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Detection and Differentiation of In Vitro-Spiked Bacteria by Real-Time PCR and Melting-Curve Analysis

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We introduce a consensus real-time PCR protocol for the detection of bacterial DNA from laboratory-prepared specimens such as water, urine, and plasma. This prototype detection system enables an exact Gram stain classification and, in particular, screening for specific species of 17 intensive care unit-relevant bacteria by means of fluorescence hybridization probes and melting-curve analysis in a one-run experiment. One strain of every species was tested at a final density of 10^6 CFU/ml. All bacteria examined except *Staphylococcus aureus* and *Staphylococcus epidermidis* could be differentiated successfully; *S. aureus* and *S. epidermidis* could only be classified as "*Staphylococcus* species." The hands-on time for preparation of the DNA, performance of the PCR, and evaluation of the PCR results was less than 4 h. Nevertheless, this prototype detection system requires more clinical validation.

The early detection and adequate treatment of bacterial infections are critical for successful outcomes for patients with systemic infections (5).

Efforts aimed at the species-specific detection of bacterial DNA have been made (1–4, 6, 8, 9). This approach would require several PCR experiments in series or in parallel, which would be expensive and time-consuming. Therefore, the aim of this study was to introduce a prototype system for the detection of bacterial DNA that enables a hands-on time of less than 4 h, including the time for the preparation of DNA and evaluation of the PCR results. For this purpose we developed a prototype rapid real-time PCR protocol for the amplification of bacterial DNA from biological fluids. This approach enables Gram stain classification with the goal of the reliable detection and differentiation of significant pathogens in the intensive care unit (ICU) by means of fluorescence hybridization probes with calculated mismatches and melting-curve analysis in a one-run experiment.

MATERIALS AND METHODS

Whole organisms of 17 ICU-relevant bacteria species (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus vulgaris*, *Haemophilus influenzae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia marcescens*, *Bacteroides fragilis*, *Acinetobacter baumannii*, *Legionella pneumophila*, *Stenotrophomonas maltophilia*, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pyogenes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*) were spiked in different biological fluids (water, plasma, urine), and the DNA was extracted and used for subsequent experiments. A quantitative PCR was performed on a real-time PCR instrument (LightCycler; Roche) with broad-range primers for amplification of a highly conserved region of the bacterial 16S DNA locus. The PCR products were hybridized with two fluorescence dye-labeled hybridization probes that specifically bind only to either gram-positive or gram-negative bacteria. The different fluorescence signals (640 and 705 nm, respectively) were detected by the LightCycler instrument and enabled differentiation of bacteria according to their Gram stain classification (Fig. 1)

Melting-curve analysis was performed to identify the melting temperatures of the hybridization probes as well as of the entire PCR product for the further differentiation of the bacterial DNA. Intra- and interassay analyses were carried out to determine the reproducibilities of the melting points. In total, 10 tests were performed with each sample. The standard deviations and the coefficients of variation (CVs) were calculated.

Oligonucleotide primers and hybridization probes. Oligonucleotide primers were manufactured by Biosource Europe (Nivelle, Belgium). The primers amplify conserved regions of the bacterial 16S rRNA gene. Primers PLK1 (5′-TAC GGG AGG CAG CAG T-3′) and PLK2 (5′-TAT TAC CGC GGC TGC T-3′) are highly conserved in different groups of eubacteria (7). These primers synthesize a 187-bp fragment. PLK2 is internally labeled with fluorescein.

Fluorescence dye-labeled hybridization probes ISN2 (5′-CCG CAG AAT AAG CAC CGG CTA ACT CCG T-3′) and ISP2 (5′-CCT AAC CAG AAA GCC ACG GCT AAC TAC GTG-3′) emit light at different wavelengths (640 and 705 nm, respectively) and can be used for detection of bacterial DNA, differentiation of isolates by Gram stain classification (7), and melting-curve analysis (see below).

DNA extraction. DNA extraction was performed with an automatic preparation device (MagNA Pure LC; Roche, Mannheim, Germany) according to the preparation protocol of the manufacturer. The density in the extracted samples was 10^6 CFU/ml. Therefore, 10 μl of an inoculum of 10^7 CFU/ml was spiked into 90 μl of water, plasma, or urine. Thereafter, bacterial DNA was extracted from a total volume of 100 μl. The DNA was used for subsequent experiments.

When testing serial dilutions with a known quantity of plasmid DNA (1, 10, 100, or 1,000 pg of plasmid DNA per ml), we noticed only slight differences in the amount of PCR inhibition between water, plasma, and urine when using the MagNA Pure automatic preparation device. The rate of inhibition was calculated with respect to that for highly purified water (double-distilled, deionized, nucleic acid free) as a noninhibitory fluid. Results for the different serial dilutions of spiked plasma were as follows: 0.96 pg/ml, 4% inhibition; 9.77 pg/ml, 2.3% inhibition; 95.6 pg/ml, 4.4% inhibition; and 964.7 pg/ml, 3.5% inhibition. Results for the serial dilutions of spiked urine were as follows: 0.94 pg/ml, 6% inhibition; 9.65 pg/ml, 3.5% inhibition; 97.4 pg/ml; 2.6% inhibition; and 955.3 pg/ml, 4.5% inhibition.

PCR amplification. PCR amplification was performed by using a real-time PCR system (LightCycler; Roche). The PCR mixture (20 μl) contained FastStart DNA Master Hybridization Probes (a ready-to-use hot-start reaction mixture for PCR containing FastStart *Taq* DNA polymerase, a mixture of deoxynucleoside triphosphates with dUTP instead of dTTP, and 10 mM MgCl; Roche), 2.4 μl of MgCl (25 mM) stock solution per reaction mixture, 13.6 μl of sterile H_2O, and 2 μl of template.

PCR protocol. The PCR protocol consisted of 1 cycle of denaturation at 95°C for 10 min (FastStart activation) and 45 cycles of amplification (15 s of denaturation at 95°C, 8 s of annealing at 52°C, and 10 s of extension at 72°C).

Melting-curve analysis. The PCR step was followed by melting-curve analysis, in which the PCR product was heated from 40 to 98°C and then cooled to room temperature.

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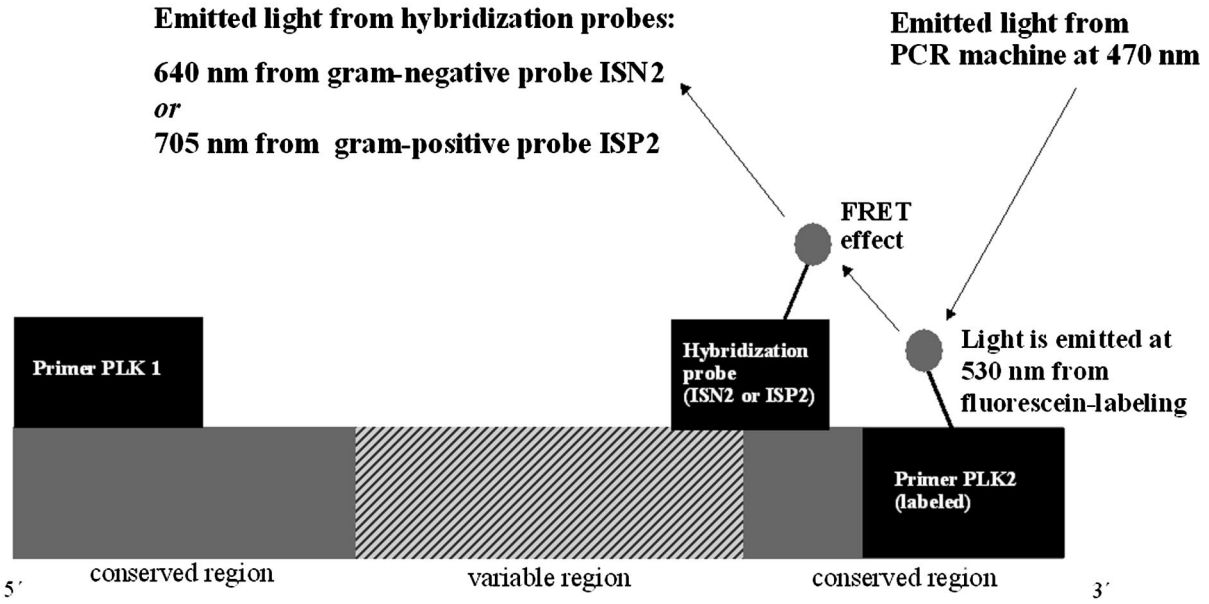

FIG. 1. Schematic overview of the amplified bacterial 16S DNA gene locus, the effect of FRET, and the locations of the primers and probes. Fluorescein labeling of primer PLK2 also emits light at 530 nm during melting-curve analysis and is responsible for the second melting point (see Fig. 2).

With an increase in temperature the fluorescence decreases due to the melting behavior of DNA, so the melting (dissociation) of the double-stranded DNA results in a drop in the fluorescence signal emitted. With respect to the probes, separation of the anchor and reporter fluorescence resonance energy transfer (FRET) probes also results in a drop in the fluorescence signal emitted at a probe-specific melting temperature (Fig. 2).

The method introduced here uses two melting points within one run per sample. The first melting point is generated by the hybridization probe (which is specific for gram-positive or gram-negative bacteria), with built-in mismatches in the fluorescence probes. These mismatches generate different melting points for most of the bacteria.

In addition, the melting points of the entire double-stranded PCR product are analyzed. Primer PLK2, which is internally labeled with fluorescein and which also acts as the anchor FRET probe for the PCR, generates this second melting point. At the end of the PCR the internally labeled primers have been incorporated into the PCR product. When the PCR product melts, these fluorescein-labeled primers give melting-curve signals very similar to the SYBR Green melting points of the same PCR product (nevertheless, this reaction does not contain SYBR Green) (Fig. 2).

Therefore, in addition to fluorescence (640 and 705 nm), the melting-curve analysis with two different melting temperatures per sample provides three pieces of information in a single run, and this information can be applied to the identification of the bacteria.

RESULTS

The DNAs of all 17 bacteria were successfully extracted and detected by PCR. Both fluorescence probes provided a correct Gram stain classification. The detection limit was 1 pg of bacterial DNA per ml. Melting-curve analysis enabled further species-specific differentiation. The two melting points could be identified for all samples. Thus, three informative items could be extracted from each PCR run: (i) the wavelength of the fluorescence emitted (640 nm for gram-negative bacteria and 705 nm for gram-positive bacteria), (ii) the melting temperature of the hybridization probe, and (iii) the melting temperature of the entire PCR product.

Melting points of the hybridization probes for 17 bacteria (interassay analysis). As depicted in Fig. 3a, the melting points of the hybridization probes were highly reproducible, with very low CVs. The mean CVs for all bacteria tested were 0.068% in the intra-assay analysis and 0.35% in the interassay analysis. Some bacteria could be identified directly (e.g., bacterium 6) according to the melting temperature of the hybridization probe; identification of other species also required the melting temperature of the entire PCR product (e.g., bacteria 13 to 17).

Melting points of the entire double-stranded PCR products for 17 bacteria. As depicted in Fig. 3b, the melting points of the entire double-stranded PCR products were also highly reproducible. The mean CVs for all bacteria tested were 0.039% in the intra-assay analysis and 0.085% in the interassay analysis. Bacteria that formed homogeneous groups according to the melting points of the hybridization probes (Fig. 3a) could be differentiated (Fig. 3b) by melting-curve analysis of the overall PCR product (e.g., bacteria 13 to 17).

Diagnostic criteria for gram-positive bacteria. A diagnostic flowchart could be constructed by taking the available information (Gram stain classification, hybridization probe melting points, and melting points of the entire PCR product) into consideration.

As depicted in Fig. 4a, all gram-positive bacteria tested except *S. aureus* and *S. epidermidis* could be differentiated. These two bacteria have very similar melting point characteristics, so they could only be identified as "*Staphylococcus* species." An additional internal probe is needed for further differentiation.

Diagnostic criteria for gram-negative bacteria. As depicted in Fig. 4b, all gram-negative bacteria tested could be differentiated. Some of the bacteria (*B. fragilis*, *S. maltophilia*, *P. aeruginosa*) could be identified according to the melting temperatures of the hybridization probes only, whereas other bacteria formed groups and had to be identified by considering the melting temperature of the entire PCR product.

All gram-negative bacteria tested could be correctly differentiated when the two different melting points for each sample were evaluated.

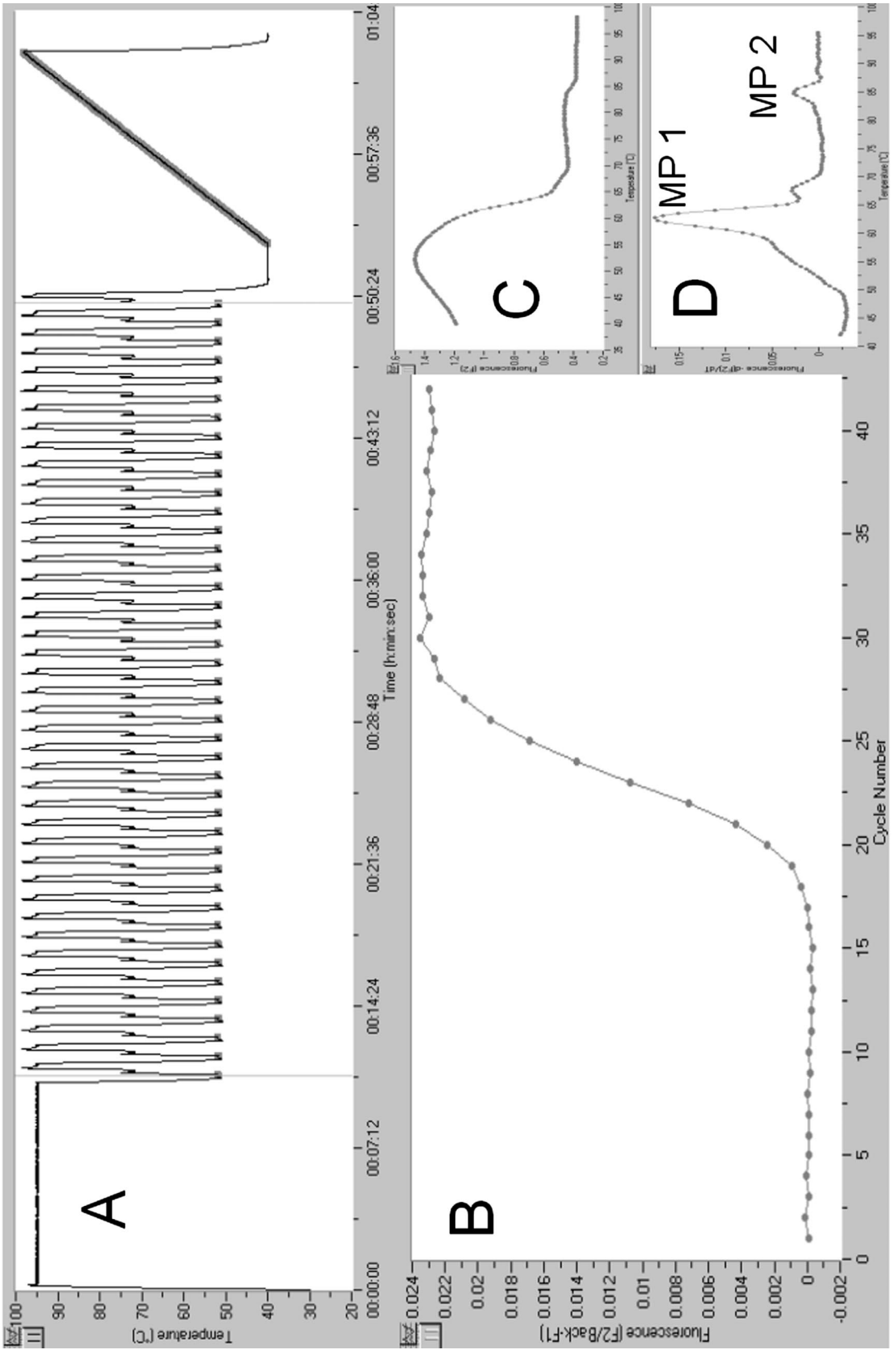

FIG. 2. Original registration with an exemplary real-time PCR and melting-curve analysis of *P. aeruginosa* (measured at 640 nm [F2]). (A) Temperature and time profile of the PCR and final melting process; (B) fluorescence intensity, which shows a log linear increase in the amount of the PCR product during the PCR process; (C) melting of PCR product and decrease in the fluorescence intensity at the first and second melting points (see panel D); (D) detection of melting points by displaying the first negative derivative ($-dF/dT$) versus the temperature of curve C. Melting point 1 (MP 1) represents the separation of the anchor and FRET reporter probes. Melting point 2 (MP 2) represents the melting temperature of the entire double-stranded PCR product.

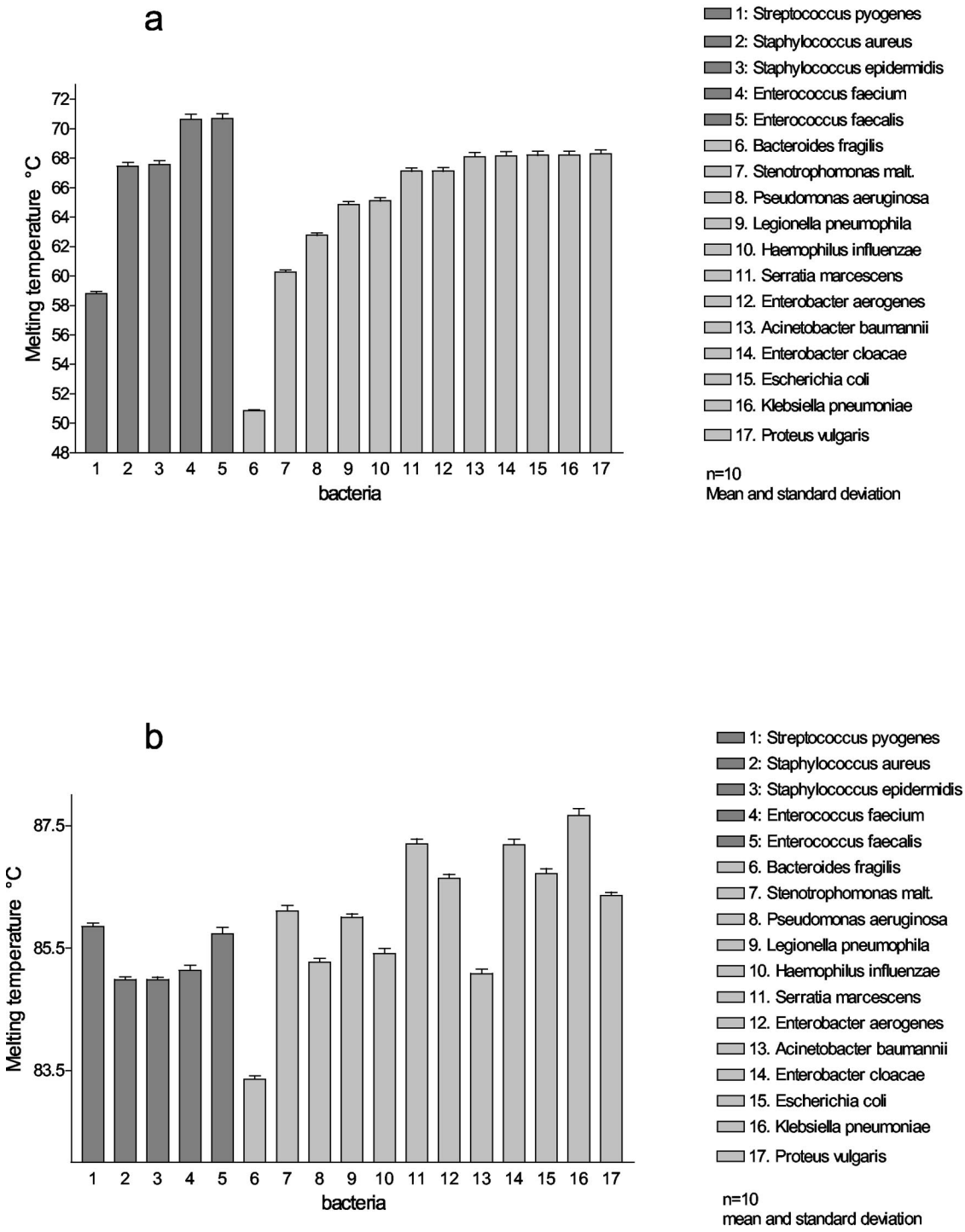

FIG. 3. (a) Interassay analysis of the melting points for 17 bacteria ($n = 10$) with hybridization probes, including the means and standard deviations; (b) interassay analysis of the melting points of the entire double-stranded PCR products for 17 bacteria ($n = 10$), including the means and standard deviations.

DISCUSSION

We previously reported on the possibility of differentiation of species by Gram stain classification by real-time PCR (7). Now we extend the application of the assay to a species-specific screen that enables us to detect bacterial DNA from biological fluids, that allows Gram stain classification, and that permits screening for specific species of ICU-relevant bacteria by means of fluorescence hybridization probes and melting-curve analysis in a one-run experiment. The real-time PCR can be performed in less than 40 min, and only one vial is needed for each sample. Seventeen different ICU-relevant bacteria have been tested with two Gram stain-specific probes containing

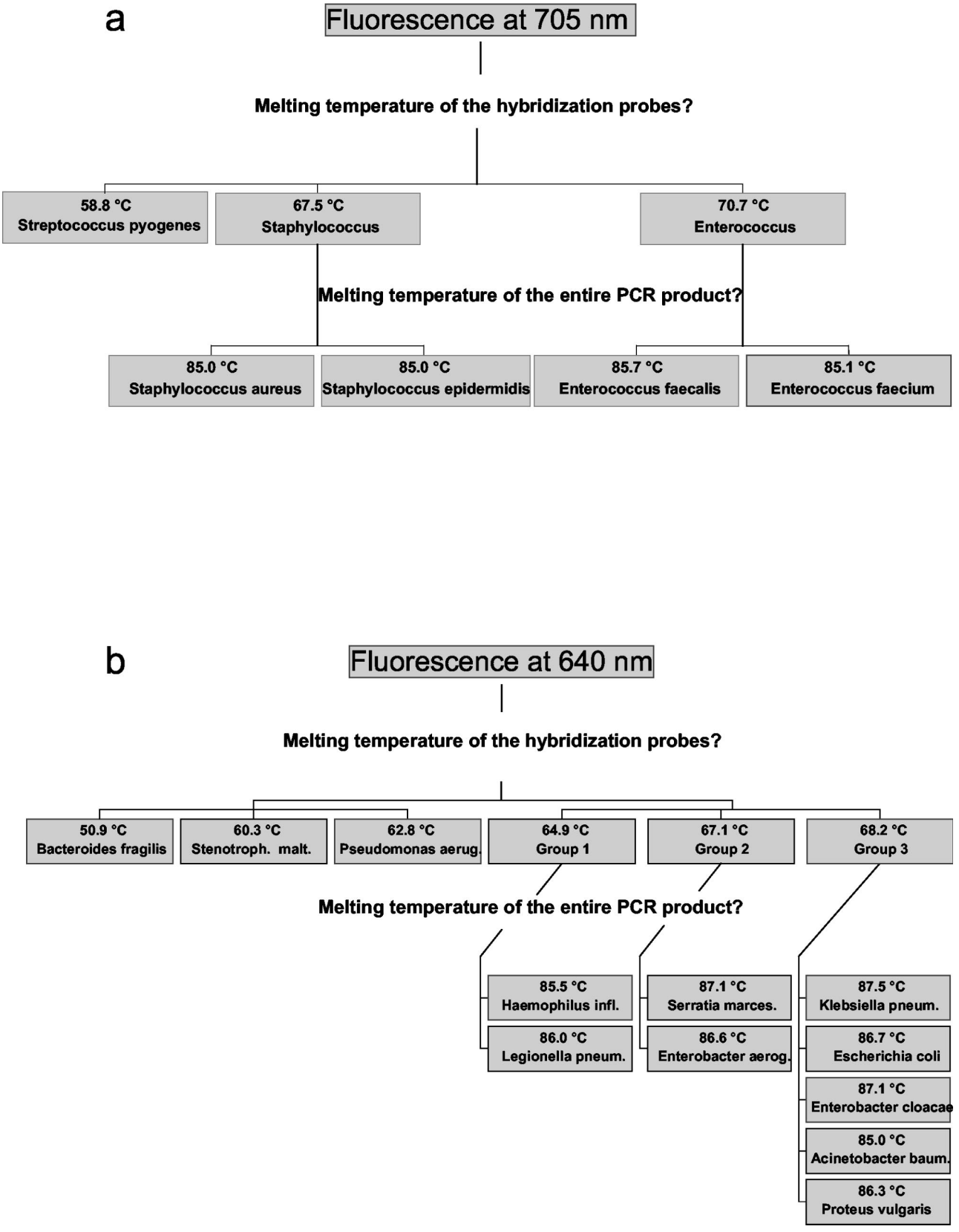

FIG. 4. Diagnostic diagrams for identification of gram-positive bacteria (a) and gram-negative bacteria (b).

calculated mismatches. All bacteria tested were correctly classified as gram positive or gram negative, but all bacteria tested except *S. aureus* and *S. epidermidis* were also specified correctly. The last two species could only be classified as "*Staphylococcus* species" because these two bacteria display very similar melting point characteristics when the probe specific for gram-positive bacteria is used. An additional species-specific probe is needed for further differentiation of these two species. It may be beneficial to test more bacteria of each species by the assay, because so far it is unknown whether other (untested) bacteria or mixed infections may lead to misinterpretations.

The time required from the time that a sample is deposited to the time that a definite result is obtained is of great relevance. The hands-on time for the preparation of DNA, performance of the PCR, and evaluation of the PCR results is less than 4 h. For use of the assay with clinical samples or in case of the detection of "*Staphylococcus* species," another PCR run of about 30 min with specific primers and probes should be done to be on the safe side. Nevertheless, this kind of screening will save time and material because only one additional analysis with a probe specific for *Staphylococcus* species is required.

In conventional microbiological methods a density of 10^6 bacteria per ml of a body fluid corresponds to about 10 organisms per oil-immersion field (×1,000) on a direct Gram stain, so the assay for the detection of specific species by melting-curve analysis described here is potentially less sensitive than direct Gram staining and the evaluation of a smear prepared by cytocentrifugation, which can reliably be used to detect 1,000 CFU of organism per ml of body fluid. Nevertheless, the initial prototype assay described here not only facilitates the fast detection of all bacteria tested and reliable Gram stain classification of 1 pg of bacterial DNA per ml, as shown previously (7), but it also enables screening for specific species of 17 ICU-relevant bacteria in spiked specimens within hours under laboratory conditions. In contrast to other PCR-based methods, the advantage of this method is the fact that testing can be performed in one tube. If a specific set of probes were used for each bacterial species, several PCR experiments in series or parallel would be required; i.e., it would be necessary to use 17 tubes for the detection of any of the possible 17 bacteria in one sample. The detection of 17 bacteria in one sample would be much more expensive if gene chips were used. Therefore, the prototype assay may hold promise for clinical use. Nevertheless, the diagnostic sensitivity must be improved and further evaluations must be done, including evaluations with clinical specimens with low densities of bacteria and mixed infections.

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