Usefulness of a real-time PCR platform for G + C content and DNA–DNA hybridization estimations in vibrios

Ana Paula B. Moreira,¹ Nei Pereira Jr² and Fabiano L. Thompson¹

Correspondence

Fabiano L. Thompson
fabiano.thompson@biologia.ufrj.br

¹Laboratory of Microbiology, Institute of Biology, Federal University of Rio de Janeiro (UFRJ), Brazil

²Laboratory of Bioprocesses, School of Chemistry, Federal University of Rio de Janeiro (UFRJ), Brazil

The aim of this study was to evaluate the utility of a real-time PCR platform to estimate the DNA G + C content (mol%) and DNA–DNA hybridization (DDH) values in the genus *Vibrio*. In total, nine vibrio strains were used to determine the relationship between genomic DNA G + C content and T_m (°C). The T_m and HPLC datasets fit a linear regression curve with a significant correlation coefficient, corroborating that this methodology has a high correlation with the standard methodology based on HPLC ($R^2=0.94$). Analysis of 31 pairs of vibrios provided a wide range of ΔT_m values, varying between 0.72 and 12.5 °C. Pairs corresponding to strains of the same species or strains from sister species showed the lowest ΔT_m values. For instance, the ΔT_m of the sister species *Vibrio harveyi* LMG 4044^T and *Vibrio campbellii* LMG 11216^T was 5.2 °C, whereas the ΔT_m of *Vibrio coralliilyticus* LMG 20984^T and *Vibrio neptunius* LMG 20536^T was 8.75 °C. The mean ΔT_m values corresponding to pairs of strains with DDH values lower than 60 % or higher than 80 % were, respectively, 8.29 and 2.21 °C (significant difference, $P<0.01$). The high correlation between DDH values obtained in previous studies and the ΔT_m values ($R^2=0.7344$) indicates that the fluorimetric methodology is a reliable alternative for the estimation of both DNA G + C content and ΔT_m in vibrios. We suggest that strains of the same *Vibrio* species will have less than 4 °C ΔT_m . The use of a real-time PCR platform represents a valuable alternative for the development of the taxonomy of vibrios.

INTRODUCTION

Prokaryotic taxonomy has been based on a polyphasic approach since the 1970s (Colwell, 1970). As it produces an accurate, reproducible, informative and pragmatic framework for species identification and classification, this is a consensus approach among microbial systematists (Vandamme *et al.*, 1996; Garrity *et al.*, 2004). DNA–DNA hybridization (DDH) plays a key role in polyphasic taxonomy and is required for the classification of novel species which share greater than 97 % 16S rRNA gene sequence similarity with known species (Tindall *et al.*, 2010; Stackebrandt *et al.*, 2002). DDH may also be required for the identification of strains that belong to complex taxonomic groups, e.g. the vibrio species core group (Thompson *et al.*, 2007), the *Burkholderia cepacia* complex (Coenye *et al.*, 2001) and the *Bacillus cereus* species group (Guinebreière *et al.*, 2008).

The taxonomy of vibrios has been under intensive analysis in recent years, with the description of several new taxa and

the improvement of tools for identification and classification (Chimetto *et al.*, 2010). At the time of writing, vibrios include six genera: *Vibrio* (Baumann & Schubert, 1984), *Photobacterium* (Baumann & Schubert, 1984), *Salinivibrio* (Mellado *et al.*, 1996), *Grimontia* (Thompson *et al.*, 2003a), *Enterovibrio* (Thompson *et al.*, 2002) and *Aliivibrio* (Urbanczyk *et al.*, 2007). Currently 128 vibrio species have been described (<http://www.vibriobiology.net/>). Vibrios are ubiquitous in the marine environment and appear to have a pivotal role in the health of marine life, most notably corals (Chimetto *et al.*, 2009; Thompson *et al.*, 2010). In general, the classification of vibrios still requires the application of DDH. In order to perform DDH experiments, dedicated equipment (e.g. microplate readers) and know-how are necessary (Ezaki *et al.*, 1989), with ΔT_m estimations following the classic methodology of De Ley *et al.* (1970). It is also necessary to determine the genomic DNA G + C content of the target strains prior to DDH experiments in order to set the temperature for optimal renaturation. This step is usually performed by HPLC (Tamaoka & Komagata, 1984) or by thermal spectrophotometry (De Ley *et al.*, 1970). Although the methodologies for DDH and T_m determination are robust, they are

Abbreviation: DDH, DNA–DNA hybridization.

A supplementary table and figure are available with the online version of this paper.

time-consuming, require several dedicated equipments and expensive reagents. ΔT_m determination has not been used frequently as a parameter in vibrio species identification and classification, in spite of its requirement for species description (Stackebrandt *et al.*, 2002). The ΔT_m is defined as the difference between the melting temperatures of the hybrid DNA and the homologous DNA under standard conditions determined as the temperature at which 50 % of the double-strand DNA is in the form of single strands. This parameter has been proven to be valuable in the taxonomy of a variety of bacterial groups (Rosselló-Mora & Amann, 2001). The ΔT_m values correlate well with the DDH values and it is expected that strains with >70 % DDH relatedness will have <5 °C ΔT_m . It is clearly possible to deduce the DDH values from the measured ΔT_m . The aim of this study was to evaluate the usefulness of a real-time PCR platform for DNA G+C content and ΔT_m determination in vibrios. Gonzalez & Saiz-Jimenez (2002, 2005) first proposed the use of a real-time PCR platform for the identification and classification of the genera *Thermococcus* and *Pyrococcus*. We now evaluate their methodology for the taxonomy of vibrios.

METHODS

The vibrio strains used in this study were taxonomically characterized in several previous studies (Supplementary Table S1, available in IJSEM Online). In total, 31 pairs of strains with DDH relatedness values between 99 and 22 % were analysed, to cover a wide range of values. This wide range allowed a robust statistical analysis of the dataset. The ΔT_m derived from the renaturation of DNA samples with remote taxonomic relationship, e.g. between strains of different genera, loses accuracy (De Ley *et al.*, 1970). Thus, pairs of strains with DDH relatedness values below 20 % were not included in this study. Total genomic DNA was extracted following the methodology described previously (Pitcher *et al.*, 1989). DNA concentration and purity were measured in a biophotometer (Eppendorf). The real-time platform ABI 7500 (Applied Biosystems) was used in all experiments following the methodology described by Gonzalez & Saiz-Jimenez (2005).

DNA G+C content (mol%) was estimated according to Gonzalez & Saiz-Jimenez (2002). The 96-well optical plates were used with appropriate optical adhesives. Initially, each well was filled with a total volume of 25 µl containing 2 µg DNA in 0.1 × SSC (standard saline citrate buffer), and SYBR Green I (SG I; Invitrogen) at a final dilution of 1:100 000. The sealed plate was inserted into the ABI 7500. Denaturation ramp was settled at the step and hold mode. Heating rate was 0.2 °C s⁻¹ with fluorescence decay measurement at each 0.2 °C step, during a 12 s hold interval, between 25 and 99.8 °C. The experiments were performed in four replicates, including wells without DNA as negative control. The T_m was obtained with the aid of the ABI 7500 software v.2.0.1. The T_m is obtained graphically as the point of inflexion (mid-point) between the lower and upper asymptotes of the sigmoid absorbance (A_{260}) curve of thermal denaturation, or as the maximum derivative of this curve (Ritz *et al.*, 1997). The same principle was applied to obtain the T_m from the thermal denaturation curve, in the presence of SG I, generated on the ABI 7500. The fluorescence/signal is maximum at the beginning of the denaturation and is minimum in the end of the denaturation. Changes in fluorescence during thermal denaturation of DNA determine a curve with the inverted profile of that determined by changes in absorbance. The curve corresponding to the first

derivative, in this case negative, of the denaturation curve was generated with the help of the ABI 7500 software v.2.0.1 (-dF/dT; where dF=derivative from fluorescence and dT=derivative from time). The T_m was considered the peak of this curve, as generated by this equation. It was considered the arithmetic mean of the results of the four replicates of each sample. The T_m obtained for each strain was correlated with the actual G+C content according to data from the literature obtained by HPLC analysis (Brenner *et al.*, 1983; Gomez-Gil *et al.*, 2004b; Hedlund & Staley, 2001; Sawabe *et al.*, 1998; Thompson *et al.*, 2003c,e). We constructed a standard curve of T_m (°C) versus G+C content, including 9 strains of pathogenic vibrios of corals with G+C contents between 41.5 and 50.4 mol%.

The methodology for ΔT_m determination consisted of three successive steps. Firstly, 2.5 µg of each test or reference DNA was dissolved in 22.5 µl 0.1 × SSC, in a 96-well plate (ABI Prism Optical Reaction Plate). The plate was covered with an optical adhesive and incubated in an ABI 9700 thermocycler. After ten minutes of denaturation at 99 °C, hybridization was performed by keeping the plate for 8 h at optimal renaturation temperature [$T_{or}=0.51 \times (\% \text{ G+C}) + 47.0$], according to De Ley *et al.* (1970). Cooling was carried out slowly, decreasing the temperature by 10 °C of the initial T_{or} each 1 h down to 25 °C. The mixture was kept at 4 °C until the next step. In the second stage, 2.5 µl SG I was added (final concentration 1:100 000). The plates were again covered with optical adhesive. The thermal denaturation of the labelled mixture (25 µl) was performed in the ABI 7500. This instrument performed heating at 0.2 °C s⁻¹ and fluorescence was measured at each 0.2 °C, during 12 s, between 25 and 99.8 °C. Thus, 375 measurements of fluorescence were obtained, given in arbitrary units of fluorescence, according to the optical calibration of the equipment and the measurements of the negative controls. The ABI 7500 software v.2.0.1 was used for data collection and processing (Applied Biosystems). Each experiment was performed in at least three replicates. Positive and negative controls for background were also included in each experiment. In the third step, the melting temperature of the hybrid DNA (ssDNA of organism A hybridized with ssDNA of organism B) and reference DNA (ssDNA of organism A hybridized with ssDNA of organism A) was obtained. The T_m is obtained as the temperature corresponding to a 50 % decrease in fluorescence. It was considered the arithmetic mean of the results of the replicates of each experiment. The ΔT_m between the hybrid and the reference DNA was obtained by calculating the difference between their T_m values. In order to obtain a relationship between the ΔT_m data and DDH data (obtained in previous studies), the two datasets were compared and a regression curve was generated.

RESULTS AND DISCUSSION

The determination of DNA G+C content (mol%) by a fluorimetric methodology using the ABI 7500 is based on the construction of standard curves correlating the T_m values of species whose G+C content is known with the respective G+C content obtained from the literature. In total, nine vibrio strains were used to determine the relationship between G+C content and T_m (°C). The vibrios had G+C contents between 41.5 and 50.4 mol%. The T_m values for *Vibrio cyclitrophicus* LMG 21359^T (41.5 mol% G+C), *Vibrio hispanicus* LMG 13240^T (42.8 mol% G+C), *Vibrio kanaloae* LMG 20539^T (44.7 mol% G+C) and *Vibrio furnissii* LMG 7910^T (50.4 mol% G+C) were, respectively, 76.9, 77.4, 78.9 and 82.2 °C. The T_m dataset fit a linear regression curve with a significant correlation coefficient (Fig. 1; $R^2=0.94$). This suggests agreement between the real-time platform data and the HPLC measurements, and

Fig. 1. Regression curve of G+C content and T_m values (expressed as mean $\pm$ SD of four replicates). The curve equation and R^2 coefficient are shown. 1, *V. cyclitrophicus* LMG 21359^T (41.5 mol% G+C; mean of Hedlund & Staley, 2001, and Thompson *et al.*, 2003e); 2, *V. hispanicus* LMG 13240^T (42.8 mol% G+C; Gomez-Gil *et al.*, 2004b); 3, *V. haliotocoli* LMG 18542^T (43.1 mol% G+C; Sawabe *et al.*, 1998); 4, *V. orientalis* LMG 7897^T (44.4 mol% G+C; Thompson *et al.*, 2003e); 5, *V. chagasii* LMG 21353^T (44.6 mol% G+C; Thompson *et al.*, 2003e); 6, *V. kanaloae* LMG 20539^T (44.7 mol% G+C; Thompson *et al.*, 2003e); 7, *V. brasiliensis* LMG 20546^T (45.9 mol% G+C; Thompson *et al.*, 2003b, c); 8, *V. xuii* LMG 21346^T (46.6 mol% G+C; Thompson *et al.*, 2003c); 9, *V. furnissii* LMG 7910^T (50.4 mol% G+C; Brenner *et al.*, 1983).

demonstrates the usefulness of the standard curve obtained for G+C content estimation in vibrios. The standard curve and equations generated in this study might improve further

if species whose G+C contents have been obtained by sequencing of complete genomes were included in the regressions. In this way, one would avoid possible distortions caused by the bias of indirect methods of G+C content determination (Tamaoka & Komagata, 1984). In our hands, other factors that affect the accuracy of measurements using the real-time PCR platform include DNA quality (i.e. DNA fragmentation and purity), the type of equipment and its calibration, and the type of dye.

Typical melting curves generated for ΔT_m determination are shown in Fig. 2. Strains of *Vibrio campbellii* had less than 5 °C ΔT_m (Fig. 2a), whereas strains of different species, including closely related sister species (*Vibrio cholerae* and *Vibrio mimicus*), were distinguished by ΔT_m values greater than 5 °C (Fig. 2b and c). Analysis of the 31 pairs of vibrios, whose previous DDH values range from 22 to 99 %, provided a wide range of ΔT_m values, varying between 0.72 and 12.5 °C (Supplementary Table S1). Overall, the pairs corresponding to strains of the same species or strains from sister species showed the lowest ΔT_m values, indicating a coherent relationship between methodologies. For instance, the intraspecies ΔT_m in *V. cholerae* and *Vibrio coralliilyticus* was 1.79 and 2.76 °C, respectively, whereas the interspecies ΔT_m between the sister species *V. cholerae* and *V. mimicus* was 6.4 °C. The methodology also allowed the discrimination of the sister species *Vibrio harveyi* and *V. campbellii* (ΔT_m equal to 5.2 °C). It is important to note that these two species have around 70 % DDH relatedness. On the other hand, ΔT_m between the coral pathogen *V. coralliilyticus* LMG 20984^T

Fig. 2. Melting curves generated with ABI 7500 for ΔT_m determination between homologous and hybrid DNA. (a) Comparison between *V. campbellii* strains provided $\Delta T_m < 5.0$ (=2.4). (b) *V. campbellii* compared with *V. alginolyticus* provided $\Delta T_m > 5.0$ (=6.27). (c) $\Delta T_m > 5.0$ for *V. cholerae* and *V. mimicus* allowed species discrimination. Strain numbers and ΔT_m intervals and values are shown.

and *Vibrio neptunius* LMG 20536^T was 8.75 °C. The DDH relatedness for this pair is 64 %, indicating a good taxonomic resolution of the ΔT_m values. The original work of Gonzalez & Saiz-Jimenez (2005) included only one pair of strains in the so-called grey zone (Rosselló-Mora & Amann, 2001), corresponding to the interval between 60 and 80 % DDH relatedness. The present study included nine pairs in this zone in order to evaluate the limitations of the methodology in more detail. The ΔT_m values corresponding to pairs with DDH values below or above the grey zone (<60 or >80 % DDH) are clear-cut in distinguishing species (Supplementary Fig. S1). There is a significant difference between the mean ΔT_m values for these two intervals ($P < 0.01$).

A high correlation between DDH values obtained in previous studies by means of the Ezaki method and the ΔT_m values generated in the present study was obtained (Fig. 3). The ΔT_m values between 4 and 5 °C may not be clear-cut for distinguishing species. The limitations of the methodology for this interval (corresponding to 60 and 80 % DDH values) are well known. Several factors may be related to this limitation. Discrepancies between data obtained by different methodologies and in different laboratories may partly explain the lack of correlation of this so-called grey zone. The mean error of DDH experiments in different types of bacteria is usually as high as 7 % for Ezaki's methodology (Goris *et al.*, 1998; Willems *et al.*, 2001). Only three pairs of vibrio strains presented standard error that might have impaired analysis: *V. harveyi* LMG 4044^T and *V. campbellii* LMG 11216^T had ΔT_m of 5.2 °C (± 1.15); *Vibrio chagasii* LMG 13237 and *V. cyclitrophicus* LMG 21359^T with a ΔT_m of 5.7 °C (± 1.36); and *V. chagasii* LMG 13237 and *Vibrio splendidus* LMG 19031^T with a ΔT_m equal to 5.1 °C (± 1.01). These results may be related to DNA quality and/or quantification. The DDH versus ΔT_m curve generated with data of 111 pairs of strains, compiled by Rosselló-Mora & Amann (2001), also highlighted a range of uncertainty for the ΔT_m obtained spectrophotometrically. In addition, the range of uncertainty (grey zone) might

Fig. 3. Regression curve of the ΔT_m and DDH values for 31 strain pairs. The DDH data were obtained from the literature (Supplementary Table S1). The curve equation and R^2 coefficient are shown.

increase with the analysis of species or genomic groups that lack discriminative phenotypic features (Coenye *et al.*, 2001). In spite of the limitations of the fluorimetric measurement of ΔT_m with ABI 7500, in the presence of SG I, we showed that it is a valuable tool for vibrio species identification. Strains of the same vibrio species will have less than 4 °C ΔT_m . Strains of different vibrio species will have more than 5 °C ΔT_m .

The present study shows that the determination of ΔT_m values by means of a real-time PCR platform is a reliable alternative to the more classic methodologies of DDH experiments, allowing researchers to perform the required experiments more rapidly and efficiently with high accuracy and throughput. Real-time PCR platforms are widely used in molecular biology laboratories for a variety of applications, including microbial detection and quantification, gene expression studies, and polymorphism detection. The introduction of this approach to the polyphasic taxonomy of vibrios will help to push forward the field and allow the end-users of taxonomy to assess the untapped bacterial (vibrio) diversity in different environments.

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