATACAG-3′	54.7	124	97.12
hfq	5′-AAGATTCTGCCCTCAACTGG-3′	54.8	5′-GGGCATCGGTCAACAGTAC-3′	55.8	126	106.34

analyzed using BLASTn against the all the *A. salmonicida* completed genomes, including *A. salmonicida* subspecies *salmonicida*. The minimum *E*-values for the selected primers was $2e^{-2}$ and all the primers have 100% of identity (Table S1).

2.3. Total RNA extraction

Biological triplicate samples of *A. salmonicida* J223, grown under the previously mentioned growth conditions, were utilized for RNA extraction. Once *A. salmonicida* reached the desired growth phase, the cells were centrifuged (6000 rpm for 10 min, at 4 °C) and washed twice with Phosphate Buffered Saline (PBS, 136 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.2)) (Sambrook and Russell, 2001). The cell pellet was utilized for RNA extraction. Total RNA was extracted using TRIzol (Invitrogen), and cleaned using the RNeasy cleanup columns kit following manufacturer's instructions (QIAGEN). RNA samples were treated with TURBO DNA-free™ Kit (Invitrogen) for DNA digestion. Purified samples were quantified and evaluated for purity using a Nano-quant spectrophotometer (Genway, UK), and evaluated for integrity by agarose gel electrophoresis (Sambrook and Rusell, 2001).

2.4. cDNA synthesis

The cDNA synthesis was performed by SuperScript Vilo IV Master Mix (Invitrogen) using 500 ng of RNA, per reaction, and random hexamers according to the manufacturer's instructions.

2.5. Gradient PCR

Gradient PCR was performed to assess the optimum annealing temperature for each primer set to maximize amplification specificity and to eliminate any non-specific, secondary amplification. Ten microliters were taken from each cDNA sample and pooled, subsequently diluted to 20 ng/μL. Five microliters of diluted cDNA (20 ng/μL) was mixed with 15 μL of PCR mix, for each gene. The PCR mix was prepared with 75 μL (2×) GoTaq (Promega), 2 μL (10 μM) of forward primer and 2 μL (10 μM) of reverse primer and 71 μL of nuclease free water (Ambion). Each sample was amplified in a Bio-Rad MJ Mini personal thermal cycler, using 5 min at 95 °C, followed by 35 cycles of (40 s at 95 °C, 40 s with variable annealing temperatures, 20 s at 72 °C) and finally, 5 min at 72 °C. The annealing temperatures assessed were 60 °C, 59 °C, 58.1 °C, 57 °C, 56.1 °C, and 55 °C. Amplicons were visualized in 1% agarose gel electrophoresis.

2.6. qPCR assays

All qPCR reactions were done in a final volume of 20 μL, containing 10 μL of 1× of PowerUp™ SYBR™ Green Master Mix (Applied BioSystems), 1 μL (10 μM) of each primer, 6 μL of nuclease free water (Ambion), and 2 μL of cDNA. All samples were amplified and detected in a QuantStudio 3 (Applied BioSystems), using 2 min at 95 °C, followed by 40 cycles of 1 s at 95 °C, 30 s at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. Primer efficiencies were analyzed using a 20 ng/μL cDNA pooled sample, which was serially diluted (dilutions starting with 1 (20 ng/μL), 1:3 (6.67 ng/μL), 1:9 (2.22 ng/μL), 1:27 (0.74 ng/μL), 1:81 (0.25 ng/μL), 1:243 (0.08 ng/μL), 1:729 (0.03 ng/μL). In all cases, the primer efficiencies were calculated using the formula $E = 10^{(-1/slope)}$ (Pfaffl, 2001) and only primers with efficiencies between 90% and 110% were considered. Each individual condition was evaluated by biological triplicate. Also, technical triplicates were utilized for each sample. The controls consisted in qPCR reactions in absence of cDNA template.

2.7. Stability analyses and validation of selected reference genes

The expression stability of all seven *A. salmonicida* reference genes was determined with BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002), Norm-Finder (Andersen et al., 2004), and a delta-C_T comparison methods (Silver et al., 2006), through the RefFinder open-access portal (http://150.216.56.64/referencegene.php?type = reference). When required, the relative quantity (RQ) was calculated by using the following equation, $RQ = E^{\Delta Ct}$, where E is the specific amplification efficiency and ΔCt = (min Ct - sample Ct) (min Ct is the lowest Ct value of each gene and sample Ct is the Ct value of the sample being transformed). The highest relative quantities for each gene were set to 1 (Wang et al., 2012) (https://genorm.cmgg.be/).

2.8. Statistical analysis

All data are shown as the mean ± standard error (SE). Assumptions of normality and homogeneity were tested for the detected variances. A one-way ANOVA was used to determine significance followed by a Tukey's *post hoc* test. Differences were considered significant at $p < .05$. All statistical analyses were performed using GraphPad Prism Version 8.0.

3. Results

3.1. Selection of candidate reference gene, primer design, and amplification efficiency

The RNA samples extracted from *A. salmonicida* at different growth phases, under iron-variable conditions, and after being grown in heat-stress conditions (28 °C) showed an acceptable concentration, purity, and integrity (Table 3 and Fig. 1A). The primers designed to amplify *gapA, era, 16S, recA, fabD, rpoB*, and *hfq* (Tables 1 and 2) were verified for optimal Tm and specific amplification by gradient PCR and 1% agarose gel electrophoresis. Gradient PCR showed that all primers designed during this study, amplified a single amplicon at an optimal Tm of 57–60 °C (Fig. 1B).

The efficiency and relative specificity of each primer pair were verified by the standard curve method range between the

Table 3
Quantification and quality assessment of total RNA extracted from *A. salmonicida*.

Sample/replicate		Bacterial culture O.D. 600 nm	RNA		
			Concentration (ng/μl)	(260/280)	(260/230)
Mid-log. 15 °C	#1	0.747	2677.3	2.386	1.882
	#2	0.743	1497.9	2.373	1.903
	#3	0.733	4108.1	2.378	1.992
Late-log. 15 °C	#1	1.550	1486.6	2.527	1.887
	#2	1.512	936.07	2.379	1.886
	#3	1.507	1855.4	2.352	1.927
Stationary 15 °C	#1	2.654	606.67	2.452	2.071
	#2	2.350	593.38	2.374	1.968
	#3	2.245	2094.5	2.362	1.815
Iron-rich conditions (Mid-log; 15 °C)	#1	0.703	4147.9	2.361	1.985
	#2	0.701	3493.9	2.429	1.960
	#3	0.696	2639.0	2.834	2.002
Iron-limited conditions (Mid-log; 15 °C)	#1	0.791	1585.0	2.351	1.927
	#2	0.776	2565.5	2.368	1.987
	#3	0.775	1709.6	2.344	2.030
Heat stress conditions (Mid-log; 28 °C)	#1	0.803	4338.6	2.402	1.997
	#2	0.722	2773.7	2.399	1.991
	#3	0.687	3508.9	2.369	2.031

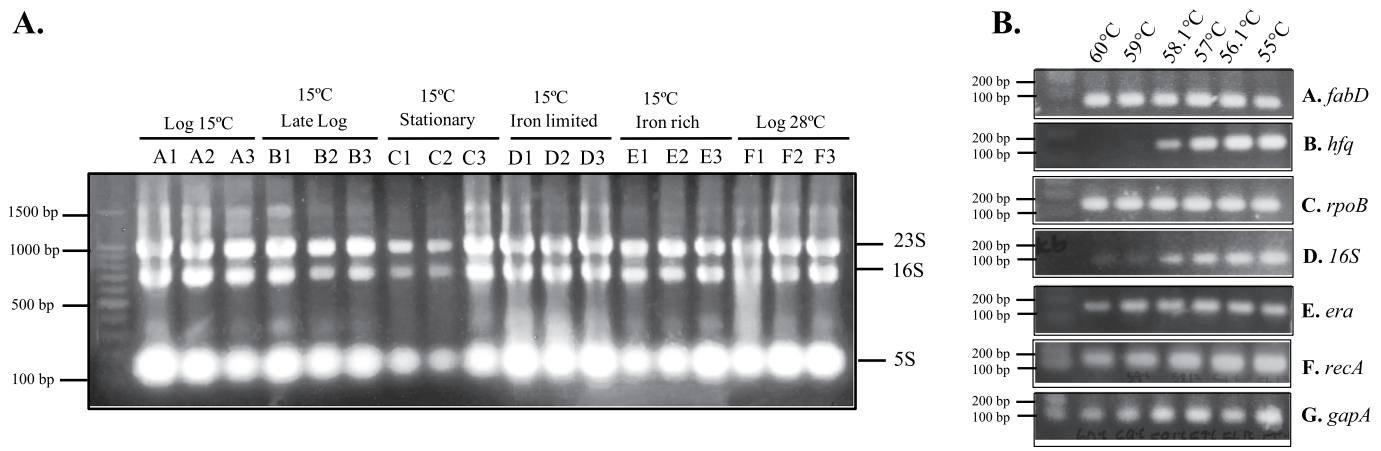

Fig. 1. RNA integrity and determination of the optimal annealing temperatures for each set of utilized primers. A. Agarose gel (1%) electrophoresis of total RNA. A1-A3. RNA extracted from *A. salmonicida* during logarithmic growth phase at 15 °C; B1-B3. RNA extracted from *A. salmonicida* during late logarithmic growth phase at 15 °C; C1-C3. RNA extracted from *A. salmonicida* during stationary growth phase at 15 °C; D1-D3. RNA extracted from *A. salmonicida* during logarithmic growth phase under heat stress conditions (28 °C); E1-E3. RNA extracted from *A. salmonicida* during logarithmic growth phase at 15 °C under iron limited conditions; F1-F3. RNA extracted from *A. salmonicida* during logarithmic growth phase at 15 °C under iron rich conditions. 100 bp molecular marker used; B. Determination of the optimal annealing temperatures. The selected temperatures for the gradient PCR were: 60 °C, 59 °C, 58.1 °C, 57 °C, 56.1 °C, 55 °C. A. *fabD*; B. *hfq*; C. *rpoB*; D. 16S; E. *era*; F. *recA*; G. *gapA*. 100 bp molecular marker used.

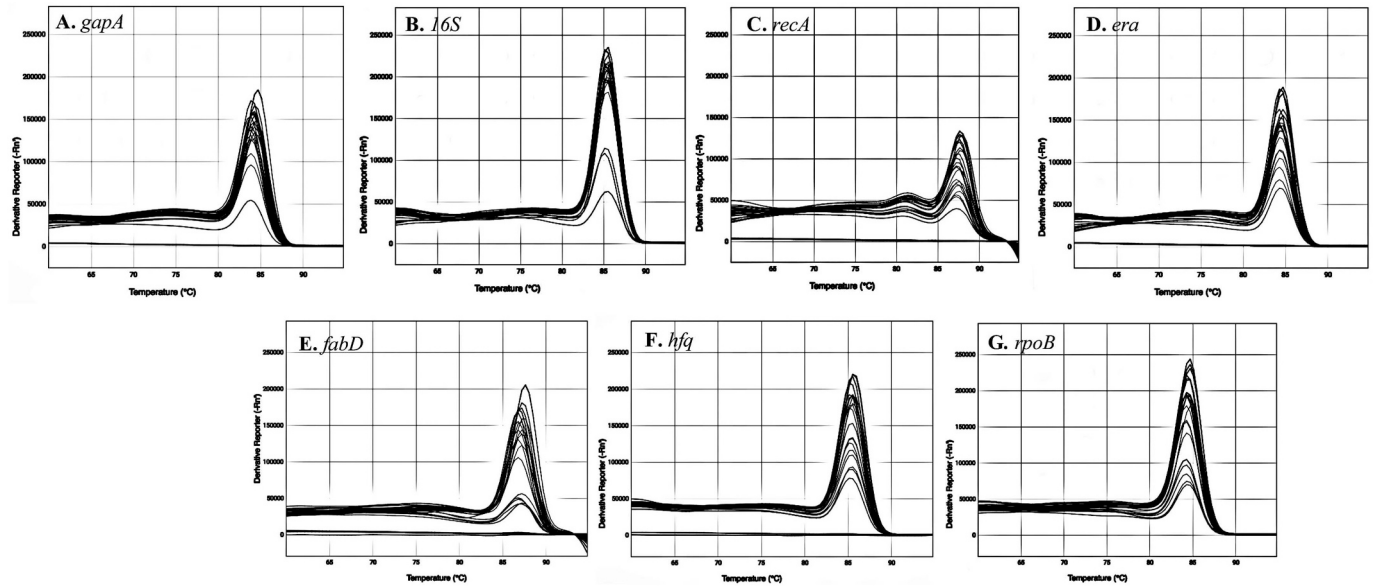

Fig. 2. qPCR melting curves for each primer set evaluated. A. *gapA*; B. 16S; C. *recA*; D. *era*; E. *fabD*; F. *hfq*; G. *rpoB*. The bottom line represents the control in absence of template to verify formation of primer dimer.

recommended values (from 90 to 110%) (Bustin and Huggett, 2017) (Table 2). The melt curves featured a single peak for each set of primers, indicating that the primers do not form dimers and were specific for a single amplicon (Fig. 2).

3.2. Expression levels of the reference genes

Analysis of C_T values indicated that the *16S* gene was expressed in high abundance in contrast to the other candidate reference genes, with C_T values ranging from 4.243 to 6.657. The other candidate reference genes produced C_T values ranging from 13.793 to 22.594 (*gapA*), 19.631 to 27.372 (*era*), 21.713 to 30.571 (*fabD*), 20.391 to 27.126 (*hfq*), 17.784 to 27.274 (*recA*), and 17.824 to 28.595 (*rpoB*) (Fig. 3). *fabD* showed the highest C_T value, indicating that its expression is the lowest of all the selected reference genes (Fig. 3). The candidate genes also displayed a stable and acceptable Ct range (between 20 and 30), independently of the condition tested (Fig. 4).

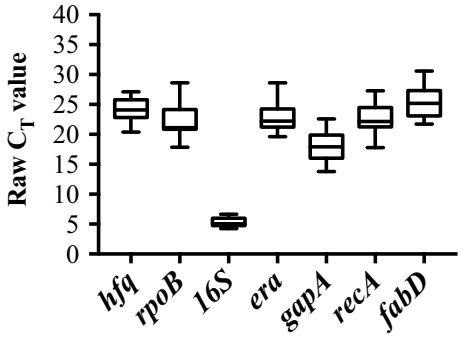

Fig. 3. Raw C_T values for seven candidate reference genes across all experimental treatments. A line across the box represents the median value. Upper and lower boxes indicate the 25th and 75th percentile, respectively, and the whisker caps represent the maximum and minimum C_T values.

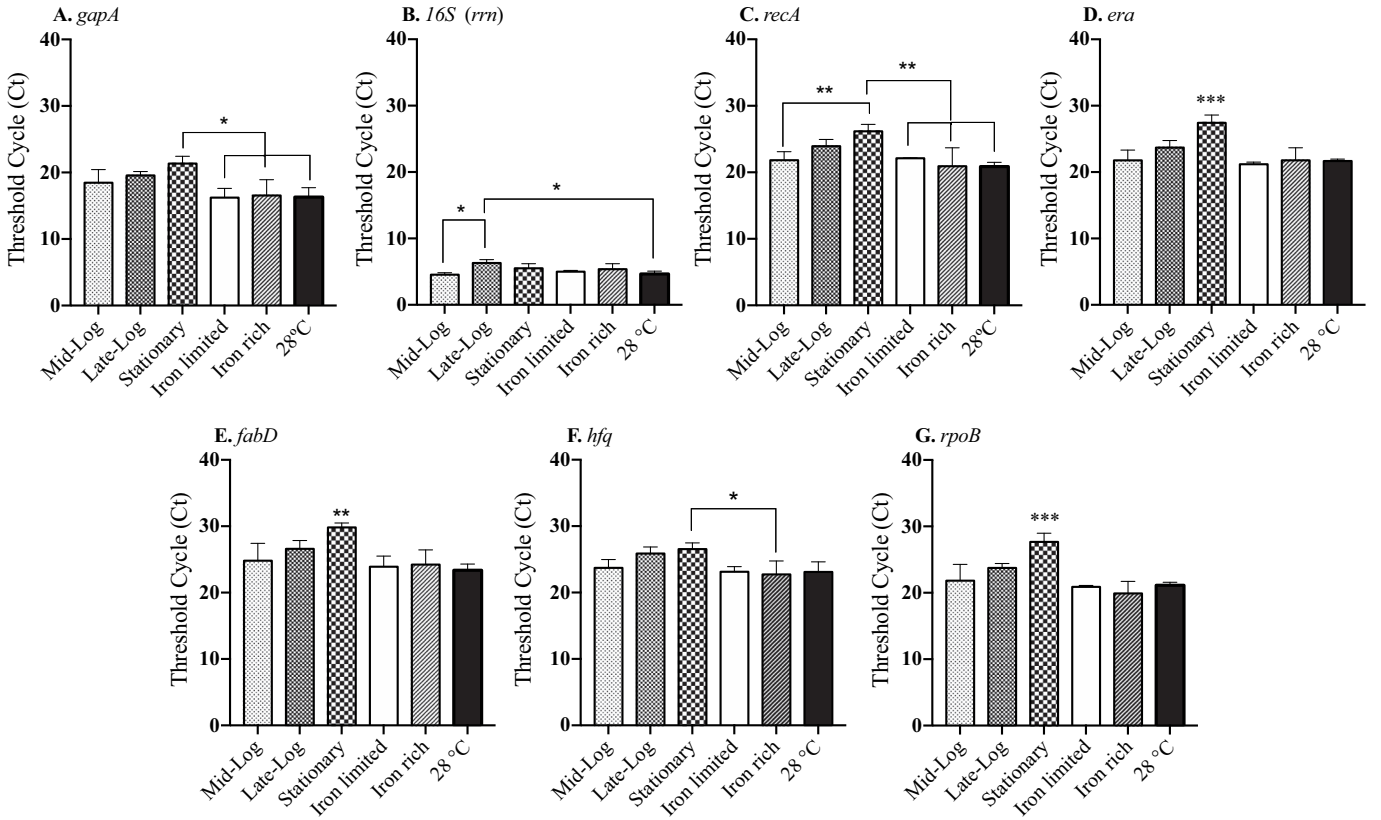

Fig. 4. Differential gene expression of *A. salmonicida* reference genes under different growing conditions. A. *gapA*; B. 16S; C. *recA*; D. *era*; E. *fabD*; F. *hfq*; G. *rpoB*. One-way ANOVA was utilized to determine statistical differences. The mean represents 3 technical replicates of each independent triplicate ± S.E.M. Different letters on the bars of the graph indicate statistical differences between the treatments, *: $p < .5$; **: $p < .01$; ***: $p < .001$.

3.3. Analysis of the gene expression stability

The expression stability of the seven candidate reference genes under different conditions were analyzed by using geNorm, BestKeeper, NormFinder, and the delta-C_T comparison program (Vandesompele et al., 2002; Andersen et al., 2004; Pfaffl et al., 2004; Silver et al., 2006) in the RefFinder open-access portal. A comprehensive ranking was generated by the RefFinder for each gene under all experimental conditions (Table 4) and individual conditions (Tables S2–S7). The genes that exhibited the most stable expression through all the tested conditions were *hfq* and *era*, followed by *recA*, with geometric mean (GM) values of 1.41, 2.21, and 2.28, respectively (Table 4). The genes that exhibited the least stable expression across all conditions assayed were *fabD* and *gapA*, with GM values of 5.23 and 6.24, respectively. This RefFinder comprehensive ranking analysis was coincident with the geNorm, NormFinder, and delta-C_T analysis, but not with the BestKeeper analysis that indicated that the *16S* gene is the most stable, followed by *era*, *recA*, and *hfq* (Table 4). However, BestKeepeer suggests that *fabD* and *gapA* are the least stable genes, coincident with RefFinder comprehensive ranking (Table 4). geNorm values were 0.686 and 0.803 for *hfq* and *recA*, respectively. These values have an acceptable range, between 0.5 and 1 according to MIQE guidelines (Bustin and Huggett, 2017).

recA and *era*, followed by *hfq*, were the most stable genes during logarithmic phase at 15 °C, with GM values of 1.682, 1.861, and 2.449, respectively (Table S2). The genes that exhibited the least stable expression under logarithm growth were *rpoB* and *fabD*, with comprehensive GM values of 5.233 and 6.236, respectively (Table S2). The most stable genes in *A. salmonicida* grown at 15 °C to late-logarithmic phase were *hfq* and *recA*, and the least stable genes were *gapA* and *fabD*

Table 4
Stability values and ranking of the seven *A. salmonicida* candidate reference genes based on geNorm, NormFinder, BestKeeper, Delta Ct, and Comprehensive ranking analysis using all the tested conditions.

Ranking	geNorm		NormFinder		BestKeeper		Delta Ct		Comprehensive ranking	
	Gene	M-value[a]	Gene	Stability value	Gene	Std dev [± CP][a]	Gene	Average of st dev[a]	Gene	GM[b]
1	*hfq/recA*	0.686	*hfq*	0.378	*16S*	0.612	*hfq*	0.902	*hfq*	1.410
2			*era*	0.525	*era*	1.051	*era*	0.970	*era*	2.210
3	*era*	0.803	*recA*	0.566	*recA*	1.187	*recA*	0.977	*recA*	2.280
4	*rpoB*	0.842	*ropB*	0.739	*hfq*	1.214	*rpoB*	1.059	*rpoB*	4.230
5	*fabD*	0.895	*fabD*	0.766	*rpoB*	1.363	*fabD*	1.072	*16S*	4.300
6	*gapA*	0.944	*rpoB*	0.924	*fabD*	1.6	*gapA*	1.162	*fabD*	5.230
7	*16S*	1.081	*16S*	1.278	*gapA*	1.622	*16S*	1.422	*gapA*	6.240

[a] Lower values indicate more stable genes. Higher ranking signifies higher expression stability.
[b] Geometric mean (GM): An average indicating the central tendency of a data set, determined by the product of their values.

(Table S3). According to the comprehensive ranking *fabD* and *hfq* were the most stable genes in cells grown to stationary phase at 15 °C, and the least stable genes were *gapA* and *recA* (Table S4).

During logarithmic phase at 28 °C, *recA* and *16S* were the most stable genes, and the least stable were *era* and *rpoB* (Table S5).

Aeromonas salmonicida grown under iron-limiting conditions, *era* and *recA* have the most stable expression, and the least stable expression were *gapA* and *fabD* (Table S6). In contrast, under iron-rich conditions the most stable expressed genes were *hfq* and *fabD*, and the least stable were *gapA* and *recA* (Table S7).

4. Discussion

A. salmonicida has a broad host range, currently affecting emergent aquaculture finfish species, including sablefish, lumpfish, cunner, turbot, Atlantic cod, halibut, and carp (Beaz-Hidalgo and Figueras, 2013; Dallaire-Dufresne et al., 2014; Lafferty et al., 2015; Powell et al., 2017). Although, several aspects of *A. salmonicida* pathogenesis have been described (Dallaire-Dufresne et al., 2014), fundamental transcriptional aspects remain unknown. As mentioned, qPCR is a precise molecular technique that can detect very slight changes in gene expression, and the appropriate choice of reference genes is essential for accurate normalization of expression data. Here, we evaluated *A. salmonicida gapA*, *hfq*, *era*, *16S*, *recA*, *fabD*, and *rpoB* as potential candidates to be used as reference genes under different *in vitro* conditions for further qPCR analysis.

Previously, *gyrB*, *proC, rpoC*, *rpoD,* and *fabD* were evaluated as candidate reference genes in *A. salmonicida* isolated from turbot, during growth at 18 °C until late-logarithmic phase (Rivera et al., 2015). According to this study, one of the least stable genes was *fabD* (Rivera et al., 2015), which is coincident with our results, *fabD* was among the least stable genes in *A. salmonicida* J223 (Table 4).

fabD, a highly conserved gene among bacteria, encodes for the D subunit of the malonyl CoA-acyl carrier protein trans-acylase, responsible for fatty acid biosynthesis, which is essential for bacterial growth and maintenance of cell membrane integrity (Zhang and Cronan, 1998; Zhu et al., 2006; Savli et al., 2003). Lipid composition plays an important role in the inner bacterial membrane integrity and function (Denich et al., 2003). Alteration of the lipid composition of the bacterial membrane is induced during stress adaptation, resulting in modification of the membrane integrity (Mihoub et al., 2012). Actually, it has been shown that alteration to the fatty acid composition is related to *fabD* up-regulation (Yun et al., 2016). Our results confirmed that expression of *fabD* is not stable in *A. salmonicida* and should not be considered as a primary candidate for reference gene selection (Table 4 and Fig. 4).

The ribosomal RNA gene (*rrn*), also called *16S*, is the most common bacterial reference gene utilized in qPCR assays (Rocha et al., 2015). However, only four of seventeen studies have validated its utilization as a stable reference gene (Rocha et al., 2015). The *16S* is also a multicopy gene, repeated 9 times in the *A. salmonicida* genome (Valderrama et al., 2017b), unsuited for transcriptional assays examining the expression of low-abundance transcripts (Rocha et al., 2015). Additionally, it has been shown that transcription of *16S* varies significantly with the environmental conditions (Rocha et al., 2015). In this study, the *16S* was utilized as a negative control for poor reference gene selection. Coincident, *16S* was ranked the least stable gene by 75% of the algorithms used (GeNorm, NormFinder, and delta-C_T method) and should not be considered as a reference gene for *A. salmonicida*.

As mentioned previously, RefFinder is an open-access portal that compiles the most popular algorithms used to assess the stability of candidate reference genes, including geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and the comparative delta-C_T method (Silver et al., 2006), and provides a comprehensive ranking of the candidate reference genes based on a GM.

RefFinder analysis of bacterial reference genes showed that GM values range between 1.81 and 4.30 in *A. salmonicida* (Rivera et al., 2015), 1.78–9.0 in *Klebsiella pneumoniae* (Gomes et al., 2018), 1.41–7.0 in *Erwinia amylovora* (Kaluzna et al., 2017), and 1.189–4.729 in *Piscirickettsia salmonis* (Flores-Herrera et al., 2018). We obtained comparable GM values for this set of genes in *A. salmonicida* J223, ranging between 1.41 and 6.24 (Table 4). According to the comprehensive ranking, *hfq* maintained the most stable expression across the conditions assayed in this study, followed by *era,* and *recA*.

hfq encodes for the RNA-binding chaperone protein, found exclusively in bacterial organisms (Moller et al., 2002) and its primary function is to regulate post-transcriptional RNA-RNA interactions and protein folding (Moller et al., 2002). Constitutive transcription of this gene is therefore required for cellular homeostasis (Moller et al., 2002). Failure to express this gene typically results in adverse pleiotropic phenotypes, including slow growth, abnormal cell length, and sensitivity to U.V. radiation (Moller et al., 2002). Although *hfq* has not been previously evaluated as a reference gene, given its cellular importance and our results, it can be inferred that *hfq* is an ideal reference gene candidate for qPCR assays.

era is a highly conserved GTPase that is partially responsible for the coordination of cellular processes, including translation and protein trafficking, signal transduction, cell motility, chromosome partitioning, growth, intracellular transport, and ribosome biogenesis (Gibbs and Fredrick, 2018). *era* is also one of the most stably expressed genes in mesophilic bacteria, like *Escherichia coli* (Rocha et al., 2015). Coincident, we found that *era* is one of the most stable reference genes for *A. salmonicida* J223.

geNorm, specifically ranks reference genes on their average expression stability, where the lowest M-value denotes the most stably expressed gene (Vandesompele et al., 2002). M-values exceeding 0.15 are considered unstable, indicating that additional reference genes are required (dos Santos et al., 2015). However, according to MIQE guideline, acceptable M-values range from 0.5 to 1 (Bustin and Huggett, 2017). Previously studies showed that M-values for reference genes typically range between 0.60 and 0.77 in *A. salmonicida* (Rivera et al., 2015), 0.44–0.99 in *K. pneumoniae* (Gomes et al., 2018), 0.550–0.747 in *E. amylovora* (Kaluzna et al., 2017), and 0.571–1.466 in *P. salmonis* (Flores-Herrera et al., 2018). In this study, the M-values in *A. salmonicida* J223 ranged between 0.686 and 1.081, similar range compared to other bacterial reference genes (Table 4). According to geNorm most stable genes were *hfq* and *recA*, followed by *era*, with an M value of 0.686, 0.686, and 0.803, respectively (Table 4). However, RefFiender open portal considered a primer efficiency of 100% for the geNorm algorithm. Using the RQ, we recalculated the geNorm M-values using the excel open access geNorm file. Although we determined that the M-values were underestimated by RefFinder portal, the most stable genes according to the excel geNorm open access platform were also *hfq* (M-value, 0.882) and *recA* (M-value, 0.882). According to our results, the most stable *A. salmonicida* genes under the tested conditions were *hfq*, *era*, and *recA* (Table 4).

NormFinder algorithm identifies optimal reference genes by determining both inter-group and intra-group variations and combining these results into a stability value for each candidate reference gene (Andersen et al., 2004). Previously studies have showed that NormFinder stability values for reference genes range between 0.12 and 0.26 in *A. salmonicida* (Rivera et al., 2015), 0.296–0.776 in *K. pneumoniae* (Gomes et al., 2018), 0.077–0.163 in *E. amylovora* (Kaluzna et al., 2017), and 0.599–1.197 in *P. salmonis* (Flores-Herrera et al., 2018). In this study, the NormFinder stability values in *A. salmonicida* J223 ranged between 0.378 and 1.278, similar range compared to other bacterial reference genes (Table 4). According to NormFinder the most stable genes were *hfq*, *era*, and *recA*, concordant with RefFinder comprehensive ranking, GeNorm M values, and the delta-C_T analysis (Table 4).

The delta-C_T analysis compares the relative expression of candidate

reference genes within each sample to identify the most stable expressed genes. If the delta-C_T value of the genes fluctuates when analyzed in a different set of samples, it means that these genes are variably expressed. If the delta-C_T value remains constant, these genes are stably expressed between the samples (Silver et al., 2006). Previously studies showed that delta-C_T stability values for reference genes range between 0.92 and 1.31 in *K. pneumoniae* (Gomes et al., 2018), 0.550–0.750 in *E. amylovora* (Kaluzna et al., 2017), and 1.233–1.579 in *P. salmonis* (Flores-Herrera et al., 2018). In this study, the delta-C_T values in *A. salmonicida* J223 ranged between 0.902 and 1.422, similar range compared to other bacterial reference genes (Table 4).

BestKeeper determines stability rankings based on the standard deviation (SD) values, which is inversely proportional to the expression stability of the genes (Pfaffl et al., 2004). In other studies BestKeeper stability values typically range between 0.88 and 1.55 in *A. salmonicida* (Rivera et al., 2015), 0.46–0.67 in *E. amylovora* (Kaluzna et al., 2017), and 0.874–1.138 in *P. salmonis* (Flores-Herrera et al., 2018). In this study, the Bestkeeper stability values in *A. salmonicida* J223 ranged between 0.612 and 1.622, similar range compared to other bacterial reference genes (Table 4). In contrast to other algorithms, BestKeeper suggested that 16S is the most stable gene in *A. salmoncida* J223. Similar results were found in *A. salmonicida* using different sets of reference genes, where *rpoD* was evaluated as the least stable gene by geNorm, NormFinder, and RefFinder comprehensive ranking, but as the most stable by BestKeeper (Rivera et al., 2015).

In concordance with other studies, we fail to identify a universal reference gene for *A. salmonicida*. We noticed that in specific conditions, like thermal stress and iron-rich or limited conditions, different genes showed high stability, likely due to the versatile nature of bacterial singles cell physiology. *recA* and *era,* followed by *hfq,* were the most stable genes during logarithmic phase at 15 °C (Table S2). The most stable genes during late logarithmic phase were *hfq* and *recA* (Table S3). During stationary phase at 15 °C the most stable genes was *hfq* (Table S4). These results suggest that *recA*, *hfq*, and *era*, are stable during *A. salmonicida* cell division. During stationary phase, nutrient limitation, stress response, and mortality are encounter by the bacterial population. *hfq* was the most acceptable reference genes for *A. salmonicida* during stationary phase. In contrast, *recA* was among the least stable gene, which correlates with its role in DNA repair, genetic recombination, and initiation of the SOS-response (Cox, 2007), required during the stress response.

Selection of reference genes for *A. salmonicida* under thermal stress is complex. According to the comprehensive ranking, *recA* and *gapA* were the most stable genes, and according to the geNorm M-value, *hfq* and *gapA* were the most stable genes, and the least stable genes were *era* and *rpoB* (Table S5).

gapA is one of the most popular reference genes used for microbial qPCR experiments (Rocha et al., 2015). *gapA* encodes for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme, which catalyzes the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate in the sixth step of glycolysis, and also is an virulence factor for some bacterial pathogens (Li et al., 2011; Liu et al., 2007; Oliveira et al., 2012; Terrasse et al., 2012). Since GAPDH is vitally for bacterial homeostasis and *gapA* has a highly conserved sequence, this gene is extensively utilized as a reference gene. Our results suggest that glycolysis is a stable process during thermal stress in *A. salmonicida* and *hfq*, *recA*, and *gapA* genes should be considered as reference genes for this stress condition.

rpoB one of the most frequently tested and validated microbial reference genes (Rocha et al., 2015), was between the 3rd and 7th most stable gene among the seven tested genes (Table 4, Tables S2–S7). *rpoB* encodes for the DNA-directed RNA polymerase, which polymerized ribonucleotides into functional RNA molecules (Vos et al., 2012). Perhaps, due to its role in transcriptional regulation and gene expression, *rpoB* is not a good candidate for *A. salmonicida* gene expression studies, especially during thermal stress.

A. salmonicida grown under iron-limited conditions showed that *era* and *recA* have the most stable expression (Table S6). In contrast, *A. salmonicida* grown under iron-rich conditions the most stable expressed gene was *hfq,* followed by *era* and *rpoB*, and the least stable were *gapA* and *recA* (Table S7).

The use of *recA* as an endogenous control to normalize differential expression data is common in bacterial gene expression studies (Rocha et al., 2015). *recA,* which encodes the recombinase subunit A, is required for essential processes, including DNA repair, genetic recombination, and initiation of the SOS-response (Cox, 2007). *recA* also plays a critical role in DNA replication / DNA metabolic processes enzymes and is one of the most stably expressed genes in mesophilic bacteria (Rocha et al., 2015). Our results under iron-rich conditions suggest that DNA damage response is required in presences of excess of iron, making *recA* unsuitable as a useable reference gene for these conditions.

The results of this study provided with an additional list of reliable reference genes for *A. salmonicida* gene expression studies using qPCR, including *hfq*, which was among the most stable tested genes that could be also considered for other bacterial species.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.05.011.

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