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METHOD PAPER

Reference genes for real-time RT-PCR expression studies in an Antarctic *Pseudomonas* exposed to different temperature conditions

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Abstract
Psychrophilic and psychrotolerant bacteria from permanently cold environments may be the most abundant extremophiles on Earth and yet little is known on how they cope with temperature stress. Real-time reverse transcription PCR (RT-qPCR) is a powerful technique that could shed light on this matter but it requires pre-validated reference genes for normalization of data to get accurate results. In this study, we assessed the expression stability of eight candidate genes for the psychrotolerant Antarctic isolate *Pseudomonas* sp. AU10 during exponential growth under 4 °C and 30 °C, and after a cold-shock. Using the software programs BestKeeper and geNorm we validated *recA*, *ftsZ*, *16S rRNA*, and *rpoD* as reference genes and we suggested the combination of *recA* and *ftsZ* for qPCR data normalization. Our results provide a starting point for gene expression studies in Antarctic *Pseudomonas* concerning temperature-related physiology and also for the validation of reference genes in other cold-adapted bacterial species.

Keywords Reference genes · Validation · RT-qPCR · *Pseudomonas* · Psychrotolerant · Cold-shock

Introduction

Antarctica is considered to be one of the harshest and most pristine permanently cold ecosystems in the world where cold-adapted microorganisms thrive (Hughes et al. 2015). These microbes are able to grow close to 0 °C and are usually differentiated by their cardinal growth temperatures as truly psychrophilic or psychrotolerant. While the former predominates in permanently cold marine ecosystems, the latter is particularly adapted to environments with important temperature fluctuations, such as the Antarctic continent (Russell et al. 1990; De Maayer et al. 2014).

Antarctica's extreme low temperatures and frequent temperature downshifts impose several physicochemical constraints to its living microbiota, having effects on diffusion rates, membrane fluidity, structure and topology of nucleic acids, protein folding and enzyme kinetics (Rodrigues and Tiedje 2008; De Maayer et al. 2014). In particular, a temperature downshift of several degrees, named cold-shock, has been found to induce an arrest in bacterial growth and bulk protein synthesis (Jones et al. 1987; Graumann et al. 1996; Michel et al. 1997; Weber and Marahiel 2003). Under these conditions, an immediate "cold-shock response" takes place, where a set of "cold-inducible proteins" are transiently overproduced, allowing the cell physiology to re-attain homeostasis. After this period, the low-temperature-adapted cells resume growth at a slower rate, while the expression of the cold-inducible proteins declines and bulk protein synthesis restarts.

The cold-shock response has been particularly studied in mesophilic bacteria and was differentiated from cold-adapted growth, where a different set of "cold-acclimation proteins" are produced (Graumann and Marahiel 1996; Weber and Marahiel 2003). For psychrophilic bacteria from permanently cold environments, particular emphasis has been given to cold acclimation protein profiles,

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but cold-shock responses were scarcely addressed (Roberts and Inniss 1992; Berger et al. 1996; Piette et al. 2012). Therefore, studying changes in gene expression which occur during the cold-shock response and during growth at permanent low temperatures is one possible strategy to shed light on the mechanisms that Antarctic bacteria evolved to cope with the cold.

Reverse transcription quantitative real-time PCR (RT-qPCR) is at present the method of choice for analyzing changes in gene expression in biological samples. It has outbalanced any other methodology for measuring RNA levels in terms of sensitivity, specificity, reproducibility, speed and dynamic range of analysis (Bustin 2002; Pfaffl et al. 2004; Bustin et al. 2005). Notwithstanding this, any variability introduced in the downstream processing of samples—including the number and physiological state of cells, efficiency of the RNA extraction and performance of reverse transcription—leads to significant errors and misleading results. It is therefore essential to minimize such variations by normalizing the expression levels of a target gene against that of a set of reference genes. The requirement that every reference gene must meet is to remain stably expressed across all the conditions being compared (Huggett et al. 2005; Bustin et al. 2009; Kozera and Rapacz 2013). However, in bacteria no standard set of reference genes has been demonstrated to exist, leading many authors to underline the importance of pre-validating multiple candidate genes for the strains to be used and in the particular experimental conditions to be assayed (Huggett et al. 2005; Bustin et al. 2009; McMillan and Pereg 2014; Rocha et al. 2015). In fact, it has been demonstrated how the use of a single non-validated reference gene distorts the relative quantification results, leading to false conclusions (Dheda et al. 2005; Reiter et al. 2011). Therefore, to get accurate and reliable expression results, the general recommendation is to normalize data using two or three of the best pre-validated gene candidates (Vandesompele et al. 2002; Takle et al. 2007; Brudal et al. 2013).

The genus *Pseudomonas* has been considered the most diverse and ecologically significant group of bacteria on the planet, displaying large metabolic capabilities and wide physiological versatility (Spiers et al. 2000). Members of this genus have usually been recovered from different Antarctic environments, most of which represent native psychrotolerant species described in the past two decades (Reddy 2004; López et al. 2009). This makes Antarctic Pseudomonads good candidates for studying adaptive strategies to cope with cold stress. In this work, we aimed to assess the suitability of eight candidate reference genes for mid-log cells of the Antarctic isolate *Pseudomonas* sp. AU10 growing at different temperatures and when subjected to a cold-shock.

Materials and methods

Culture conditions and growth profiles

The strain used in this work, *Pseudomonas* sp. AU10, was isolated from a freshwater body at the Fildes Peninsula (King George Island, Antarctica) (Martínez-Rosales and Castro-Sowinski 2011; Martínez-Rosales et al. 2012). Axenic cultures of this strain were always grown on LB medium (1% Tryptone, 0.5% Yeast Extract, 1% NaCl) at different temperature conditions. Flasks filled with culture media up to 10% of their volume were inoculated with over-night precultures to an estimated initial optical density at 600 nm (OD_{600}) of 0.05. Then, cultures were incubated in an orbital shaker under aerobic conditions (150 rpm) at 30 °C or 4 °C. At the end of each growth experiment, aliquots were streaked on agar plates to rule out contamination.

For the cold-shock experiments, cultures were grown at 30 °C until mid-log phase ($OD_{600} = 0.5$). At that point, flasks were transferred to a cold-water bath (4 °C) for six minutes and then were incubated at 4 °C in an orbital shaker as stated above. Those six minutes in the water bath were sufficient for the temperature to drop from 30 °C to 4 °C. A cold-shock induction time of 75 min (including the water-bath time) was selected based on other studies (Michel et al. 1997; Czapski and Trun 2014).

Growth profiles at different temperatures were obtained by turbidimetry at 600 nm and also by viable cell counts on LB agar following the Miles-Misra method (Hedges et al. 1978).

Sampling, RNA extraction and cDNA synthesis

RNA was extracted from cultures with an OD_{600} of 0.5, corresponding to three different conditions assayed: i) mid-log cells growing at 30 °C, ii) mid-log cells growing at 4 °C, and iii) cold-shocked cells (30 °C to 4 °C, 75 min, as explained above). Based on total counts performed on a Petroff-Hausser counting chamber, approximately 5×10^8 cells were harvested for each culture and then centrifuged 5 min at 5000 rpm at 30 or 4 °C, following the original culture temperature. Three independent cultures were processed in parallel for each condition (methodological replicates).

Total RNA was extracted using a ZR Fungal/Bacterial RNA MiniPrep kit (Zymo Research) with a previous step of pellet resuspension and incubation for two min in a 0.8 mg/mL lysozyme solution in 30 mM Tris–HCl, 5 mM

EDTA, 50 mM NaCl buffer (pH 7). Two digestions using the rigorous protocol from the DNA-free Kit (Ambion) were necessary to remove genomic DNA. RNA samples were quantified and assessed using a NanoDrop spectrophotometer. $A_{260/280}$ ratios above 2.05 were considered as acceptable. RNA integrity was assayed by electrophoretic analysis of the 23S and 16S RNAs on 1% agarose gels under denaturing conditions (0.6% sodium hypochlorite) (Aranda et al. 2012).

First-strand complementary DNA (cDNA) was synthesized from 500 ng RNA through random hexamer primed reactions using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's specifications for GC rich templates. Controls omitting the reverse transcriptase (RT^-) were carried out to rule out residual genomic DNA contamination, by performing qPCR with the *recA* primer pair. Genomic DNA contamination was considered negligible since RT^- controls showed no amplification or presented Ct values at least thirteen times greater than those observed for RT^+ templates, which means that genomic DNA would represent no more than 0.012% of total template DNA.

Selection of candidate genes and primer design

Eight candidate reference genes (*atpA*, *ftsZ*, *gap*, *gyrA*, *recA*, *rpoD*, *16S rRNA* and *secA*) were selected based on previous selections for other bacterial species (Rocha et al. 2015). Coding sequences were obtained from a draft-genome of the *Pseudomonas* strain under study (our unpublished results). All primers used in this study were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) following the general recommendations for qPCR and are listed in Table 1 (Bustin et al. 2009). The amplification efficiency of each primer pair was determined using tenfold dilution series of a genomic DNA template. Primer specificity was determined by melt curve analysis and by polyacrylamide gel electrophoresis.

Quantitative PCR experiments

Reverse-transcribed quantitative PCRs (RT-qPCR) were carried out in a StepOne Plus thermocycler (Applied Biosystems) using the SensiFAST SYBR Hi-ROX kit (Bioline). Each reaction contained 2 µL of 1:25 cDNA diluted template in a total volume of 10 µL, with each primer at a 400 nM concentration. Because of the high abundance of 16S rRNA, cDNA had to be diluted a further 100 more times when analysing *16S rRNA* expression. Cycling conditions included a 3 min hot start at 95 °C, followed by 40 amplification cycles (10 s at 95 °C and 30 s at 60 °C, with a single fluorescent measurement) and a final ramp from 60 °C to 95 °C for the melt curve analysis. Samples from three independent experiments (methodological replicates) were analysed in duplicate (technical replicates), with negative controls included in each assay.

Table 1 Candidate reference genes and primer pairs used for RT-qPCR

Gene	Gene product	Primer pairs (5′–3′)	Tm (°C)	Amplicon (pb)	Efficency (%)	r^2
16S rRNA	16S ribosomal RNA	**F:** GTGTAGCGGTGAAATGCGTAG	61.5	78	92.9	0.994
		R: CTCAGTGTCAGTATTAGTCCAGGT	61.2			
atpA	ATP synthase subunit alpha	**F:** TGAACAATACCGAGACCGATG	59.7	68	90.2	0.994
		R: GTCTACCGACTTACGCCAGA	60.7			
ftsZ	Cell division protein FtsZ	**F:** GGTATCTCCGACATCATCAAG	57.5	83	95.5	0.999
		R: ATGCCCATTTCGCTCATCAC	60.8			
gap	Glyceraldehyde-3-P dehydrogenase	**F:** TTCCAGTGACTTCAACCATAAC	58.1	93	90.3	0.998
		R: TACCAAGCCAGCACTTTCAG	59.9			
gyrA	DNA gyrase subunit A	**F:** GGCGTGAAGGATGAGGACTA	60.7	85	93.4	0.993
		R: CTTTGCCTTTGCTGGAGAAC	59.4			
recA	Recombination protein RecA	**F:** GCGGTTAAAGAAGGTGACGAG	61.1	120	99.9	0.995
		R: GTAAATACCCTTGCCGTAGAG AATC	61.2			
rpoD	RNA polymerase sigma factor RpoD	**F:** CATCGCCAAGAAGTACACCA	59.7	88	99.3	0.996
		R: CTTGTCCACAGCCTTCATCA	59.6			
secA	Protein translocase subunit SecA	**F:** TATTCCGCCACAGTCCCTGC	64.1	119	93.9	0.994
		R: ACAGGTGGTCGTCTTCGTCC	63.6			

F forward primer, *R* reverse primer, *Tm* melting temperature, r^2 coefficient of determination

Data analysis

Expression data for the candidate reference genes were obtained in the form of threshold cycle (Ct) values. The amplification efficiencies and determination coefficients were calculated using the StepOne Plus Software v2.3 (Applied Biosystems). To determine the most stably-expressed set of reference genes, the Ct raw data output was analyzed on the EXCEL-based program BestKeeper (Pfaffl et al. 2004) and with the geNorm program (Vandesompele et al. 2002) available in the software Biogazelle qBase + (Hellemans et al. 2007).

Results and discussion

Selection of candidate reference genes, qPCR amplification efficiencies and primer pair specificity

In this study, we investigated the suitability of eight candidate reference genes for proper normalization of RT-qPCR gene expression analysis from mid-log cells of the Antarctic isolate *Pseudomonas* sp. AU10 incubated at different temperatures (4 °C and 30 °C) and subjected to a cold-shock (30–4 °C, 75 min). These genes had been frequently selected as reference genes in previous studies (Rocha et al. 2015), being seven of them protein-coding genes whose products are involved in different primary metabolic activities (*atpA, ftsZ, gap, gyrA, recA, rpoD* and *secA*) and the last one the *16S rRNA* gene (Table 1). The efficiencies of the primer pairs specific for these eight genes fell in the range of 90–100%, with coefficients of determination higher than 0.993 (Table 1). These values are within the recommended ones in the literature for qPCR relative quantification (Taylor et al. 2010). Moreover, melt peak analysis showed a single homogeneous peak for all the primer pairs and polyacrylamide gel electrophoresis of the amplified products revealed single bands of the expected molecular weight, indicating specific amplification in all cases (Online Resource 1A and 1B).

Growth behavior in relation to temperature

By following the cultural behavior of this strain in relation to temperature, we could determine that it is able to grow at temperatures close to 0 °C, presents an optimal growth temperature of 28 °C and no growth at 37 °C. With these cardinal growth temperatures, *Pseudomonas* sp. AU10 must be considered a psychrotolerant microorganism. In particular, growth was followed at 30 °C and 4 °C by viable cell counts and turbidimetry (Fig. 1a, b). At both temperatures, cultures remained in log-phase until an OD_{600} of ca. 2, but

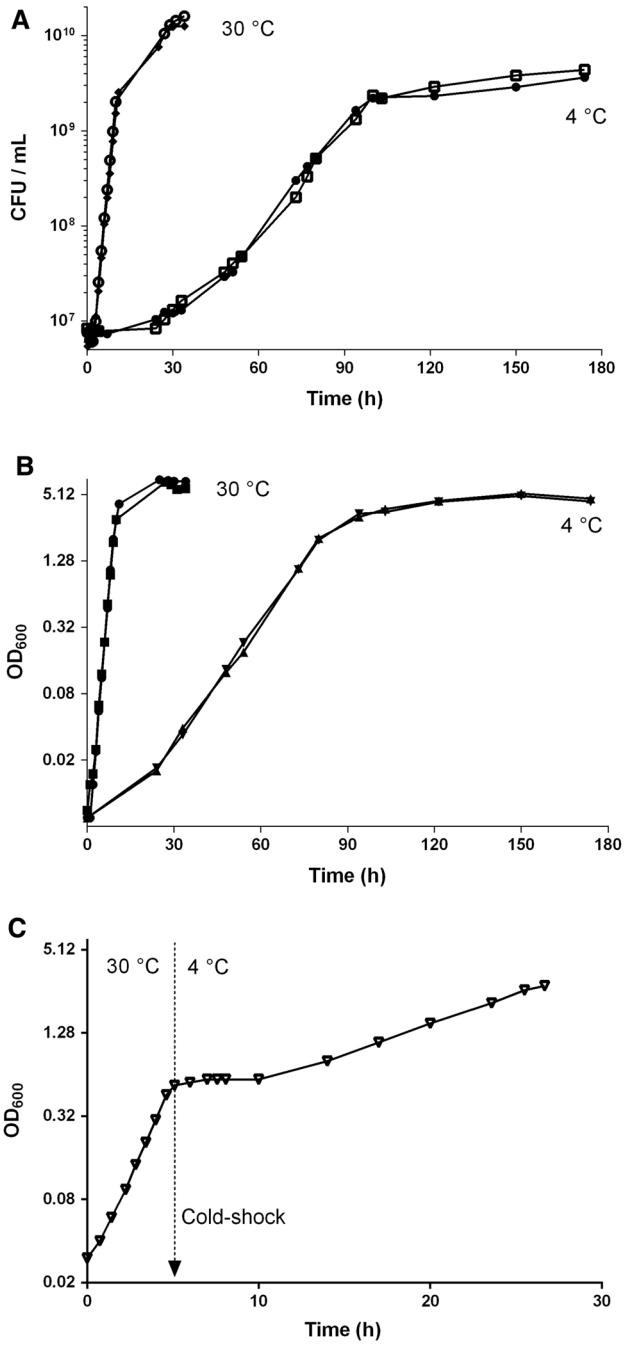

Fig. 1 Growth curves of *Pseudomonas* sp. AU10 incubated at 4 °C, 30 °C and subjected to a cold-shock. **a** Growth followed by viable cell counts at 30 and 4 °C in two independent assays. **b** growth at 30 and 4 °C followed by turbidimetry at 600 nm in two independent assays. **c** growth followed by turbidimetry before and after a cold-shock induction from 30 to 4 °C when the culture reached an OD_{600} of 0.5. The *y* axis is shown in $\log_{10}$ scale for **a** and $\log_2$ scale for **b** and **c**

the estimated generation times were quite different: 1 h at 30 °C and 8 h at 4 °C.

In addition, when mid-log cells growing at 30 °C were subjected to a cold-shock at 4 °C, a 5 h-latency phase

ensued, followed by resumption of logarithmic growth at a slower rate (Fig. 1c). Thus, this strain presents the typical growth arrest observed in mesophiles and cold-adapted bacteria, following a significant temperature downshift (Michel et al. 1997; Piette et al. 2012). For studying gene expression during the cold-shock response we selected a window of 75 min following the temperature downshift, based on the results of other authors. For instance, in *Pseudomonas fragi* it was observed that the majority of the variations at the proteomic level occurred during the first hour following the downshifts (Michel et al. 1997). Furthermore, in *Escherichia coli*, an accumulation of transcripts of several cold-shock genes appeared during the first hour after the cold-shock (Czapski and Trun 2014) and the highest rate of cold-inducible protein synthesis occurred after 2 h (Jones et al. 1987).

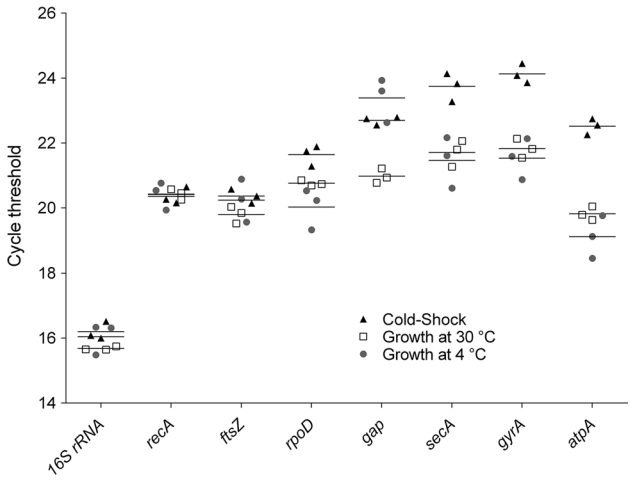

Fig. 2 Expression levels of the candidate reference genes across the temperature-related conditions assayed. Gene expression levels are represented by Ct values for the three independent methodological replicates of each condition. Lines indicate median values for each condition

Expression and stability of candidate reference genes

The transcriptional levels of the eight candidate reference genes were quantified in cultures grown at 30 °C, at 4 °C and after the cold-shock treatment. Raw Ct values ranged from 15.5 (*16S rRNA*) to 24.5 (*gyrA*) and important differences across the conditions assayed were observed for several genes while others seemed to be similarly expressed (Fig. 2). For instance, the cold-shock samples tend to have higher Ct values indicating a certain degree of repression of gene expression for *rpoD*, *gyrA*, *secA* and *atpA*. This goes in line with the generalized repression observed when a cold-shock was induced in other bacterial species (Jones et al. 1987; Graumann et al. 1996). Also, the *gap* gene, which codifies for a key enzyme of carbon metabolism, seems to be induced in cultures growing at 30 °C with respect to the lower temperature conditions. More important for the purpose of this work is that *recA*, *16S rRNA* and *ftsZ* appeared to be stably expressed across all conditions.

RT-qPCR data were analyzed with the statistical programs BestKeeper and geNorm to validate the expression stability of the candidate reference genes. The former is an Excel-based spreadsheet application that analyzes data as a whole regardless of the conditions assayed and provides a series of descriptive statistics that are shown in Table 2. Only those genes with a Ct standard deviation (SD) lower than 1 are considered good candidates for qPCR data normalization (Pfaffl et al. 2004). From our set of eight gene candidates, only *recA*, *16S rRNA*, *ftsZ*, *rpoD*, *gap* and *secA* met this criterion, ordered from the most to the least stably expressed. Conversely, *atpA* and *gyrA* showed SD values higher than 1 and thus were discarded as possible reference genes. It should be noted that *gap* and *secA* presented SD values close to the cut-off and the minimal and maximal fold-change expression with respect to the geometric mean was around threefold (Table 2). These results seem poor for good reference genes.

Table 2 BestKeeper descriptive statistics for the gene expression analysis of the candidate reference genes

Statistics	*ARNr 16S*	*atpA*	*ftsZ*	*gap*	*gyrA*	*recA*	*rpoD*	*secA*
Geometric mean (C_t)[a]	15.97	20.43	20.13	22.33	22.47	20.40	20.80	22.28
Minimum C_t	15.49	18.46	19.52	20.78	20.87	19.94	19.33	20.61
Maximum C_t	16.52	22.75	20.89	23.93	24.45	20.77	21.89	24.14
Standad deviation ($\pm C_t$)[b]	0.31	1.36	0.35	0.92	1.09	0.22	0.56	0.96
Minimum *x*-fold	−1.40	−3.92	−1.53	−2.93	−3.02	−1.38	−2.77	−3.18
Maximum *x*-fold	1.46	4.98	1.69	3.04	3.96	1.29	2.13	3.63
Standard deviation (±*x*-fold)	1.24	2.56	1.27	1.89	2.13	1.16	1.48	1.95

[a] C_t threshold cycle

[b] Genes with a standard deviation < 1 are considered stably expressed (highlighted in bold)

The second program, geNorm, calculates the average pairwise variation of a particular candidate gene with all other tested genes as the gene-stability measure *M*. Thus, genes with the lowest *M* values have the most stable expression. geNorm developers have considered genes with *M* values below 0.5 as good reference genes of high stability (Vandesompele et al. 2002). In this study, the analyzed genes that fulfilled this rule were *16S rRNA*, *ftsZ*, *recA* and *rpoD*, ordered from the most to the least stable (Fig. 3a). In addition, geNorm infers an optimal number of reference genes needed for normalization by calculating a statistic *V*. The developers define the geometric averaging of the *n* most stable genes, based on their *M* values, as a Normalization Factor (NF_n) and consider *n* as the optimal number of genes when this value does not change significantly upon adding the next most stable gene (NF_{n+1}). This is reflected in a pairwise variation between the two sequential NFs ($V_{n/n+1}$) lower than 0.15 (Vandesompele et al. 2002). In the present study, geNorm infers that the two most stable reference genes, *16S rRNA* and *ftsZ*, suffice to obtain trustworthy results, since $V_{2/3}$ is already under the cut-off value (Fig. 3b).

Both programs showed consistent results and agreed to recognize *16S rRNA*, *recA*, *ftsZ* and *rpoD* as good endogenous controls for normalization, despite the slight differences observed in the order in which they were arranged. These genes have also been found as suitable normalizers in other bacteria and experimental conditions. The *recA* gene was validated for plant growth-promoting bacteria such as *Azospirillum brasilense* (McMillan and Pereg 2014) and *Herbaspirillum seropedicae* (Pessoa et al. 2016), for the plant pathogens *Pectobacterium atrosepticum* (Takle et al. 2007) and *Pseudomonas syringae* (Smith et al. 2018) and for the human pathogens *Klebsiella pneumoniae* (Darrieux et al. 2018), *Streptococcus agalactiae* (Florindo et al. 2012) and *Vibrio parahemoliticus* (Ma et al. 2015). Although less frequently used, the other two protein-coding genes validated herein were also found to be stably expressed across different experimental conditions: *ftsZ* for *Oenococcus oeni* (Sumby et al. 2012) and several *Francisella* strains (Brudal et al. 2013), and *rpoD* for *K. pneumoniae* (Darrieux et al. 2018), *Gluconoacetobacter diazotrophicus* (Galisa et al. 2012), *P. syringae* (Smith et al. 2018) and *P. aeruginosa* (Savli 2003). Likewise, the *16S rRNA* gene has been widely used for qPCR normalization in bacterial models, though a prior validation was properly carried out only a few times (Rocha et al. 2015). Such is the case of the studies conducted for *Clostridium difficile* (Metcalf et al. 2010), *Flavobacterium psychrophilum* (Hesami et al. 2011), *Shewanella psychrophila* (Liu et al. 2018), *Saccharopolyspora spinosa* (Zhang et al. 2015) and *Leptospira* spp. (Carrillo-Casas et al. 2008). Nonetheless, several authors pointed out that the *16S rRNA* gene might not be the best option for qPCR normalization because of its stability, which makes it not comparable to the fast-decaying mRNA population, and because of its high expression rate, which makes it less reliable for normalizing lower-expression genes such as protein-coding genes (Takle et al. 2007; Kozera and Rapacz 2013; Rocha et al. 2015). In fact, in the present study the cDNA templates had to be diluted a hundred times more for the *16S rRNA* gene than for the other evaluated genes to be quantified in the appropriate range of Cts.

Regarding the genus *Pseudomonas*, there are a few other reports in the literature devoted to reference gene validation. Savli et al. (2003) studied the expression stability of six housekeeping genes in several clinical strains of *P. aeruginosa* with diverse resistance phenotypes. They proposed the geometric average of *proC* and *rpoD* as a good normalizing factor for qPCR analysis. Another study on *P. aeruginosa*

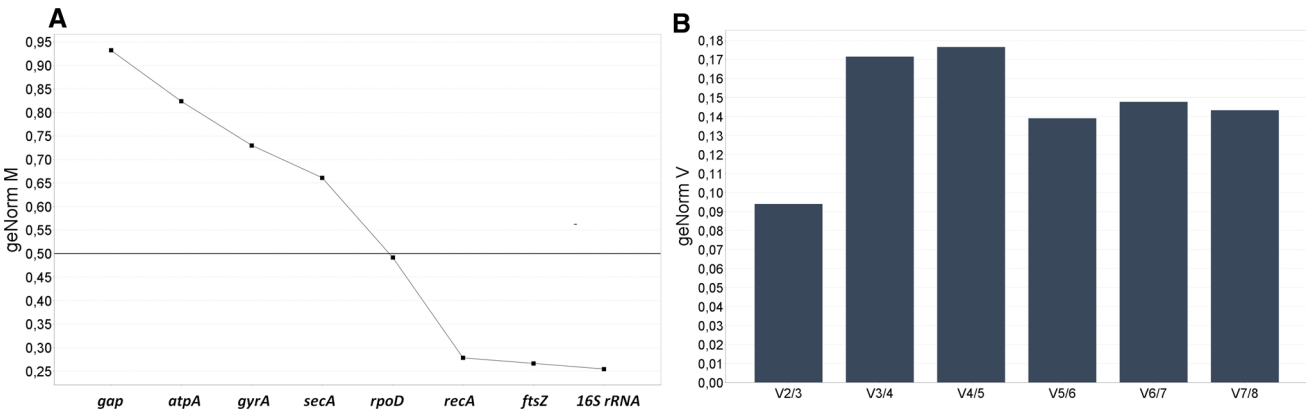

Fig. 3 geNorm analysis of the candidate reference genes. **a** ranking of candidate gene expression stability based on the *M* value. Genes with the lowest *M* values have the most stable expression and are positioned on the right side of the diagram. Cut-off value of 0.5 represented by horizontal solid line; **b** determination of the optimal number of reference genes required for reliable normalization. When $V_{n/n+1}$ is lower than 0.15, then *n* would be the appropriate number of reference genes to be used. Genes are included one by one starting with those of lower *M* values

focused on the expression of 13 candidate reference genes under carbon starvation and nutrient-replete conditions (Alqarni et al. 2016). They concluded that *rpoS* was the only stably expressed gene. Likewise, for *P. putida* eight housekeeping genes were quantified under conditions of p-xylene degradation, having validated *rpoN*, *rpoD*, *16S rRNA* and *atkA* as suitable normalizers (Chang et al. 2009). Studies were also carried out *in planta* for *P. syringae* by inoculating leaves of *Arabidopsis thaliana*. In this case, *oprF*, *leuD*, *recA* and *rpoD* were found to be stably expressed (Smith et al. 2018). Although all these results might seem disjointed, *rpoD* emerges as a common reference gene not only in three of the above-mentioned studies but also in the present one, and despite the dissimilar experimental conditions assayed.

To the best of our knowledge, only a couple of reports dealt with gene stability assessment in cold-adapted bacteria across different temperature conditions. For *Flavobacterium psychrophilum*, the etiological agent of cold-water disease in fish, nine commonly used reference genes were evaluated at 8 °C and 20 °C, including the genes *gyrA*, *recA* and *16S rRNA* selected in the present study (Hesami et al. 2011). The authors found *16S rRNA* to be the candidate gene with less variable expression and discarded *gyrA* and *recA* because of their upregulation in the cold. Another study recently published aimed to select reference genes in the psychrophilic and piezophilic strain *Shewanella psychrophila* WP2 isolated from west Pacific deep-sea sediment (Liu et al. 2018). The authors analyzed seven candidate genes, including *16S rRNA* and *gyrA*, at different growth temperatures (4 °C, 15 °C and 22 °C). In contrast to our results, they found *gyrA*, together with *gyrB*, as the most stable reference genes. Neither of these reports nor any other study of which we have knowledge has dealt with the stability-analysis of gene expression in Antarctic bacteria or with regard to the particular stressful condition of a cold-shock. Therefore, this work could serve as a guide for the selection of reference genes when growth has to be evaluated in relation to temperature stress in other psychrophilic and psychrotolerant species from the Antarctic environment.

Since using more than one pre-validated reference gene has been recommended (Vandesompele et al. 2002; Brudal et al. 2013), we would advise the use of a combination of two of the four genes validated in this study, *recA* and *ftsZ*, as endogenous controls for qPCR experiments concerning the temperature related physiology of the Antarctic isolate *Pseudomonas* sp. AU10. Both were well ranked in expression stability according to BestKeeper and geNorm and, unlike *16S*, they do not present the above-mentioned disadvantages of using rRNA genes.

To sum up, in this work we report the first study on the validation of reference genes under different temperature conditions, including low temperatures and cold-shock response, for the genus *Pseudomonas*. Our results may provide a starting point for the study of differential gene expression in Antarctic *Pseudomonas* and also for the validation of reference genes in other cold-adapted bacterial species. Antarctic bacteria are not expected to respond to cold in the same way as their temperate or tropical counterparts. Therefore, studying changes in gene expression during cold-shock and during sustained cold or warm temperatures is of paramount importance to shed light on how the most abundant extremophiles on Earth cope with temperature stress in a planet threaten by global climate change. **Online Resource 1. Specificity of the primer pairs used for RT-qPCR amplification.** A: melt curves obtained for the eight primer pairs; B: 10% polyacrylamide gel electrophoresis of the qPCR products corresponding to the candidate reference genes (MWM: HyperLadder 25bp, Bioline).

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