Using the iCycler iQ® Detection System to Estimate Microbial DNA Base Composition From Melting Curves

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Introduction

Scientists aim to name and unambiguously describe every species. To support this process, a set of basic rules for the description of microorganisms is periodically revised and published (Wayne et al. 1987). Recent trends point to the need for a "polyphasic" taxonomic approach (Vandamme et al. 1996), which means that a variety of techniques should be used to describe and name a novel isolate. Thus, both physiological and genomic properties should be analyzed. Potential methods of genomic characterization of microorganisms are DNA/DNA hybridization to analyze relatedness between species, ribosomal RNA sequence analysis, and quantitation of G+C levels. Some of these analyses are difficult to carry out in nonspecialized laboratories. Thus, simple methodologies for performing these analyses are greatly useful and widely appreciated.

DNA base composition, or the mole percentage of G+C (expressed with the unit mol%) in a microorganism's genome, is a recommended characteristic for the standard description of microbial species (Rosselló-Mora and Amann, 2001). DNA base composition varies between 24 and 76% among prokaryotes (Vandamme et al. 1996). Although similar G+C content does not imply that two species are highly related, we can confirm that two microbial species having different DNA base composition belong to different species. It is generally accepted that microorganisms differing by more than 5 mol% do not belong to the same species and that members of the same genus are within a range of 10 mol% (Wayne et al. 1987).

Several techniques have been used to assess the percentage G+C in the genomes of microorganisms. Sequencing an entire genome solely for the purpose of obtaining G+C content is infeasible. The two most common approaches are high-performance liquid chromatography (HPLC) and thermal denaturation. HPLC techniques are accurate but require an HPLC system set up specifically for this purpose, which is costly and only worthwhile if these determinations are frequently performed (Tamaoka and Komagata 1984, Mesbah et al. 1989). Measurements of absorbance during thermal denaturation of DNA have been used as an alternative to rapidly estimate G+C content in the DNA of microorganisms. DNA melting temperatures and their G+C percentages follow a linear relationship (Marmur and Doty 1962, De Ley et al. 1970). Following the progressive denaturation of the DNA at increasing temperatures by absorbance measurements is a commonly used strategy that requires a spectrophotometer with a thermal controller. Fluorometric determinations of DNA denaturation should be a much more sensitive method for assessing G+C mol% content. Thermal cyclers for quantitative real-time PCR are becoming common in most laboratories because of the widespread use of real-time PCR techniques. In this study, we propose a simple and rapid high-throughput fluorometric technique using the iCycler iQ detection system for melt-curve analysis to estimate the DNA base composition of microorganisms. The method uses a fluorescent double-stranded DNA-specific dye, SYBR Green I, and the melting temperature analysis capabilities of the iCycler iQ detection system. The method was calibrated using the relationship between G+C mol% and melting temperature of completely sequenced genomes.

Methods

SYBR Green I (Molecular Probes, Eugene, Oregon) is a dye that fluoresces strongly upon binding double-stranded DNA. With this dye, double-stranded DNA molecules can be selectively quantitated during heat denaturation experiments. SYBR Green I shows maximum fluorescence at excitation and emission wavelengths of 497 and 520 nm, respectively. These peaks are coincident with the FAM filter set provided with the iCycler iQ system.

Microbial DNA was extracted following standard procedures (Marmur 1961). DNA concentration was determined spectrophotometrically. Each reaction mixture contained 1–5 µg genomic DNA, 0.1x SSC (final concentration), and SYBR Green I at 1:100,000 dilution (final concentration).

Final volume of each reaction was 50 µl. Reactions were prepared in triplicate on 96-well PCR plates. An iCycler iQ real-time detection system was used to obtain melt curves and fluorescence measurements during thermal denaturation of the DNA. The thermal ramp was from 25°C to 100°C at 1°C/min. Fluorescence measurements were performed at each step (0.2°C increase per 12 sec step) during this ramp. Well factors were obtained from an external well factor plate containing 0.5 µM fluorescein. The experimental samples were not placed in the instrument until the reaction block reached 25°C. Thermal denaturation was performed in 0.1x standard saline citrate as recommended by De Ley et al. (1970). The pH of this solution in the range of the denaturing temperatures was typically stable at around 8.0, which allows maximum fluorescence of SYBR Green I.

We used a number of microbial species from both Archaea and Bacteria for the standard calibration curve (Table 1). These strains were selected because their genomes have been completely sequenced and are available in public databases, and they are nonpathogenic. Calibration curves were obtained from the melting temperature (T_m) of total genomic DNA as described above and the G+C mol% determined from the genomic sequence, where G+C mol% = [G+C]/[A+T+C+G] x 100. Genome sequences were obtained from Entrez Genomes at http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html (NCBI web site). T_m was calculated from the minimum value of the slope tangent to the melt curve of fluorescence vs. temperature. Data were transferred to a Microsoft Excel spreadsheet, and least-squares linear regression analysis was performed according to Sokal and Rohlf (1981).

Table 1. Genomic DNA G+C content and mean T_m for microbial species used to construct a calibration curve.

Species	Strain	Domain	G+C mol%	T_m ± SD
Clostridium acetobutylicum	ATCC 824	Bacteria	30.93	71.1 ± 0.1
Lactococcus lactis lactis	IL1403	Bacteria	35.33	72.5 ± 1.3
Pyrococcus furiosus	DSM 3638	Archaea	40.77	75.1 ± 1.5
Pyrococcus horikoshii	DSM 12428	Archaea	41.88	74.6 ± 0.2
Bacillus subtilis	168	Bacteria	43.52	77.1 ± 0.9
Pyrococcus abyssi	GE5	Archaea	44.71	77.0 ± 0.6
Archaeoglobus fulgidus	DSM 4304	Archaea	48.58	79.7 ± 0.1
Escherichia coli	K-12 (MG1655)	Bacteria	50.79	80.7 ± 0.1
Corynebacterium glutamicum	ATCC 13869	Bacteria	53.81	81.2 ± 0.2
Pseudomonas aeruginosa	PAO1	Bacteria	66.56	87.5 ± 0.1
Deinococcus radiodurans	R1	Bacteria	66.63	88.5 ± 0.1

Results and Discussion

In this study we used 11 strains with completely sequenced genomes for the calibration of the method. DNA base composition for these strains ranges between 30.9% and 66.6%. Melting temperatures for these strains' genomic DNA ranged from approximately 71 to 89°C (Table 1), and G+C mol% showed a positive relationship with the melting temperatures. The obtained regression line ($n = 11$, $r^2 = 0.99$, $P << 0.001$) was G+C mol% = $1.98T_m - 106.91$. This relationship can be used to estimate the G+C mol% or DNA base composition of novel microbial isolates (Figure 1). Melt-curve data were exported from the iCycler iQ software to Microsoft Excel software in order to convert the y-axis values from relative fluorescence units to percent maximum fluorescence. This enables visualization of all curves on the same scale without changing the calculated T_m.

The regression lines for the standard calibration lines obtained above are universal (i.e., valid for every microorganism) as previously shown with similar calibration curves using spectrophotometric methods (De Ley et al. 1970). De Ley et al. (1970) obtained a regression coefficient of 2.44, which is slightly higher than our value of 1.98. This can be explained by our adoption of different methodology. De Ley et al. (1970) used spectrophotometry, and in this study we followed a novel fluorometric protocol. In addition, differences in G+C content estimates can result from DNA base composition values obtained with diverse techniques or from previously published values. In our study, we obtained a calibration curve using microorganisms with their genome completely sequenced and available to the scientific community. The availability of the full genome sequence allows method-independent, precise calculation of the G+C mol% in the DNA sequence under study.

The method proposed in this study represents an easy and rapid technique to estimate the G+C mol% or DNA base composition of a microorganism, and it could be carried out by any nontaxonomist interested in classifying a novel microorganism. In addition, the outlined protocol describes an additional application of the iCycler iQ detection system besides diverse quantitative real-time PCR strategies.

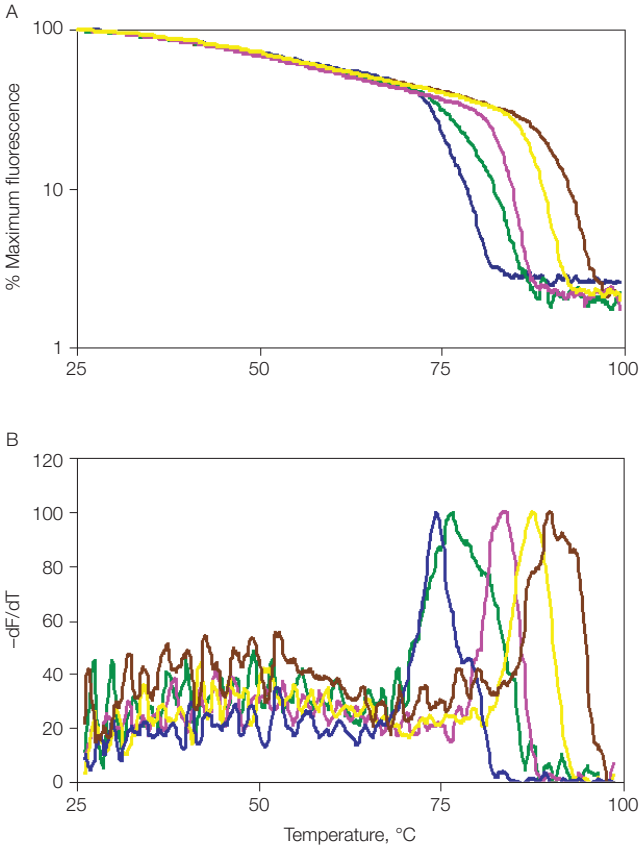

Fig. 1. Melt curves (A) and first derivative plots (B) for genomes of five bacterial isolates. Bacterial strains, in order of increasing melting temperatures (estimated G+C content in parentheses) are: blue, *Bacillus weihenstephanensis* strain P2-14 (39.6%); green, *Acinetobacter baumannii* strain AVP2-4 (44.4%); pink, *Pseudomonas* strain AGP3-7 (58.4%); yellow, *Streptomyces lateritius* strain CSC113.1 (66.1%); brown, *Nocardopsis* strain F101 (73.1%). The sharp decrease in fluorescence during the melt curve corresponds to denaturation of the double-stranded DNA (A), and the major peaks in the –dF/dT plots indicate the melting temperature for each bacterial strain.

Acknowledgements

The authors acknowledge support from the Spanish Ministry of Science and Technology, Ramón y Cajal program and projects REN2002-00041/GLO and BTE2002-04492-C02-01, and the European projects CATS (EVK4-CT-2000-00028) and COALITION (EVK4-CT-1999-20001).

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Information in this tech note was current as of the date of writing (2003) and not necessarily the date this version (Rev A, 2004) was published.

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Bulletin 3084 US/EG Rev A *03-929 0204 Sig 1103*