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Expression stability of 13 housekeeping genes during carbon starvation of *Pseudomonas aeruginosa*

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ABSTRACT

Quantitative real-time polymerase chain reaction (qRT-PCR) is a reliable technique for quantifying mRNA levels when normalised by a stable reference gene/s. Many putative reference genes are known to be affected by physiological stresses, such as nutrient limitation and hence may not be suitable for normalisation. In this study of *Pseudomonas aeruginosa*, the expression of 13 commonly used reference genes, *rpoS*, *proC*, *recA*, *rpsL*, *rho*, *oprL*, *anr*, *tipA*, *nadB*, *fabD*, *ampC*, *algD* and *gyrA*, were analysed for changes in expression under carbon starvation and nutrient replete conditions. The results showed that *rpoS* was the only stably expressed housekeeping gene during carbon starvation. In contrast, other commonly used housekeeping genes were shown to vary by as much as 10–100 fold under starvation conditions. This study has identified a suitable reference gene for qRT-PCR in *P. aeruginosa* during carbon starvation. The results presented here highlight the need to validate housekeeping genes under the chosen experimental conditions.

1. Introduction

One of the current, leading methods for defining the genetic response pathways of organisms upon exposure to different conditions is based upon the analysis of total mRNA, or transcriptomics. This technique is powerful in defining global changes in RNA expression levels. Nonetheless, such studies almost always require confirmation by more sensitive techniques where the method of choice is commonly quantitative reverse transcriptase PCR (qRT-PCR), especially for the analysis of relatively few gene targets and where sample material is limited. Sample preparation for qRT-PCR involves several steps that may introduce technical biases, and the accuracy of relative gene expression by qRT-PCR ultimately depends on an appropriate normalisation method to distinguish between true biological variation and that introduced by sample processing (Bustin et al., 2010; Radonic et al., 2004). Normalisation is typically performed using an internal control gene, often referred to as a housekeeping gene, which requires that the expression of the housekeeping gene to be constant under the experimental conditions used (Bustin et al., 2005; Huggett et al., 2005; Vandesompele et al., 2002). Many studies have indicated that even the most stably expressed housekeeping genes are differentially regulated under certain experimental conditions. Therefore, validation of the housekeeping gene is vital for robust analysis of gene expression.

Pseudomonas aeruginosa is a ubiquitous environmental microorganism and an opportunistic pathogen of concern that serves as a model organism for studying biofilm formation and quorum sensing signaling (De Kievit et al., 2001; Finnan et al., 2004; Lyczak et al., 2000; Stover et al., 2000). Genomic analysis suggests that about 5% of the *P. aeruginosa* genome is dedicated to regulatory functions (Finnan et al., 2004; Stover et al., 2000). The high proportion of regulatory genes may in part explain how *P. aeruginosa* manages to adapt to such a broad range of growth conditions and as such, considerable effort has be invested in defining the global response networks of *P. aeruginosa* under a range of conditions, including quorum sensing, biofilm development, adaptation to the host and viable but non nonculturable (VBNC) cells (Ramamurthy et al., 2014; Sauer et al., 2002; Schuster et al., 2003; Wagner et al., 2003; Welsh and Blackwell, 2016). VBNC is a state of dormancy where cells lose the ability to grow under standard culture conditions, whilst retaining features of viability, including a membrane potential, membrane barrier, nucleic acids etc. and can be induced by a range of stressors (Gonzalez-Escalona et al., 2006; McDougald et al., 1999; Ramamurthy et al., 2014; Vora et al., 2005). Whilst VBNC cells retain nucleic acids that can be detected by methods such as qRT-PCR, the mRNA, rRNA and have different degradation rates and hence this needs to be considered with investigating bacteria that exhibit such a state of dormancy (Weichart et al., 1997). RNA-

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based genotypic approaches targeting the detection of stress related genes in VBNC has been used in *Salmonella* spp. in biosolid qRT-PCR, for detecting the VBNC state of *Vibrio cholera Escherichia coli*, *Pseudomonas putida* and *Legionella pneumophila* (Buse et al., 2013; Dunaev et al., 2008; González-Escalona et al., 2006; Gunasekera et al., 2002; Vora et al., 2005; Yaron and Matthews, 2002). To facilitate those studies, several housekeeping genes have been identified for the opportunistic human pathogen *P. aeruginosa* under various experimental conditions. For example, a hallmark study investigated a panel of *P. aeruginosa* clones that exhibited various antibiotic resistance phenotypes, and found the *proC* and *rpoD* genes displayed the most stable expression (Savli et al., 2003b). Subsequently, many other genes have been reported to serve as a stably expressed internal controls under various conditions, including *rpoS* (Savli et al., 2003b), *proC* (Savli et al., 2003b), *recA* (Records and Gross, 2010; Takle et al., 2007; Zhao et al., 2011), *rpsL* (Dumas et al., 2006; Weir et al., 2008), *rho* (Theis et al., 2007), *oprL* (Joly et al., 2005), *anr* (Trunk et al., 2010), *tipA* (Theis et al., 2007), *nadB* (Ghosh et al., 2011), *fabD* (Savli et al., 2003b), *ampC* (Savli et al., 2003b), *algD* (Bragonzi et al., 2005) and *gyrA* (Bragonzi et al., 2005). Notably, several of these genes are either involved in, or affected by, metabolic processes, suggesting that their stable expression may not extend to experimental conditions that involve nutritional changes. While growth in the laboratory usually involves nutrient replete conditions, the vast majority of bacteria must also survive periods of nutrient deprivation, which has often been called the starvation response. We have recently described that carbon starvation provides a signal for *P. aeruginosa* to alter its cellular physiology from being part of a multicellular community, termed a biofilm, to actively disperse as planktonic single cells (Huynh et al., 2012). In some cases, the environmental or physiological trigger for the induction of biofilm dispersal has been determined.

In this study, we applied qRT-PCR under nutrient limiting conditions to quantify changes in the expression of 13 previously used housekeeping genes for *P. aeruginosa*, *rpoS* (Savli et al., 2003b), *proC* (Savli et al., 2003b), *recA* (Records and Gross, 2010; Takle et al., 2007; Zhao et al., 2011), *rpsL* (Dumas et al., 2006; Weir et al., 2008), *rho* (Theis et al., 2007), *oprL* (Joly et al., 2005), *anr* (Trunk et al., 2010), *tipA* (Theis et al., 2007), *nadB* (Ghosh et al., 2011), *fabD* (Savli et al., 2003b), *ampC* (Savli et al., 2003b), *algD* (Bragonzi et al., 2005) and *gyrA* (Bragonzi et al., 2005). Initially, testing and analysis of these putative reference genes showed that all but *rpoS* and *rpsL* were strongly repressed following 30 min of carbon starvation. More detailed analysis revealed a strong temporal regulation of *rpsL* during carbon starvation, and confirmed that *rpoS* represented the housekeeping gene of choice for qRT-PCR based analysis of carbon starvation. Finally, the housekeeping gene *rpoS* was used to quantify the differential expression of previously used controls. Thus, careful selection and validation of the house-keeping gene under the experimental conditions of the study must be considered.

2. Materials and methods

2.1. Growth conditions

P. aeruginosa and mutant strains were grown in Lysogeny-Broth (LB) (10 g L^{-1} Tryptone 5 g L^{-1} Yeast extract, 10 g L^{-1} NaCl) or maintained on LB plates containing 1.5% agar (*w*/*v*). Prior to each experiment, cultures were inoculated from freshly-streaked LB agar plates and grown overnight in M9 medium (50 mM K_2HPO_4, 50 mM KH_2PO_4, 0.25 mM NH_4Cl, 2 mM $MgSO_4$ and 100 μM $CaCl_2$) supplemented with 10 mM glucose as the sole carbon source and trace elements (final concentrations: 1.3 mM Na_2EDTA, 0.71 mM $FeCl_2$, 0.036 mM $ZnCl_2$, 0.015 mM $MnCl_2$, 0.004 mM H_3BO_3 and 0.084 mM $CoCl_2$) (Schleheck et al., 2009).

2.2. Sample preparation

Overnight cultures of wild-type *P. aeruginosa* strains were diluted to an OD_{600} = 0.01 into 10 mL fresh M9 medium supplemented with 10 mM glucose at 37 °C with shaking at 200 rpm (Ratek Orbital Mixer Incubator). These cultures were grown to an OD600 = 0.5 and collected by centrifugation (10 min 9000 × *g* at 4 °C), and washed three times with phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KH_2PO_4 and 2.7 mM KCl, pH 7.2). Bacterial cultures were then resuspended in 9 mL of either fresh M9 medium supplemented with 10 mM glucose, or with M9 salts without glucose, separately. Samples were harvested for the quantification of mRNA immediately, called here ($t = 0$), and following 30 min incubation at 37 °C with shaking 200 rpm ($t = 30$) (Fig. 1). To harvest mRNA, 1 mL of the cultures were resuspended in 2 mL of RNA protect bacterial reagent (Qiagen, Germany) and stored at −80 °C for RNA extraction later. Experiments were performed using independent biological triplicates (separate experiments), $n = 3$, in addition to technical replicates, for statistical analysis.

2.3. RNA extraction and purification

RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol (enzymatic lysis protocol) with on-column DNase digestion. Purified RNA was treated with a second round of DNase to remove any residual genomic DNA using a Turbo DNA-free kit (Ambion, U.S.A) at 37 °C for 30 min. RNA was analysed with a NanoDrop ND1000, to determine RNA concentration (A260/A280 ratio), and purity (A230/A260 ratio). RNA quality was verified using a Bioanalyzer (Agilent Technologies, Ramaciotti Centre for Genomics, Sydney, Australia).

2.4. Quantification of housekeeping gene expression by qRT-PCR

cDNA synthesis was performed using 1 μg purified total RNA using the iScript cDNA synthesis kit as recommended by the manufacturer (Bio-Rad, U.S.A). A negative control (NCT) was included for each sample by performing a reaction without the addition of the reverse transcriptase enzyme to control for contaminating genomic DNA. PCR reactions were performed using a CFX96 quantitative PCR detection system (Bio-Rad, U.S. A). PCR reactions contained 1 μL DNA template, 12.5 μL SsoFast Evagreen supermix (Biorad, U.S.A), 7.5 pmol of each primer and H_20 to a total volume of 20 μL in black, hardshell, 96 well plates containing white wells (Biorad, U.S.A). The reaction conditions were as follows: an initial activation cycle of 3 min at 98 °C, followed by 30 cycles of 60 °C for 5 s, 72 °C for 10 s with data acquisition, and 98 °C for 10 s. A melt curve analysis was performed using the temperature range 60 °C to 98 °C at 0.5 °C intervals to confirm the specific amplification of a single PCR product.

Unless otherwise indicated, the primers were designed for real-time PCR using NCBI primer-Blast (Table 1). Initially, the reaction efficiencies were calculated for each amplicon using the equation $E = 10^{-1/\text{slope}}$ from the slope of each calibration curve that was generated using a 500-fold serial dilutions of cDNA (Livak and Schmittgen, 2001), and primer pairs with equivalent efficiencies were used for analysis. Cycle-threshold (Ct) values were generated by the CFX manager™ software (Biorad CFX, U.S.A) and used to quantify the stability of the potential housekeeping genes by the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). Also, the Ct values were used to quantify the relative levels of gene expression by the $2^{-\Delta\Delta Ct}$ method with the *rpoS* gene as an internal control (Livak and Schmittgen, 2001). Data represent the mean value of three biological replicates, each measured as independent replicates ($n = 3$) with corresponding standard deviations. GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) was used to determine the statistical significance between the $2^{-\Delta\Delta Ct}$ of housekeeping genes by one-way ANOVA followed by Tukey's multiple comparison for differences. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $^{****}P < 0.0001$.