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NOTE

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A simple fluorimetric method for the estimation of DNA–DNA relatedness between closely related microorganisms by thermal denaturation temperatures

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Abstract Determination of whole-genome DNA–DNA similarity is today a standard technique for species delineation in microbial taxonomy. However, these studies demand hard-to-perform and time-consuming experiments. Herein, we present an easy and rapid fluorimetric method to estimate DNA–DNA relatedness between microbial strains from differences of the thermal denaturation temperatures of hybrid and homologous genomic DNA. Double-stranded DNA was specifically stained with SYBR Green I, and its thermal denaturalization was followed by measuring a decrease in fluorescence. A quantitative, real-time PCR thermocycler was used to perform the experiment and obtain fluorescence determinations at increasing temperatures. The proposed method was validated by comparing species of the hyperthermophilic genera *Pyrococcus* and *Thermococcus*. The method proves to be an easy, rapid, and inexpensive alternative to estimate DNA–DNA relatedness between closely related species.

Keywords DNA homology · DNA hybridization · DNA relatedness · Melting curve · *Pyrococcus* · SYBR Green I · Thermal denaturation temperature · *Thermococcus*

Today, prokaryote taxonomists agree that a reliable classification can only be achieved by the exploration of a wide variety of characteristics in what is generally known as the ''polyphasic approach'' (Vandamme et al. 1996). This approach implies that both the genomic and phenotypic properties of a strain should be investigated. Genomic information is gained from the nucleic acids, either directly through sequencing or indirectly through parameters like DNA–DNA similarity or DNA base composition (G + C mol%). The classification of prokaryotic species can only be achieved by the recognition of genomic distances between related taxons; related taxons are identified by either physiological characteristics or 16S rRNA sequencing. Unlike other cell components, the DNA of a microbial cell does not depend on growth conditions, and so it is independent of environmental factors. In addition, the most complete genomic source of information is the entire microbial genome. One interesting approach is the comparison of genomic similarities by DNA–DNA pairing studies. Among these techniques, DNA–DNA hybridization techniques have proved to be very valuable in unraveling relationships between microorganisms (De Ley et al. 1970; Johnson 1989; Stackebrandt and Goebel 1994).

The determination of DNA–DNA similarity is today the standard technique for species delineation (Vandamme et al. 1996; Roselló-Mora and Amann 2001). A characteristic property of DNA is its ability for the reassociation or hybridization of complementary sequences to form a double-stranded DNA molecule (Turner 1996). If DNA of two microorganisms is mixed and denatured, then a solution mixture of single-stranded DNA molecules results. Under control conditions, DNA reassociation occurs resulting in hybrid molecules. The overall pairing of DNA strands depends on the similarity between the nucleotide sequences. Comparisons between the results obtained with the mixture of DNA and the pure reference DNA yield in a quantification of the genomic relatedness of these microbes.

Currently, there are two major experimental strategies to measure the degree of relatedness or similarity between microbial strains. They are based on assessing the degree of binding by hybridization (Popoff and Coynault 1980; Ezaki et al. 1989; Jahnke 1994) or the differences in thermal denaturation midpoints (De Ley et al. 1970; Torsvik et al. 1995). The binding strategy

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consists of fixing single-stranded, high-molecular-weight DNA on a solid support (generally, nitrocellulose or nylon membranes), which will be incubated in the presence of single-stranded, low-molecular-weight, labeled DNA. There are multiple-labeling procedures, and they use either radioisotopes or biotin-based, non-radioactive labeling methods (Ausubel et al. 1992). Using this procedure, the degree of binding or hybridization between the labeled DNA and the membrane-fixed DNA can be measured. Although extremely useful, this strategy presents major disadvantages: (1) labeled DNA from the reference strain must be prepared, which is both time-consuming and expensive; (2) the number of comparisons between strains or species is usually very limited for practical reasons, and so, complete matrices of comparisons for hybridization values between related species are extremely rare in the literature (Vandamme et al. 1996; De Ley et al. 1970). In addition, often it is unclear whether hybridizations are performed under optimal, stringent, or suboptimal conditions, which would have critical effects on the results (Grimont et al. 1980; Roselló-Mora and Amann 2001).

The thermal denaturation temperature approach represents an alternative method, which is used to estimate the thermal stability of the hybrid DNA duplexes versus homologous DNA. Under established conditions, double-stranded DNA denatures mainly depending on temperature (De Ley et al. 1970). The temperature at which 50% of the initial double-stranded molecules have denatured (into single-stranded DNA) is called the melting temperature or the thermal denaturation midpoint (T_m). The parameter used to estimate DNA–DNA relatedness, ΔT_m, is the difference between the T_m of the reference strain and the T_m of the hybrid DNA. There is no direct transformation between percentage of binding and ΔT_m. However, the results of both analyses are generally well correlated (Roselló-Mora and Amann 2001). The advantage of the ΔT_m over the measures of degree of binding is that ΔT_m estimates are independent of the method used for hybridization (as far as the hybridization buffer is maintained), while the degree of binding can be greatly affected by different hybridization conditions (Grimont et al. 1980).

To estimate the ΔT_m, first, purified total genomic DNA and mixtures of DNA from related species are denatured and allowed to renature at the optimum temperature for renaturation [(T_{or}) De Ley et al. 1970]. T_{or} can be estimated from the G + C mol% of the strains under study as described by De Ley et al. (1970). The single-strand and hybrid DNA is then progressively heated, and the absorbance of the solution is estimated. An inconvenience of the method is that absorbance measurements are usually too insensitive. In this study, we propose the use of fluorescent dyes highly specific for double-stranded DNA. This method results in an easy, rapid, inexpensive, high-throughput, fluorimetric technique to estimate DNA–DNA relatedness or similarity between DNA from closely related microbial strains. This is achieved by using a fluorescent, double-stranded-DNA-specific dye and the melting temperature software and hardware capabilities of modern quantitative, real-time PCR thermocyclers. Among the hyperthermophiles, the species of the genus *Pyrococcus* and *Thermococcus* have been reported as being closely related based on physiological and genomic information (Zillig 1992; Gonzalez et al. 1998; Maeder et al. 1999). The techniques proposed in this study have been used to compare strains and species within the Thermococcales.

Microorganisms used in this study were the hyperthermophilic Archaea *P. abyssi* (strain GE5^T) (Erauso et al. 1993); *P. endeavori* (strain ES4^T) (Pledger and Baross 1991); *P. furiosus* (DSM 3638^T) (Fiala and Stetter 1986); *P. furiosus* subsp. *woesei* (DSM 3773) (Zillig et al. 1987; Kanoksilapatham et al. 2004); *P. horikoshii* (DSM 12428^T); *Thermococcus celer* (DSM 2476^T) (Zillig et al. 1983); *T. litoralis* strains NS-C^T and A3 (DSM 5473^T and DSM 5474, respectively); and *T. peptonophilus* strains OG1^T and SM2 (JCM 9653^T and JCM 9654, respectively, Gonzalez et al. 1995). Cultures were prepared as described by Gonzalez et al. (1998). DNA was extracted using a modification of the method described by Marmur (1961). Briefly, cells were harvested in early stationary phase of growth by centrifugation (8,000 *g* for 15 min). Cell pellets were suspended in TNE buffer (Tris-HCl, 10 mM, pH 8.0; NaCl, 100 mM; EDTA, 1 mM). Cell lysis was achieved by 0.01% sodium sarcosine, 0.1% sodium dodecylsulfate, and lysozyme treatment (0.35 mg/ml) at 37°C for 30 min. Protease digestion (1.50 mg/ml) was performed for 2 h at 50°C and followed by two phenol extractions. Chloroform–isoamyl alcohol extractions were performed twice, and the DNA was precipitated with 99.5% ethanol at −20°C and rinsed twice with 70% ethanol at room temperature. DNA pellets were suspended in TE buffer. DNA concentrations were estimated fluorimetrically as described by Gonzalez and Saiz-Jimenez (2003). DNA was adjusted to the same concentration prior to hybridization and thermal denaturation experiments.

Thermal denaturation was quantified from fluorescence measurements, using an iQ iCycler (Bio-Rad, Hercules, California) and the capabilities of the accompanying software to perform melting curves. SYBR Green I (Molecular Probes, Eugene, Ore., USA) is a fluorescent dye showing high fluorescence when binding double-stranded DNA. By using this dye, double-stranded DNA can be estimated in the presence of single-stranded DNA molecules during denaturation experiments. SYBR Green I shows maximum fluorescence at excitation and emission wavelengths of 497 and 520 nm, respectively. Thermal denaturation was performed in a solution of 0.1× SSC (De Ley et al. 1970). The pH of this solution at denaturing temperatures was typically stable at around 8.0, which is the optimum pH resulting in maximum fluorescence of the dye SYBR Green I.

Thermal conditions consisted on a denaturation step of 99°C for 10 min, followed by an annealing period of

8 h at T_{or}, which was approximated according to De Ley et al. (1970), using the equation $T_{or} = 0.51(\%GC) + 47.0$. It was followed by progressive 60-min steps, each at 10°C below the previous one, until room temperature was reached (approximately 25°C). The hybrid and homologous DNA was preserved at 4°C until the quantitative thermocycler was available. Prior to performing the thermal denaturation experiments of the hybrids and homologous DNA, the SYBR Green I solution (1:100,000 final concentration) was added to those wells containing DNA solutions on a 96-well PCR plate. The experiment started with a period of 15 min at room temperature (25°C), followed by a ramp from 25–100°C at 0.2°C/s. Fluorescence measurements were performed at each step during this ramp. Melting curves were performed at least in duplicate. T_m values of total genomic DNA from homologous and hybrid DNA solutions were calculated as the temperatures corresponding to a 50% decrease in fluorescence. ΔT_m values between homologous and hybrid DNA of 5°C or higher were considered as corresponding to distinct microbial species (Wayne et al. 1987; Roselló-Mora and Amann 2001).

Although the concept of microbial species is in need of further refinements and is currently generating debate among microbiologists (Wayne et al. 1987; Vandamme et al. 1996; Roselló-Mora and Amann 2001; Stackebrandt et al. 2002), DNA hybridization is acknowledged as the reference method to establish boundaries between species. Wayne et al. (1987) defined bacterial species as a group of strains (including a type strain) sharing 70% or greater DNA–DNA relatedness with ΔT_m of 5°C or lower (Wayne et al. 1987; Vandamme et al. 1996; Roselló-Mora and Amann 2001). The current microbial species concept requires improved and easy methods to estimate DNA–DNA relatedness, as it is a requirement for distinguishing and classifying microbial species. In this study, we present a novel technique to estimate DNA–DNA relatedness based on established thermal denaturation experiments. The proposed protocol greatly facilitates the experimental procedures to be carried out with respect to previous methodologies.

Most modern real-time PCR thermocyclers allow performing of melting temperature experiments. We used an iQ iCycler real-time thermocycler to perform melting curves of genomic, reference, and hybrid DNA in order to determine the DNA–DNA relatedness between pairs of microbial species. For the detection of double-stranded DNA, we used a double-stranded-specific fluorescent dye, SYBR Green I (Akey et al. 2001; Gonzalez and Saiz-Jimenez 2002, 2003). At increasing temperatures, double-stranded DNA starts to denature, and single-stranded DNA appears in the solution, which is reflected by a decrease in fluorescence.

Figure 1 shows an example of the melting curves obtained from DNA of two different but closely related species of the genus *Pyrococcus* (*P. horikoshii* and *P. endeavori*) and their hybrid DNA mixture. Variability of T_m estimates showed coefficients of variation lower

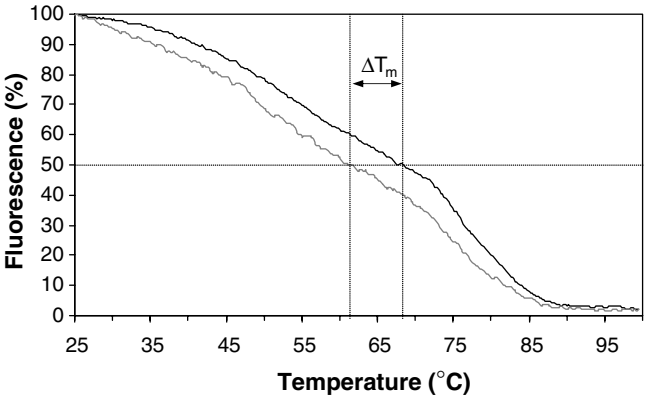

Fig. 1 Thermal denaturation of genomic DNA from *Pyrococcus horikoshii* (*black curving line*) and the *P. horikoshii* and *P. endeavori* hybrid DNA mixture (*light gray curving line*), following the proposed protocol

Table 1 Comparison of DNA–DNA relatedness (ΔT_m), which is the difference between the melting temperature of the reference strain and the melting temperature of the hybrid DNA. ΔT_m values from this study and percentage of similarity for some Archaea of the genera *Pyrococcus* and *Thermococcus*

DNA species	ΔT_m (°C)[a]	Percentage of similarity[b]
P. furiosus–P. horikoshii	8.7	50/63[f]
P. furiosus–P. abyssi	13.1	45/36[f]
P. furiosus–P. endeavori	7.4	ND[h]
P. horikoshii–P. abyssi	8.3	36/32[f]
P. horikoshii–P. endeavori	6.9	ND
P. abyssi–P. endeavori	9.3	ND
P. furiosus–T. celer	6.3	50[f]
P. horikoshii–T. celer	10.2	25[f]
P. abyssi–T. celer	10.5	36[f]
P. endeavori–T. celer	7.8	ND
P. furiosus subsp. *woesei–P. furiosus*	2.7	83/87[d]
T. peptonophilus OG1–*T. peptonophilus* SM2	2.1	90[e]
T. litoralis NS-C–*T. litoralis* A3	3.4	86/89[c]
T. celer–T. litoralis NS-C	7.6	10[c]
P. abyssi–T. litoralis A3	9.0	11[d]
P. furiosus–T. litoralis A3	12.9	6[d]
T. celer–T. peptonophilus OG1	12.8	9[e]
P. abyssi	0.4	100[g]
P. endeavori	0.6	100[g]
P. furiosus	0.4	100[g]
P. horikoshii	0.6	100[g]
P. furiosus subsp. *woesei*	0.4	100[g]
T. celer	0.5	100[g]
T. litoralis NS-C	0.2	100[g]
T. litoralis A3	0.6	100[g]
T. peptonophilus OG1	0.4	100[g]

[a]Data from this study following the proposed protocol
[b]Values separated by an *en dash* are from different hybridization experiments published in the literature
[c]Data from Neuner et al. (1990)
[d]Data from Erauso et al. (1993)
[e]Data from Gonzalez et al. (1995)
[f]Data from Gonzalez et al. (1998)
[g]Value corresponds to a single species
[h]*ND* Not determined

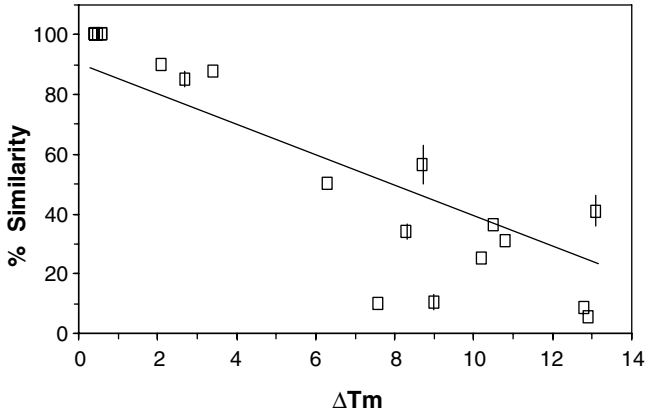

Fig. 2 Approximated relationship between DNA–DNA relatedness (ΔT_m), which is the difference between the melting temperature of the reference strain and the melting temperature of the hybrid DNA, and the percentage of similarity proposed by Roselló-Mora and Amann (2001) showing data obtained from the technique proposed in this study. Data obtained from homologous genomic DNA from single species and hybrid DNA from species of the genus *Pyrococcus* and *Thermococcus* are represented

than 1%. Thus, ΔT_m values equal to or higher than 5°C (i.e., the species threshold) represented highly significant differences. Table 1 shows ΔT_m values resulting from a matrix of comparisons between several *Pyrococcus* and *Thermococcus* species obtained using the proposed methodology and their comparison to the corresponding results by Neuner et al. (1990), Erauso et al. (1993), and Gonzalez et al. (1995, 1998), from DNA–DNA dot-blot hybridization using [^{32}P] labeling of DNA by nick translation. Although *Pyrococcus* and *Thermococcus* spp. have been reported to show high physiological and genomic similarity (Zillig 1992; Maeder et al. 1999), the proposed method has been tested by differentiating *Pyrococcus* and *Thermococcus* species. The method was also able to confirm strains of *T. peptonophilus* (OG1 and SM2 strains), *T. litoralis* (NS-C and A3 strains), and *P. furiosus* subspecies [*P. furiosus* and *P. furiosus* subsp. *woesei* (Kanoksilapatham et al. 2004)] as belonging to the same species. Thus, the method proves to be successful to discriminate among phylogenetically related strains belonging to different microbial species and to group strains of a single species.

Several studies have reported agreement between percentage of similarity and ΔT_m estimates (Wayne et al. 1987; Vandamme et al. 1996; Roselló-Mora and Amann 2001). Specifically, Marteinsson et al. (1999) have mentioned this agreement for the hyperthermophilic genera *Pyrococcus* and *Thermococcus*. Figure 2 shows a comparison of the ΔT_m data (this study) and dot-blot hybridization results by Neuner et al. (1990), Erauso et al. (1993), and Gonzalez et al. (1995, 1998) together with the approximate relationship proposed by Roselló-Mora and Amann (2001).

The technique presented in this study shows several advantages over previous methods to estimate DNA–DNA similarity. The method proposed in this study is very easy to perform, rapid, and inexpensive, allowing high-throughput comparisons. Previous methods (i.e., DNA–DNA dot-blot hybridizations) were expensive and time-consuming, restricting the comparisons to be carried out to a low number and to be performed only by specialized laboratories. The proposed method allows a full comparative matrix to be easily carried out for the related species that need to be tested. This is increasingly important due to the fact that some genera are getting an elevated number of phylogenetically close species, which need to be analyzed for comparisons of DNA–DNA relatedness. In addition, the proposed method is not, as are other thermal denaturation temperature strategies for assessing DNA–DNA homologies, affected by the stringency of the experimental conditions (Grimont et al. 1980; Vandamme et al. 1996). As a limitation, it is recommended that thermal denaturation DNA–DNA relatedness analyses between microbial species showing distant genetic relationships (i.e., those showing not significant hybridization levels) be done with care, since the denaturation experiments might only provide information on monospecific DNA rather than the desired denaturation of a hybrid DNA mixture.

The novel strategy to estimate DNA–DNA relatedness proposed in this study is based on established DNA denaturation techniques (De Ley et al. 1970; Torsvik et al. 1995) and the methodology recently described by Gonzalez and Saiz-Jimenez (2002) to estimate DNA base composition in microorganisms. Now, using a similar methodology, one could determine the G+C mol% content in a novel microorganism and its relatedness to phylogenetically related species. The proposed approach represents a great advantage for non-taxonomist microbiologists in need of describing and classifying a novel bacterial strain. Consequently, this and other strategies to be developed are certainly to facilitate the procedure to classify microorganisms and specifically, novel extremophiles.

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