

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Identification and validation of reference genes to study the gene expression in *Gluconacetobacter diazotrophicus* grown in different carbon sources using RT-qPCR

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ARTICLE INFO

Article history:

Received 9 April 2012

Received in revised form 3 July 2012

Accepted 3 July 2012

Available online 16 July 2012

Keywords:

Gluconacetobacter diazotrophicus

Endophytic diazotrophic

Reference gene

ABSTRACT

Gluconacetobacter diazotrophicus strain PAL5 is a nitrogen-fixing endophytic bacterium originally isolated from sugarcane and later on was found to colonize other plants such as rice, elephant grass, sweet potato, coffee, and pineapple. Currently, *G. diazotrophicus* has been considered a plant growth-promoting bacterium due to its characteristics of biological nitrogen fixation, phytohormone secretion, solubilization of mineral nutrients and antagonism to phytopathogens. Reverse transcription followed by quantitative real-time polymerase chain reaction (RT-qPCR) is a method applied for the quantification of nucleic acids because of its specificity and high sensitivity. However, the decision about the reference genes suitable for data validation is still a major issue, especially for nitrogen-fixing bacteria. To evaluate and identify suitable reference genes for gene expression normalization in the diazotrophic *G. diazotrophicus*, mRNA levels of fourteen candidate genes (*rpoA*, *rpoC*, *recA*, *rpoD*, *fabD*, *gmk*, *recF*, *rho*, *ldhD*, *gyrB*, *gyrC*, *dnaG*, *lpxC* and *23SrRNA*) and three target genes (*matE*, *omp16* and *sucA*) were quantified by RT-qPCR after growing the bacteria in different carbon sources. The geNorm and Normfinder programs were used to calculate the expression stabilities. The analyses identified three genes, *rho*, *23SrRNA* and *rpoD*, whose expressions were stable throughout the growth of strain PAL5 in the chosen carbon sources. In conclusion our results strongly suggest that these three genes are suitable to be used as reference genes for real-time RT-qPCR data normalization in *G. diazotrophicus*.

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1. Introduction

Gluconacetobacter diazotrophicus is a strict aerobic N₂-fixing endophyte, originally isolated from roots and shoots of sugarcane plants grown in Brazil (Cavalcante and Dobereiner, 1988) and in other countries such as Argentina, Uruguay, Mexico, Cuba, United States, India and Canada (Dong et al., 1994; Baldani et al., 1997; Loganathan and Nair, 2003). *G. diazotrophicus* has been mainly found colonizing plants such as *Pennisetum purpureum* Schum (Reis et al., 1994; Baldani et al., 1997), *Ipomea batatas* Lamb (Paula et al., 1991), *Coffea arabica* L (Jimenez-Salgado et al., 1997), *Ananas comosus* L. (Tapia-Hernández et al., 2000), *Polycarpon succulentum* L., *Fagonia arabica* L., and *Malva parviflora* L. (Madhaiyan et al., 2004). Recently, the existence of *G. diazotrophicus* was reported in the rhizo-and-phylo spheres of a number of desert plants in Sinai environment (Hanna et al., 2012). This bacteria species is capable of oxidizing ethanol to acetic acid, tolerate high concentrations of glucose (Kesters et al., 2006; Nakano

and Fukaya, 2008) and to convert N₂ to NH₃ through biological nitrogen fixation (Cavalcante and Dobereiner, 1988; Kim and Rees, 1994). In addition, *G. diazotrophicus* can also promote biomass gain induced by hormone and/or siderophore production (Urquiaga et al., 1992; Logeshwaran et al., 2009). In 2009 the genome of *G. diazotrophicus* strain PAL5 was completely sequenced by Bertalan et al. (2009) and genes involved in nitrogen fixation, sugar metabolism, transport systems, biosynthesis of polysaccharides, quorum sensing and auxin biosynthesis were identified. The genome data are an important source of information that can be used to understand plant–bacteria interactions which could improve productivity of sugarcane and other biotechnological applications.

Different methods have been used to study gene expression in bacteria, RNase protection assays, cDNA-AFLP, northern hybridization, microarray and reverse transcription quantitative polymerase chain reaction (RT-qPCR) that allow the identification of differentially expressed genes and it is best applied when there are many genes to be tested in many conditions (Freeman et al., 1999).

Several studies in recent years have undoubtedly shown that stability assessment of internal reference genes for each experimental

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condition is a prerequisite for valid normalization of transcriptional data and reliable gene expression results (Pfaffl et al., 2004; Dheda et al., 2005). Although several studies have proposed the use of two or more genes, especially if only small changes are to be detected (Theis et al., 2007; Reiter et al., 2011), several reference genes have been employed to analyze gene expression either in Gram-negative or in Gram-positive bacteria (Nielsen and Boye, 2005; Nieto et al., 2009; Metcalf et al., 2010; Thomas et al., 2011; Hommais et al., 2011).

Previous studies with other bacteria have highlighted the difficulty and relevance of finding a good internal control for RT-qPCR experiments and transcript level measurements (Desroche et al., 2005). Several steps are required to determine such a good control, at least three are very important: (a) choose presumed reference control genes; (b) ensure that their expression is not affected by experimental condition; and (c) ensure that PCR efficiency is over 90% for internal control for all tested genes (Ginzinger, 2002). However, recent studies have shown that the control in RT-qPCR experiments is influenced by metabolic, growth phase or experimental conditions (Dheda et al., 2004; Huggett et al., 2005).

The evaluation of a reference gene is therefore mandatory and has been performed for several genes from different bacteria including *Acidithiobacillus pleuropneumoniae* (Nielsen and Boye, 2005), *Acidithiobacillus ferrooxidans* (Nieto et al., 2009), *Lactobacillus plantarum* (Fiocco et al., 2008), *Clostridium difficile* (Metcalf et al., 2010), *Zobellia galactanivorans* (Thomas et al., 2011), *Dickeya dadantii* (Hommais et al., 2011), *Bacillus cereus* (Reiter et al., 2011), *Escherichia coli* (Zhou et al., 2011) and others. However, these studies have not revealed an ideal universal reference gene. Moreover, the use of an inappropriate internal control has led to a significant difference and even incorrect results (Dheda et al., 2005). Therefore, each internal control gene needs to be validated within a given experimental condition before it can be used for normalization of RT-qPCR data (Dheda et al., 2005).

Alqueres et al. (2010) used the 23S rRNA for normalization of the relative expression of its respective mRNAs when investigated the influence of reactive oxygen species production and detoxification in *G. diazotrophicus*. Conversely, there is evidence showing differential expression of ribosomal RNA gene (*rrs*) under cellular stress (Stevenson and Weimer, 2005). In addition, use of *rrs* as a reference gene has been challenged because it is a very abundant species of RNA present at concentrations far outside of the calibration ranges indicating that the stability of this gene has not been validated.

In this study, we aimed to identify genes to be used as internal reference controls envisaging studies of gene expression in the diazotrophic bacteria *G. diazotrophicus* cultivated under different grown conditions.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Gluconacetobacter diazotrophicus strain PAL5 (BR11281) was obtained from Embrapa Agrobiologia Culture Collection. The bacteria were grown in LGI medium (Magalhães et al., 1983) and LGI medium modified by replacing crystal sugar by 0.5% of mannitol, glycerol and glucose. The cells were incubated at 30 °C, at 200 rpm for 48 h (non-nitrogen fixing condition). Cells (5.5×10^7 cells/ml) were centrifuged at $4,000 \times g$ for 5 min and the pellet was used for total RNA extraction.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated with Trizol according to the manufacturer's protocol. RNA purity and integrity appropriate for downstream RT-qPCR applications were confirmed by measuring the ratio of absorbance at 260 nm/280 nm and 260 nm/230 nm and by visualization on a 0.8% agarose gel. First-strand cDNA synthesis was carried out using the

Superscript™ III Reverse Transcriptase kit (Invitrogen) performed according to the manufacturer's manual. Reverse transcription reactions were carried out with 3 µg of total RNA and 250 ng random primers according to the manufacturer's protocol. The cDNA was diluted 1:20 and then used in RT-qPCR reaction.

2.3. Choice of reference gene candidates and primer design

The candidate reference genes were chosen from various functional classes present in many bacteria, in order to minimize the risk of co-regulation. Fourteen genes belonging to nine different pathways were selected (Table 1). These genes were used to identify the orthologous genes in the *G. diazotrophicus* genome, and only the sequences with high similarity with the query reference genes were selected as potential candidates.

Primers for RT-qPCR were designed in order to have a length of about 20–25 bases, a G/C content of over 50%, T_m of 60 °C and the length of the PCR product ranged between 102 and 150 bp. Primer designer software (Primer3 plus) was used to select primer sequences (Rozen and Skaletsky, 2000).

2.4. RT-qPCR experiment

RT-qPCR was performed on the 7500 Fast Real Time PCR system using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR reaction consisted of 7.5 µl of SYBR Green PCR Master Mix, different concentrations of forward and reverse primers were utilized (Table 2), and 5.0 µl of 1:20-diluted cDNA template in a total volume of 15 µl. Cycling was performed using the default conditions of the 7500 Software v 2.0.5: 2 min at 95 °C, followed by 40 cycles of 20 s at 95 °C and 30 s at 58 °C. All RT-qPCR assays were carried out using three technical replication and non-template control, as well as three independent cDNA syntheses.

2.5. Analysis of gene expression stability and relative quantification

The C_q values from the fourteen reference genes evaluated in this study, obtained from the Miner software (Vandesompele et al., 2002), were imported by the qBase software v1.3.5 (Hellemans et al., 2007) and converted into non normalized relative quantities, corrected by

Table 1
Function of genes used for determination of an internal control.

Gene name	Definition	Known essential functions
<i>23S rRNA</i>	Ribosomal RNA subunit 23S	Structural component of the 23S ribosomal subunit
<i>dnaG</i>	Primase	Initiation of chromosomal DNA replication
<i>fabD</i>	Malonyl CoA-acyl carrier protein transacylase	Fatty acid biosynthesis
<i>Gmk</i>	Guanylate kinase	Nucleotide metabolism
<i>gyrB</i>	Gyrase subunit B	DNA replication, supercoiling of chromosomal DNA
<i>gyrBC</i>	Gyrase subunit B	DNA replication, supercoiling of chromosomal DNA
<i>ldhD</i>	D-Lactate dehydrogenase	Sugar catabolism pathway
<i>lpxC</i>	UDP-3-O-acyl N-acetylglucosamine deacetylase	Catalyzes the second step in lipid A biosynthesis
<i>recA</i>	DNA recombination	Catalyzes the pairing of ssDNA with complementary regions of dsDNA
<i>recF</i>	DNA recombination	DNA replication and repair protein
<i>Rho</i>	Rho termination factor	Transcription termination factor
<i>rpoA</i>	RNA polymerase Sigma α factor	Initiation of transcription
<i>rpoC</i>	RNA polymerase, β subunit	Initiation of transcription
<i>rpoD</i>	RNA polymerase Sigma factor RpoD	Binding of RNA polymerase and initiation of transcription

Table 2
Primer sequences design for candidate normalization genes.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon length (bp)	Concentration utilized (pmol)
23S _{rRNA}	GTGAGACAGTTCGGTCCTATC	CGATTATCCCCTCAGACTTAG	150	3
<i>dnaG</i>	GCAGCCGAAATACCTCAACG	ATGTAGCCTTCGACCACCAC	147	9
<i>fabD</i>	GAATAACGAGGACGCCATGC	TCGACTTCTGGAACACCTC	126	9
<i>Gmk</i>	GACGTCATCAGCCTGTTCTG	GATCAAATTCCTGCCAGTGC	145	9
<i>gyrB</i>	CGCAAGGACCTGAAAGACAC	GACCAGGAAGATCTCGGTCTC	114	11
<i>gyrBC</i>	GAGACGATGCTGTGCTTCAC	AGGGACTGGCTGCTCTTCT	137	11
<i>ldhD</i>	AACATCACCGCCCAGTACCT	GGATCGTGCCCTTAGGTTG	130	9
<i>lpxC</i>	TGACCCTGTCCATCGACTTC	CAGGGTGAAGGTCCGACAAT	113	9
<i>recA</i>	CTGATCCCTTCGCCATACAC	TCAAGGACAAGGACGAGGTC	115	9
<i>recF</i>	TTCAGGAACTACCGGCACCT	CGACAGGGCTTCAGAAGAT	102	9
<i>Rho</i>	GCAACTCGGAACTCATCTCT	GGACCCACATCTTCGACAGT	132	11
<i>rpoA</i>	GGTCGACGAACTGGAACGT	CATCTCTGCTCGGTCTTCT	109	11
<i>rpoC</i>	GCCGTTTACTACTCGAAGC	TCCAGGATGTCCCATACCTC	105	11
<i>rpoD</i>	ACAACGACACCACTCTGCTG	CTCCGACGACATCTGATCCT	131	9

the amplification efficiency values for each gene, using the formula $Q = E^{\Delta Cq}$, where: E corresponds to the gene amplification efficiency and ΔCq is the sample with the lower expression between the data group subtracted from the Cq value of the referred sample. These non-normalized data were independently imported to geNorm Software v3.5 and NormFinder Software (Andersen et al., 2004), in order to determine the most suitable reference genes for the experimental conditions applied.

The expression profile analysis of the target genes (*omp16*, *matE* and *sucA*) was carried out with the same cDNA samples used to evaluate the reference genes. The RT-qPCR condition was performed following the same criteria used for the reference genes. Analysis for relative quantification (RQ), the Cq and efficiency values were determined using the Miner software (Zhao and Russel, 2005). These values were used to calculate the relative expressions on qBase software v1.3.5.

3. Results

3.1. Expression level of candidate reference genes

The total RNA was isolated from *G. diazotrophicus* grown in four carbon sources and the cDNA were used to compare the expression level of fourteen different chosen reference genes.

The C_T value (average of three replicates) for each reference gene is shown in Fig. 1. C_T values were in a range of 10.2 to 31.2 indicating quite different expression levels. The 23S_{rRNA} was the highest expressed gene as compared to *gmK*, which showed very high C_T values. The

rpoD, *rpoA*, *rpoC* and *rho* genes showed intermediate expression levels with C_T values varying between 15 and 20 (Fig. 1).

3.2. Expression stability of candidate reference genes

The expression stability of the candidate reference genes in *G. diazotrophicus* cultivated in LGI with different carbon sources was calculated by the geNorm as shown in Fig. 2. The genes 23S_{rRNA} and *rho* showed the lowest average expression stability (M) (0.23) and were therefore indicated to be the two most stable reference genes according to the geNorm analysis. The genes *dnaG*, *rpoC* and *gmK* were considered the least stable ones among the fourteen genes analyzed. However, the average expression stability M value for all genes was below 1.5, a cut-off value suggested by Vandesompele et al. (2002). The lowest value indicates the more stable gene across the experimental assay samples (Fig. 2).

The geNorm algorithm analyzes two parameters for the most suitable gene determination: a) the average expression stability value (M) after stepwise exclusion rounds and, b) a normalization factor (NF) based on geometric mean of the expression values of the set of reference genes tested (Fig. 2). The pair-wise variation (V_n/V_{n+1}) is assessed, which calculates the effect of the next most stable gene addition on NF and determines the optimal number of genes for accurate normalization (Fig. 3). The pair-wise variation results showed values in the range of 0.06 for $V_2/3$ and 0.15 for $V_4/5$. Furthermore, the genes 23S_{rRNA} and *rho* were insufficient gene numbers to gene expression normalization since the $V_2/3$ value was 0.06 below the cut-off from which it is not necessary to include additional reference gene.

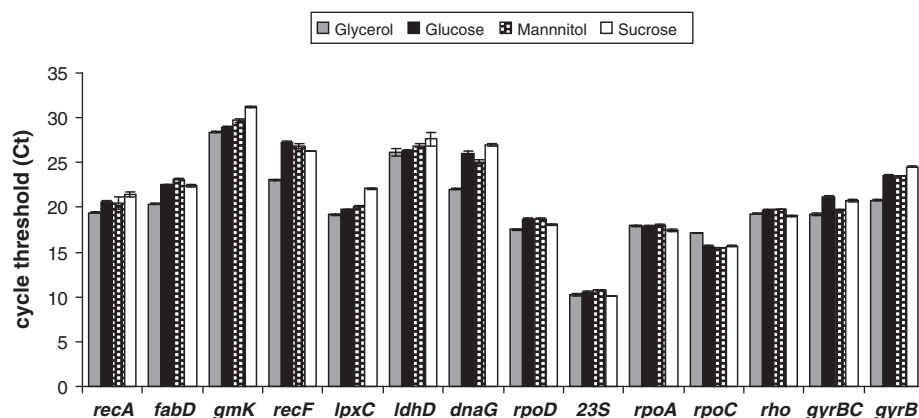


Fig. 1. The cycle threshold (C_T) of fourteen presumed reference genes determined in *G. diazotrophicus* strains Pa15 grown at four carbon sources. For each condition, C_T was measured from three independent cDNAs.

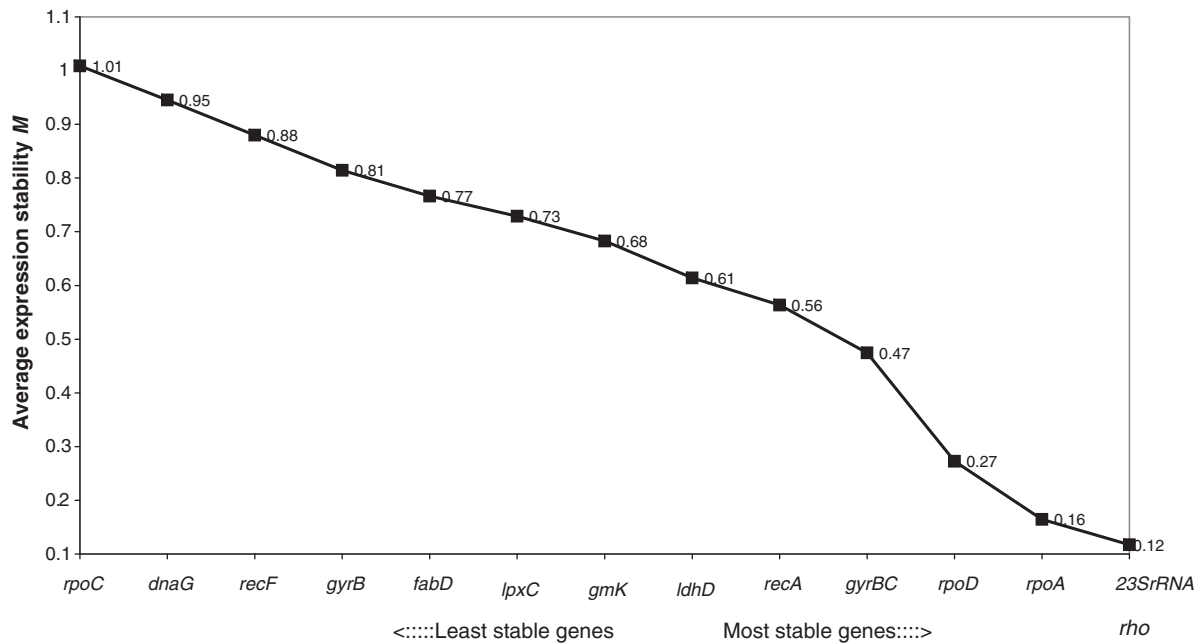


Fig. 2. Gene expression stability analysis of the fourteen candidate reference genes using the geNorm software. Average expression stability values (M), starting from the least stable gene at the left, ending with the two most stable genes on the right.

Altogether, the pair-wise variations showed consistency with all genes below the cut-off value, as suggested by geNorm developers ($V_n/V_{n+1} \leq 1.5$) (Vandesompele et al., 2002). Taken together, the M value of all genes presents not only acceptable stability, despite some differences on its expression evaluated by geNorm, but also a great pair-wise variation results for a suitable NF calculation.

Additionally to geNorm analyses, the same data set was also analyzed using the NormFinder software (Andersen et al., 2004) to determine the expression stability of candidate reference genes. NormFinder suggested *recA* and *rpoD* as the most suitable reference genes (Fig. 4). Although the geNorm and NormFinder results have shown small differences in the reference genes rank, the genes *recA*, *rpoD*, and *GyrBC* were always

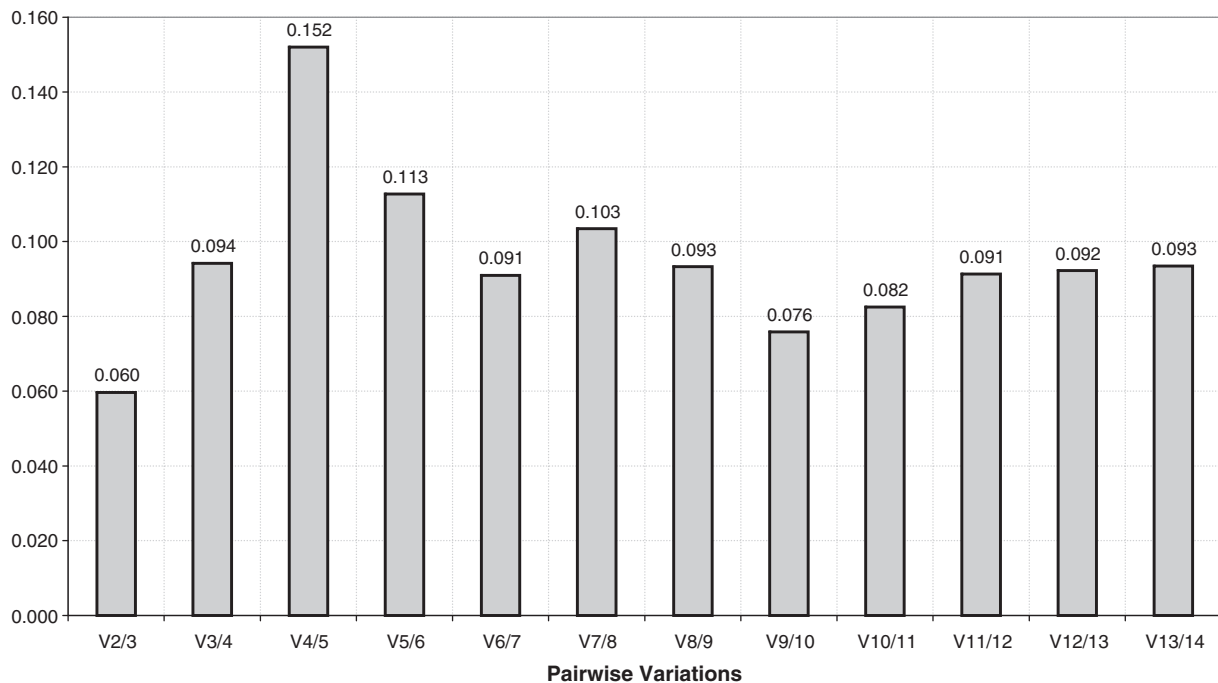


Fig. 3. Determination of optimal number of control genes for normalization. Each bar represents changes in normalization accuracy when stepwise adding more reference genes according to ranking in Fig. 2. V2/V3, pair wise variation between the two most stable genes (23SrRNA and *rho*), plus the third most stable gene (*rpoA*). To meet the recommended cut-off value of 0.15 two or three genes would be satisfactory for normalization.

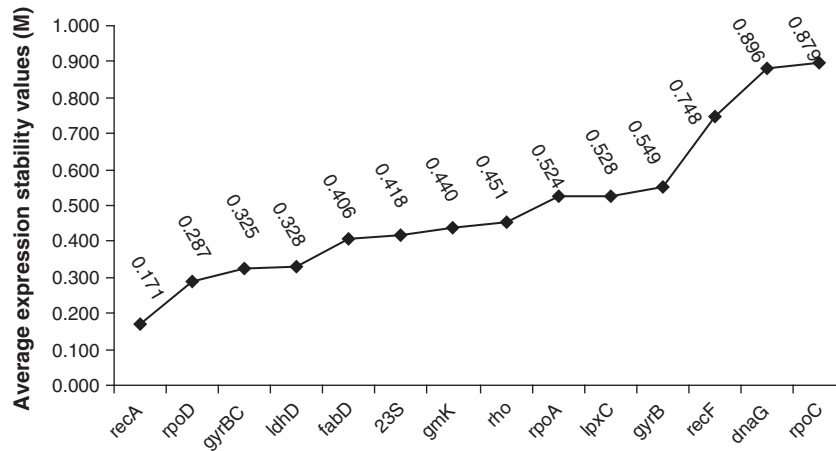


Fig. 4. Gene expression stability for the fourteen candidate reference genes using the NormFinder software.

within the five most stable genes in both algorithms, which gives consistency to the analyses.

3.3. Relative quantification (RQ) of *matE*, *omp16* and *sucA*

The isolation of *G. diazotrophicus* transcript-derived fragments differentially expressed by cDNA amplified fragment length polymorphism (cDNA-AFLP) technique, previously performed, allowed the identification of genes homologous to multi antimicrobial extrusion protein (*matE*) (GDI_1282 ORF), a gene homologous to outer membrane lipoprotein (*omp16*) (GDI_0807 ORF) and a gene homologous to gene 2-oxoglutarate dehydrogenase (*sucA*) (GDI_1416 ORF) (Unpublished data). The transcript levels of these three genes were measured by RT-qPCR during the exponential growth phase of the strain PAL5. The *rho* and *rpoD* genes were used as the reference for data normalization. Sucrose was used as calibrator (RQ=1.0) to relative quantification (Fig. 5).

The expression levels of all genes were affected by the carbon sources as compared to the sucrose control. When *G. diazotrophicus* was cultivated in LGI medium supplemented with glycerol as carbon source, the expression level of *matE* gene was increased about 3.5 times. Similar results were observed with mannitol and glucose, however the transcript level was only increased 1.5-fold when compared to sucrose. On the other hand, the *sucA* expression profile was down regulated throughout all treatments (Fig. 5).

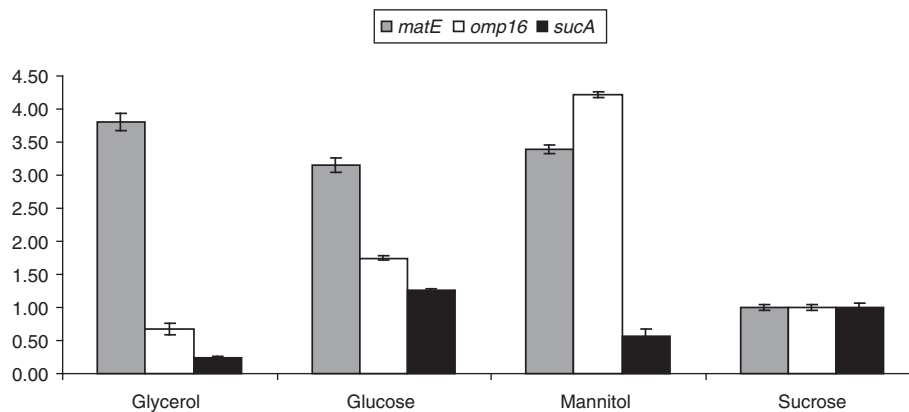


Fig. 5. Relative expression of *G. diazotrophicus* cultivated in LGI medium supplemented with different carbon sources. Gene expression was quantified using RT-qPCR and the comparative threshold ($\Delta\Delta C_T$) method using the qBase software v1.3.5. The *rho* and *rpoD* genes were used as reference genes for data normalization, and sucrose expression was used as the calibrator (control) taken as 1. Bars indicate standard error (SE).

4. Discussion

The *G. diazotrophicus* whole genome sequence (Bertalan et al., 2009) opens new opportunities for post-genomic investigations mainly to better understand the plant–bacteria interaction as a means to improve sugarcane crop production and other biotechnological applications.

RT-qPCR is the method of choice for nucleic acid quantification because it is a specific and high sensitive technique (Bustin et al., 2009). In the field of functional genomics, this technique has been intensively used for gene expression investigation in diverse biological systems such as plants (Long et al., 2011) and bacteria (Theis et al., 2007; Ritz et al., 2009; Oliveira et al., 2010; Alqueres et al., 2010). However, to overcome the experimental variations which lead undoubtedly to incorrect interpretation of the results, the choice and use of appropriate reference gene are extremely important (Vandesompele et al., 2002).

The use of reference genes which have a stable expression between the compared groups is crucial for gene expression investigation (Nieto et al., 2009; Cinar et al., 2012). Previous studies have shown that different reference genes can modify the outcome and conclusions of a specific study (Glare et al., 2002; Dheda et al., 2004; Kriegova et al., 2008).

To select a suitable reference gene to *G. diazotrophicus* we assayed the expression stability of fourteen genes.

The pair-wise variation between these genes defines the variable V, and the lower variable V is the lower is the variation. The results have shown that the pair-wise variation values (V_n/V_{n+1}) for fourteen genes were below the cut-off of 0.15 in all situations (V_n/V_{n+1})

except the combination V4/5 where the value is 1.52 (Fig. 3), which is suggested by the geNorm developers (Vandesompele et al., 2002). These results suggest that the use of the optimal number of reference genes and implies that the employment of the two most stable reference genes (*rpoD* and *rho*), or *23S*rRNA and *rho* plus the third most stably gene (*rpoA*) is sufficient to result in a reliable gene expression normalization of target genes.

Taking into account both the geNorm and NormFinder results we recommend the use of the three most stably expressed reference genes *rho*, *23S*rRNA and *rpoA* as V2/3 (0.06) or only (*rho*, *rpoD*) which has the expression stability *M* value of 0.12, below the 1.5 cut-off suggested by Vandesompele et al. (2002). Similar results have been observed by Theis et al. (2007). These authors observed that a group of genes, including *rho* gene was less affected by rhodamine 6G and crystal violet, however when *Staphylococcus aureus* culture was grown in the presence of ethidium or berberine, while the gene *rho* lost stability.

The *23S*rRNA was shown to be stably expressed in *G. diazotrophicus* and was used before to evaluate the expression of the influence of ROS production and detoxification in these nitrogen-fixing bacteria. However the initial analyses of *23S*rRNA *C_T* values revealed a very high expression level and take into account that most of the target genes do have not similar transcript abundance, the use of *23S*rRNA should be used with care to avoid underestimation of the target gene (Desroche et al., 2005; Theis et al., 2007; Ritz et al., 2009; Nieto et al., 2009; Reiter et al., 2011).

rpoC and *dnaG* have previously been selected as promising reference genes in *Acidithiobacillus ferrooxidans* (Nieto et al., 2009) and *Zobellia galactanivorans* gene expression (Thomas et al., 2011), respectively. Our results showed that *rpoC* and *dnaG* were highly unstable in *G. diazotrophicus* cultivated in LGI medium containing different carbon sources. Indeed, reference genes may vary substantially depending on the experimental conditions, especially in the human organism (Vandesompele et al., 2002; Dheda et al., 2004; Dheda et al., 2005; Theis et al., 2007; Piehler et al., 2010). In bacteria previous studies clearly showed that there is no consensus for which gene is the best internal control and the selection of specific reference genes for nitrogen-fixing bacteria such as *G. diazotrophicus* has not been so far accomplished.

Our results underline the importance of previous studies to select valid endogenous controls suitable for normalization of RT-qPCR data. The genes *matE*, *omp16* and *sucA* previously characterized as differentially expressed in *G. diazotrophicus* cultivated in different carbon sources (unpublished data) allowed the validation of the *rho* and *rpoD* genes selected by geNorm software as suitable reference genes for the experimental conditions tested.

Interestingly, the expression profile of the target genes varied with the carbon source present in LGI medium. The *matE* gene showed the highest expression on glycerol, whereas *omp16* and *sucA* were more expressed on mannitol and sucrose, respectively.

Studies on other bacteria have showed that the genes *matE*, *omp16* and *sucA* may be regulated depending on the environmental and/or experimental condition (Kaatz et al., 2005; Baldo et al., 2010; Pasquevich et al., 2012; Liedert et al., 2012). The results suggested that there is a specific regulation in the energetic metabolism of the cell which could explain the changes observed for each gene in its preferential carbon source. It is already known that *G. diazotrophicus* is able to metabolize different carbon source including glucose, glycerol and mannitol.

5. Conclusions

In this work, a RT-qPCR was applied as a rapid and sensitive method to determine the change of transcript level in the N₂-fixing endophyte *G. diazotrophicus*. The *rho*, *rpoD* and *23S*rRNA genes of *G. diazotrophicus* are suggested as good candidates to evaluate the growth of the bacteria in different carbon sources. Therefore, this study provides a set of reference genes for *G. diazotrophicus* gene that can be applied to evaluate the

expression of target genes in this bacteria cultivated in different physiological growth conditions and eventually in other diazotrophic organisms closely related to *G. diazotrophicus*.

Acknowledgments

We thank Robert Michael Boddey for previous English reading of this manuscript. This work was supported by Embrapa Agrobiologia and the projects: Universal (479968/99-8), and INCT-FBN (573828/2008-3).

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