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HSP60 Gene Sequences as Universal Targets for Microbial Species Identification: Studies with Coagulase-Negative Staphylococci

SWEE HAN GOH,[1,2,3]† SHEILA POTTER,[1,2] JULIAN O. WOOD,[1,2] SEAN M. HEMMINGSEN,[4] ROBERT P. REYNOLDS,[1,2] AND ANTHONY W. CHOW[1,2,3,5]*

Departments of Medicine[1] and Microbiology and Immunology,[5] Division of Infectious Diseases, University of British Columbia, the Vancouver Hospital Health Sciences Centre,[2] and the Canadian Bacterial Diseases Network,[3] Vancouver, British Columbia, and the Canadian National Research Council Plant Biotechnology Institute, Saskatoon, Saskatchewan,[4] Canada

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A set of universal degenerate primers which amplified, by PCR, a 600-bp oligomer encoding a portion of the 60-kDa heat shock protein (HSP60) of both *Staphylococcus aureus* and *Staphylococcus epidermidis* were developed. However, when used as a DNA probe, the 600-bp PCR product generated from *S. epidermidis* failed to cross-hybridize under high-stringency conditions with the genomic DNA of *S. aureus* and vice versa. To investigate whether species-specific sequences might exist within the highly conserved HSP60 genes among different staphylococci, digoxigenin-labelled HSP60 probes generated by the degenerate HSP60 primers were prepared from the six most commonly isolated *Staphylococcus* species (*S. aureus* 8325-4, *S. epidermidis* 9759, *S. haemolyticus* ATCC 29970, *S. schleiferi* ATCC 43808, *S. saprophyticus* KL122, and *S. lugdunensis* CRSN 850412). These probes were used for dot blot hybridization with genomic DNA of 58 reference and clinical isolates of *Staphylococcus* and non-*Staphylococcus* species. These six *Staphylococcus* species HSP60 probes correctly identified the entire set of staphylococcal isolates. The species specificity of these HSP60 probes was further demonstrated by dot blot hybridization with PCR-amplified DNA from mixed cultures of different *Staphylococcus* species and by the partial DNA sequences of these probes. In addition, sequence homology searches of the NCBI BLAST databases with these partial HSP60 DNA sequences yielded the highest matching scores for both *S. epidermidis* and *S. aureus* with the corresponding species-specified probes. Finally, the HSP60 degenerate primers were shown to amplify an anticipated 600-bp PCR product from all 29 *Staphylococcus* species and from all but 2 of 30 other microbial species, including various gram-positive and gram-negative bacteria, mycobacteria, and fungi. These preliminary data suggest the presence of species-specific sequence variation within the highly conserved HSP60 genes of staphylococci. Further work is required to determine whether these degenerate HSP60 primers may be exploited for species-specific microbic identification and phylogenetic investigation of staphylococci and perhaps other microorganisms in general.

Coagulase-negative staphylococci have emerged as predominant pathogens in hospital-acquired infections (3, 16, 17). In light of this, it has become increasingly important to accurately identify these isolates to the species level in reference laboratories in order to further define the epidemiology and clinical significance of these microorganisms in the hospital setting. Several commercial kits are now available for the identification of coagulase-negative staphylococci to the species level (13, 21). Unfortunately, the overall accuracy of these systems, which are based on phenotypic discrimination, has been low, ranging from 50 to 70%. Genotypic methods of identification, either directed at unique genes specific for a microbial species or directed at unique sequences of ubiquitous genes such as 16S rRNA, may provide better results in terms of both sensitivity and specificity (14, 16a, 23). Several nucleic acid-based methods for species identification of staphylococci have been reported and are primarily based on restriction analysis of the 16S rRNA genes (4, 7, 8, 10, 20, 27). Besides being labor-intensive, a potential drawback with these procedures is within-species microheterogeneity, presumably because there may be multiple, evolutionarily diverged rRNA gene copies in some microorganisms (23). A more ideal universal DNA target for microbial identification to the species level would be one which has well-conserved DNA sequences within a given species, but with sufficient sequence variation to allow for species-specific identification. Here, we present preliminary data that the ubiquitous and highly conserved, single-copy 60-kDa heat shock protein (HSP60) [also known as GroEL]) genes may be an alternate DNA target for species-specific identification of staphylococci and perhaps other microorganisms in general.

We have designed and synthesized a set of universal degenerate primers based on highly conserved regions within the HSP60 genes from different microorganisms reported in the published literature. These degenerate primers amplified by PCR an anticipated 600-bp product from both *Staphylococcus aureus* and *Staphylococcus epidermidis*. However, when used as a DNA probe, the 600-bp PCR product generated from *S. epidermidis* failed to cross-hybridize under high-stringency conditions with the genomic DNA of *S. aureus* and vice versa (data not shown). This suggested to us that there may be variable sequences within the highly conserved HSP60 genes that may

* Corresponding author. Mailing address: Division of Infectious Diseases, G. F. Strong Research Laboratory, Vancouver Hospital Health Sciences Centre, 2733 Heather St., Vancouver, British Columbia, Canada V5Z 3J5. Phone: (604) 875-4148. Fax: (604) 875-4013.

† Present address: Department of Pathology and Laboratory Medicine, University of British Columbia, and the Provincial Laboratory, British Columbia Centre for Disease Control, Vancouver, British Columbia, Canada V5Z 1L8.

be useful for species-specific microbic identification among different staphylococci. To test this hypothesis, DNA probes were prepared from 600-bp PCR products generated by the degenerate HSP60 primers from six reference staphylococcal species (*S. aureus* 8325-4, *S. epidermidis* 9759, *S. haemolyticus* ATCC 29970, *S. lugdunensis* CRSN 850412, *S. saprophyticus* KL122, and *S. schleiferi* ATCC 43808). These were used in dot blot hybridization studies to identify a set of 58 reference and clinical isolates of *Staphylococcus* and non-*Staphylococcus* species in a coded fashion. The results, which demonstrated 100% accuracy in species identification of staphylococci compared with that of the reference method of Kloos and Lambe (17), strongly suggest that species-specific sequence variation exists within the highly conserved HSP60 genes of staphylococci.

MATERIALS AND METHODS

Bacterial isolates. The bacterial isolates used in this study consisted of 35 reference strains of various *Staphylococcus* species obtained from the American Type Culture Collection and from W. Kloos, North Carolina State University, Raleigh, N.C., and 20 clinical isolates from our own collection identified in the Clinical Microbiology Laboratory of the Vancouver Hospital and Health Sciences Centre, Vancouver, British Columbia, Canada (Table 1). In addition, three non-staphylococcal reference isolates, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Bacillus subtilis* ATCC 12432, were included as negative controls. All cultures were grown in brain heart infusion (BHI) broth and subcultured on BHI plates for examination of purity and colony characteristics. Clinical isolates that gave either false-positive or false-negative results when probed with the 600-bp HSP60 staphylococcal species probes were coded and sent to the reference laboratory, the Provincial Laboratory of the British Columbia Centre for Disease Control, Vancouver, for reidentification according to the method of Kloos and Lambe (17).

Isolation of genomic DNA. Genomic DNA of pure cultures after overnight growth in BHI broth were prepared by the standard sodium dodecyl sulfate (SDS)-proteinase K-cetyl trimethyl ammonium bromide-phenol-chloroform method (2). For staphylococci, lysostaphin (from Sigma, or a recombinant product from Applied Microbiology Inc., New York, N.Y.) was substituted for lysozyme in facilitating cell lysis. DNA was resuspended in TE buffer (10 mM Tris, [pH 8.0], 1 mM EDTA), the concentration was determined by UV spectroscopy at A_{260}, and the purity was estimated by the A_{260}/A_{280} ratio.

Input DNAs of mixed staphylococcal species for PCR experiments were prepared by pooling of 50 μl of overnight cultures in BHI broth from different organisms in various combinations. Crude DNA was prepared with the Instagene purification matrix (Bio-Rad) according to the manufacturer's instructions. Uninoculated BHI broth was mock processed and used as a negative control. The final extract was in 500 μl of purification matrix, and 20 μl of the matrix extract was used as the target DNA for PCR.

PCR amplification. The PCR mixture contained (in final concentration) 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate (dNTP), 50 ng of genomic DNA or 20 μl of Instagene extract, 2 U of *Taq* DNA polymerase (GIBCO), and 0.5 μg of each of the degenerate HSP60 primers, in a final volume made up to 100 μl with distilled H_2O. The sequences of the 5′ and 3′ HSP60 primers, designated H279 and H280, are 5′-GAATTC GAIIIIGCIGGIGA(TC)GGIACIACIAC-3′ and 5′-CGCGGGATCC(TC)(T G)I(TC)(TG)ITCICC(AG)AAICCIGGIGC(TC)TT-3′, respectively. Inosine (I) was used to reduce the degeneracy of the primers. The thermal cycling conditions were 3 min at 95°C for 1 cycle, followed by 40 cycles of 1 min at 94°C, 2 min at 37°C, and 5 min at 72°C. The last cycle was for 10 min at 72°C. After PCR amplification, 10 μl of each reaction mixture was analyzed on a 2.0% TAE (Tris-acetate-EDTA) agarose gel. The DNA fragments were visualized and photographed under UV light after ethidium bromide staining.

Purification and digoxigenin labelling of 600-bp HSP60 PCR products. The 600-bp PCR products amplified from genomic DNA prepared from *S. aureus* (8325-4), *S. epidermidis* (9759), *S. haemolyticus* (ATCC 29970), *S. lugdunensis* (CRSN 850412), *S. saprophyticus* (KL 122), and *S. schleiferi* (ATCC 43808) were purified by electrophoresis on a 2.0% low-melting-point agarose gel and extracted with either β-agarase I (New England Biolabs) or the QIA Quick Gel extraction kit (Qiagen Inc.) according to manufacturer's instructions. Labelling of the 600-bp fragments for use as DNA probes was carried out with digoxigenin–11-dUTP and the standard random primer method (24) according to Boehringer Mannheim protocols.

Dot blot hybridization. Genomic DNAs (300 ng, 0.4 M NaOH denatured) of pure cultures or PCR-amplified DNAs from mixed cultures were dot blotted onto nylon membranes (Boehringer Mannheim). For mixed cultures, an overnight growth in BHI broth was first subjected to PCR amplification with the degenerate HSP60 primers as described earlier. PCR-amplified DNA was then purified with the QIA Quick-spin PCR purification kit (Qiagen Inc.). A 1:8,000 dilution of the purified PCR product in 50 μl of distilled H_2O was prepared, and 5 μl of each of the diluted samples was spotted on the filters. After baking of the filters at 120°C for 30 min, the blots were neutralized with 0.5 M Tris (pH 7.5) and then dried before use. The filters were prehybridized at 42°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2% Boehringer Mannheim blocking reagent–0.1% *N*-lauryl sarcosine–0.02% SDS for at least 1 h. Hybridization with the same prehybridization buffer and digoxigenin probes (40 to 50 ng/ml) was allowed to proceed overnight at 42°C. After hybridization, the filters were washed sequentially with 2× SSC–0.1% (wt/vol) SDS twice for 15 min at room temperature and then with 0.1× SSC–0.1% SDS twice for 15 min at 68°C. Detection of hybridization by chemiluminescence was performed as described in the Boehringer Mannheim protocols.

DNA sequencing. Direct DNA sequencing of the six *Staphylococcus* species HSP60 probes was performed by the fluorescence-based dideoxy termination method with a cycle sequencing protocol and reagents supplied by Applied Biosystems, Inc. (26). The cycle sequencing reaction mixture contained 400 mM Tris-HCl; 10 mM $MgCl_2$; 100 mM $(NH_4)_2SO_4$ (pH 9.0); 750 μM dITP; 150 μM (each) dATP, dTTP, and dCTP; 150 μM (each) dye-labeled ddNTP; 4 U of Ampli*Taq* DNA polymerase; 7 μl of template DNA; and 3.2 pM either primer H279 or primer H280, in a final volume made up with distilled H_2O to 20 μl. The thermal cycling conditions were 30 s at 96°C, 15 s at 50°C, and 4 min at 60°C for 25 cycles. Sequenced products were purified with the QIA Quick-spin PCR purification kit, ethanol precipitated, and resuspended in 4 μl of loading buffer (5:1 [vol/vol] deionized formamide–50 mM EDTA [pH 8.0]). Samples were heated at 90°C for 2 min prior to loading on a 6% (wt/vol) polyacrylamide gel containing 7 M urea for electrophoresis and sequencing in an automated DNA sequencer (Applied Biosystems model 373A). Emission data from the fluorescence-tagged reaction mixtures were collected and analyzed with the proprietary Macintosh-based software (version 1.2.0). The automatically assigned base calls were examined with the analysis software, and the sequence was edited manually when necessary. A nucleotide sequence homology search was performed through the National Center for Biotechnology Information (NCBI) BLAST Network Service according to the algorithm of Altschul et al. (1). A phylogenetic tree of the six HSP60 staphylococcal species probes was derived from multiple sequence alignments based on the Higgins-Sharp algorithm with the CLUSTAL4 package (15).

RESULTS

Dot blot hybridization of genomic DNA from pure cultures. The results of dot blot hybridization of the 58 reference and clinical isolates of *Staphylococcus* and non-*Staphylococcus* species with the six *Staphylococcus* species HSP60 probes are summarized in Table 1. An example of the dot blot results from the *S. epidermidis* HSP60 probe is shown in Fig. 1. None of the control isolates, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *B. subtilis* ATCC 12432, gave positive hybridization signals with any of the six *Staphylococcus* species HSP60 probes. The dot blot results from all 35 reference isolates of *Staphylococcus* species with the six HSP60 probes were in complete agreement regarding their species designation. In contrast, 7 of 20 (35%) clinical staphylococcal isolates gave either false-positive or false-negative results when hybridized with the six *Staphylococcus* species HSP60 probes. These seven clinical isolates included two strains identified as *S. haemolyticus* by the clinical microbiology laboratory, which did not hybridize with the *S. haemolyticus* probe but hybridized with the *S. epidermidis* probe and the *S. lugdunensis* probe, respectively; one strain identified as *S. saprophyticus*, which did not hybridize with the *S. saprophyticus* probe but hybridized with the *S. epidermidis* probe; and four other strains identified as *S. hominis*, *S. sciuri*, *S. xylosus*, and *S. capitis*, which hybridized with the *S. epidermidis* probe, the *S. aureus* probe, the *S. lugdunensis* probe, and the *S. haemolyticus* probe, respectively. These seven clinical isolates were coded and sent to the provincial reference laboratory in a blinded fashion for reidentification according to the method of Kloos and Lambe (17). The results obtained from the reference laboratory indicated that all seven isolates had been previously misidentified in the clinical microbiology laboratory and that their corrected species designation agreed completely with the hybridization results obtained with the *Staphylococcus* species HSP60 probes (Table 2). Thus, all three apparently false-negative results from the HSP60 probes were in fact true negative, and all seven apparently false-positive

TABLE 1. Order of genomic DNA used in dot blots and hybridization results

Isolate no.	Identification	Strain	Hybridization results with HSP60 probe specific for:					
			S. epidermidis	*S. aureus*	*S. haemolyticus*	*S. schleiferi*	*S. saprophyticus*	*S. lugdunensis*
1	*S. aureus*	8325-4	−	+	−	−	−	−
2	*S. aureus*	ATCC 29213	−	+	−	−	−	−
3	*S. aureus*	8387	−	+	−	−	−	−
4	*S. aureus*	SA8	−	+	−	−	−	−
5	*S. aureus*	ATCC 12600[a]	−	+	−	−	−	−
6	*S. epidermidis*	9759	+	−	−	−	−	−
7	*S. epidermidis*	ATCC 14990[a]	+	−	−	−	−	−
8	*S. epidermidis*	8469	+	−	−	−	−	−
9	*S. epidermidis*	8331	+	−	−	−	−	−
10	*S. haemolyticus*	ATCC 29970[a]	−	−	+	−	−	−
11	*S. haemolyticus*	8459	+	−	−	−	−	−
12	*S. haemolyticus*	8564	−	−	+	−	−	−
13	*S. haemolyticus*	8589	−	−	−	−	−	+
14	*P. aeruginosa*	ATCC 27853	−	−	−	−	−	−
15	*S. saprophyticus*	8523	−	−	−	−	+	−
16	*S. saprophyticus*	8524	−	−	−	−	+	−
17	*S. saprophyticus*	8638	−	−	−	−	+	−
18	*S. saprophyticus*	9761	+	−	−	−	−	−
19	*S. lugdunensis*	CRSN 850412[a]	−	−	−	−	−	+
20	*S. schleiferi*	ATCC43808[a]	−	−	−	+	−	−
21	*S. schleiferi*	BOSTON[a]	−	−	−	+	−	−
22	*S. capitis* subsp. *ureolyticus*	ATCC 49326[a]	−	−	−	−	−	−
23	*S. capitis* subsp. *capitis*	ATCC 27840[a]	−	−	−	−	−	−
24	*S. capitis*	8468	−	−	+	−	−	−
25	*S. hominis*	ATCC 27844[a]	−	−	−	−	−	−
26	*S. hominis*	9998	−	−	−	−	−	−
27	*S. hominis*	8458	+	−	−	−	−	−
28	*S. warneri*	ATCC 27836[a]	−	−	−	−	−	−
29	*S. warneri*	9290	−	−	−	−	−	−
30	*S. warneri*	8586	−	−	−	−	−	−
31	*S. xylosus*	ATCC 29971[a]	−	−	−	−	−	−
32	*S. xylosus*	8584	−	−	−	−	−	+
33	*S. sciuri*	ATCC 29061[a]	−	−	−	−	−	−
34	*S. sciuri*	10034	−	+	−	−	−	−
35	*S. simulans*	ATCC 27848[a]	−	−	−	−	−	−
36	*E. coli*	ATCC 25922	−	−	−	−	−	−
37	*S. simulans*	9852	−	−	−	−	−	−
38	*S. caprae*	ATCC 35538[a]	−	−	−	−	−	−
39	*S. auricularis*	ATCC 33753[a]	−	−	−	−	−	−
40	*S. cohnii* subsp. *cohnii*	ATCC 29974[a]	−	−	−	−	−	−
41	*S. capitis* subsp. *ureolyticus*	ATCC 49325[a]	−	−	−	−	−	−
42	*S. kloosii*	ATCC 43959[a]	−	−	−	−	−	−
43	*S. equorum*	ATCC 43958[a]	−	−	−	−	−	−
44	*S. arlettae*	ATCC 43957[a]	−	−	−	−	−	−
45	*S. carnosus*	MA[a]	−	−	−	−	−	−
46	*S. intermedius*	CFDD[a]	−	−	−	−	−	−
47	*S. delphini*	Heidy[a]	−	−	−	−	−	−
48	*S. hyicus*	ATCC 11249[a]	−	−	−	−	−	−
49	*S. chromogenes*	CDC 2[a]	−	−	−	−	−	−
50	*S. caseolyticus*	ATCC 13548[a]	−	−	−	−	−	−
51	*S. lentus*	K20[a]	−	−	−	−	−	−
52	*S. vitulus*	ATCC 51145[a]	−	−	−	−	−	−
53	*S. pasteuri*	BM 10426[a]	−	−	−	−	−	−
54	*S. gallinarum*	ATCC 35539[a]	−	−	−	−	−	−
55	*S. felis*	GD 521[a]	−	−	−	−	−	−
56	*S. schleiferi* subsp. *coagulans*	ATCC 49545[a]	−	−	−	+	−	−
57	*S. aureus*	7162	−	+	−	−	−	−
58	*B. subtilis*	ATCC 12432	−	−	−	−	−	−
59	0.4 M NaOH		−	−	−	−	−	−

[a] Isolates kindly provided by W. Kloos, North Carolina St. University, Raleigh.

results were in fact true-positive. Taken together, the dot blot results with these six *Staphylococcus* species HSP60 probes were 100% accurate when tested with the set of 58 bacterial isolates compared with those tested by the reference identification method of Kloos and Lambe (17).

Dot blot hybridization with HSP60 products from mixed cultures. The species specificity of the HSP60 probes was assessed in two sequential steps: by determination of whether the degenerate primers can amplify species-specific staphylococcal targets from mixed cultures by PCR and whether species-spe-

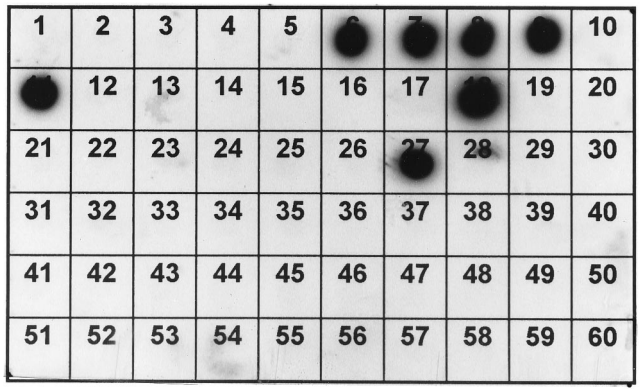

FIG. 1. Determination of the specificity of the *S. epidermidis* 9759 HSP60 probe by dot blot hybridization to bacterial chromosomal DNA. Numbers refer to the numerical order and designation of bacterial isolates listed in Table 1. Positive hybridization reactions were observed for isolates 6 (*S. epidermidis*), 7 (*S. epidermidis*), 8 (*S. epidermidis*), 9 (*S. epidermidis*), 11 (misidentified as *S. haemolyticus* by the clinical laboratory and confirmed as *S. epidermidis* by the reference laboratory), 18 (misidentified as *S. saprophyticus* by the clinical laboratory and confirmed as *S. epidermidis* by the reference laboratory), and 27 (misidentified as *S. hominis* by the clinical laboratory and confirmed as *S. epidermidis* by the reference laboratory).

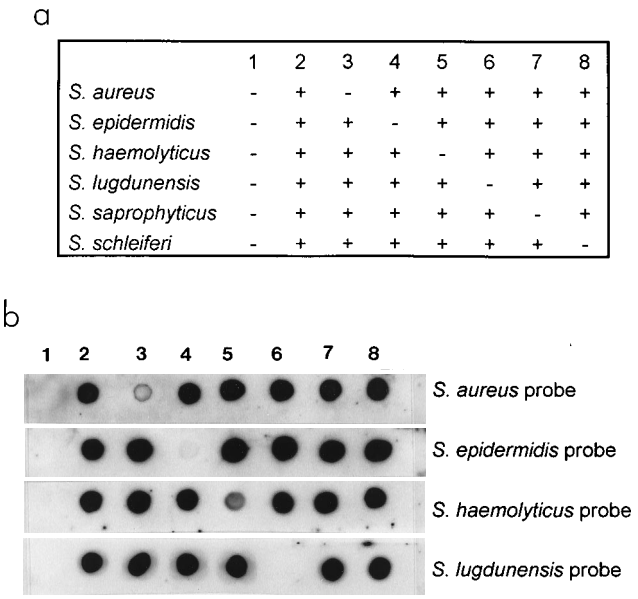

FIG. 2. Determination of the species specificity of *Staphylococcus* HSP60 probes by dot blot hybridization with PCR-amplified DNA from mixed cultures. (a) Mixed cultures consisting of the combination of different *Staphylococcus* species (represented by numbers shown at the top [+, present; −, absent]) were first prepared from overnight cultures and used as target DNA for PCR amplification with the degenerate HSP60 primers. (b) PCR-amplified DNAs from mixed cultures (corresponding to the numbers at the top in panel a) were used for dot blot hybridization with each of four *Staphylococcus* species HSP60 probes. Absent or markedly reduced signals were observed in the blots lacking the probe-specified *Staphylococcus* species DNA.

cific HSP60 probes can identify the correct target from mixed PCR products by dot blot hybridization. Mixed PCR targets containing various combinations of all but one of the six different *Staphylococcus* species (i.e., *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. saprophyticus*, and *S. schleiferi*) were prepared with the Bio-Rad Instagene purification matrix and were amplified by PCR with our degenerate HSP60 primers (Fig. 2a). PCR-amplified mixed-species DNAs were probed with each of four *Staphylococcus* species HSP60 probes (i.e., *S. aureus*, *S. epidermidis*, *S. haemolyticus*, and *S. lugdunensis*) (Fig. 2b). In all instances, when the four respective probes were used, there was either absent or markedly reduced signal in the hybridized mixed-species DNA targets lacking the probe-specified *Staphylococcus* species DNA, indicating the excellent species specificity of these HSP60 probes (Fig. 2b). Although some residual background signal can be observed in the blots hybridized with the *S. haemolyticus* and *S. aureus* probes, the signal-to-noise ratio was high with each probe compared with those of the positive and negative controls (Fig. 2b, lanes 1 and 2).

Partial DNA sequences and phylogenetic tree of HSP60 *Staphylococcus* species probes. Results from the DNA sequences of the six HSP60 *Staphylococcus* species probes are only partially complete (data not shown and to be separately reported). However, species-specific sequence variation within the 600-bp probes was clearly evident in each instance. Furthermore, nucleotide sequence homology searches of databases available through the NCBI BLAST Network Service with these partial HSP60 DNA sequences yielded the highest matching scores for both *S. epidermidis* and *S. aureus* with the corresponding species-specified probes, the only two staphylococcal species for which HSP60 gene sequence data have been published (Table 3). Finally, the partial HSP60 staphylococcal species DNA sequences were also analyzed by multiple alignments, and a tentative phylogenetic tree demonstrating the

TABLE 2. Species designation of seven clinical isolates with apparent false-negative and false-positive hybridization reactions with the six HSP60 staphylococcal species probes

Isolate	*Staphylococcus* species designation		Positive hybridization with HSP60 staphylococcal probes
	Clinical laboratory	Reference laboratory	
9761	*S. saprophyticus*	*S. epidermidis*	*S. epidermidis*
8458	*S. hominis*	*S. epidermidis*	*S. epidermidis*
10034	*S. sciuri*	*S. aureus*	*S. aureus*
8468	*S. capitis*	*S. haemolyticus*	*S. haemolyticus*
8459	*S. haemolyticus*	*S. epidermidis*	*S. epidermidis*
8584	*S. xylosus*	*S. lugdunensis*	*S. lugdunensis*
8589	*S. haemolyticus*	*S. lugdunensis*	*S. lugdunensis*

TABLE 3. Results of homology searches of *Staphylococcus* species partial HSP60 DNA sequences from databases through the NCBI BLAST Network Service

Staphylococcus species HSP60 sequence	High-scoring sequence identified from databases			
	S. epidermidis HSP60[a]		*S. aureus* HSP60[b]	
	% Similarity	Probability[c]	% Similarity	Probability[c]
S. epidermidis	95	$6.0\,e^{-136}$	78	$2.1\,e^{-78}$
S. aureus	80	$4.2\,e^{-102}$	93	$3.2\,e^{-135}$
S. haemolyticus	80	$2.0\,e^{-101}$	82	$1.8\,e^{-93}$
S. lugdunensis	80	$3.1\,e^{-85}$	82	$4.8\,e^{-80}$
S. saprophyticus	82	$2.4\,e^{-88}$	82	$2.7\,e^{-76}$
S. schleiferi	77	$7.4\,e^{-100}$	79	$1.4\,e^{-86}$

[a] GenBank accession number U13618 (12).
[b] GenBank accession number D14711 (19).
[c] Probability of a random match.

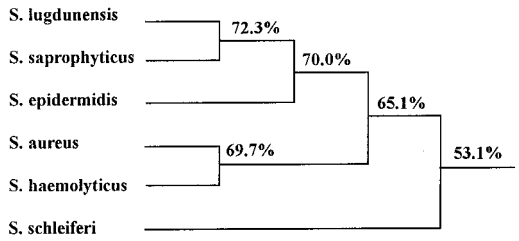

FIG. 3. Phylogenetic tree derived from multiple alignments of partial DNA sequences of six *Staphylococcus* species HSP60 probes. Match percentages are shown at branch points.

interspecies relationships among these six HSP60 probes was derived (Fig. 3). The data revealed that *S. aureus* and *S. haemolyticus* are highly related (match ratio, 69.7%). Similarly, *S. lugdunensis* and *S. saprophyticus* are highly related (match ratio, 72.3%). Both of these clusters are related to *S. epidermidis* (match ratios, 65.1 and 70.0%, respectively), while *S. schleiferi* is the least related species (match ratio, 53.1%).

DISCUSSION

The finding that our six staphylococcal HSP60 probes accurately identified all 35 reference isolates of various *Staphylococcus* species, including *S. aureus* and five species representing the most clinically important coagulase-negative staphylococci, is most encouraging. The observation that 7 of 20 clinical isolates of coagulase-negative staphylococci were misidentified by commercially available identification systems further supports the notion that existing laboratory methods based on phenotypic characterization of coagulase-negative staphylococci are unreliable.

The hybridization results from the 58 bacterial isolates in this study and the partial DNA sequence data from the six *Staphylococcus* species HSP60 probes are highly suggestive that HSP60 gene sequences can be useful targets for species identification of *S. aureus* and at least some coagulase-negative staphylococci. Whether this approach can be successfully applied to all *Staphylococcus* species and perhaps other microorganisms will require extensive study. Nonetheless, the selection of the HSP60 gene as a possible universal DNA target for microbial species identification offers a number of theoretical advantages. First, HSP60 genes are ubiquitous in both procaryotes and eucaryotes and encode highly conserved housekeeping proteins which are essential for the survival of these cells (5, 9, 18, 22, 29). These characteristics may render them less subject to random mutations or intraspecies variation. Second, these highly conserved proteins have been examined in a limited fashion for phylogenetic relationships among eubacterial lineages (6, 11, 28). Evolutionary trees drawn from the protein sequences of these molecules demonstrate remarkable similarities to those derived from 16S rRNA genes. Moreover, in some instances, these phylogenetic studies on the basis of HSP60 analysis have indicated specific relationships between certain eubacterial groups which appear not to be clearly defined or are controversial in rRNA-based phylogenetic evaluation (28). Finally, the degenerate HSP60 primers that we have developed were capable of amplifying an anticipated 600-bp HSP60 product from all 29 *Staphylococcus* species listed in Table 1 and all but 2 of 30 other microbial species, including the following gram-positive and gram-negative bacteria, mycobacteria, and candida: *Streptococcus agalactiae* and *S. pneumoniae*; *Enterococcus faecalis*; *Listeria monocytogenes*; *B. subtilis*; *E. coli*; *Salmonella typhi*, *S. typhimurium*, and *S. gallinarum*; *Yersinia pseudotuberculosis*; *Vibrio cholerae*; *P. aeruginosa*; *Haemophilus influenzae*; *Campylobacter jejuni*, *C. coli*, and *C. laridis*; *Helicobacter pylori* and *H. mustelae*; *Flexispira rappini*; *Neisseria gonorrhoeae*; *Legionella pneumophila*; *Bartonella henselae*, *B. quintana*, and *B. bacilliformis*; *Borrelia hermsii*; *Mycobacterium marinum* and *M. fortuitum*; and *Candida albicans*. The two exceptions were *Borrelia burgdorferi* and *Mycobacterium smegmatis* MC155. It is likely that the HSP60 gene targets of these two species contained mismatches with our degenerate primer set. Indeed, further optimization of our degenerate primer set in subsequent studies did result in successful amplification of the anticipated 600-bp HSP60 product from *B. burgdorferi* (data not shown). In addition, Steingrube et al. (25) were able to amplify the GroEL gene sequences of *M. smegmatis* by utilizing a different set of HSP primers. Thus, for the majority of microbial species, it would be possible to generate the 600-bp HSP60 sequences with our degenerate primers and to determine by direct sequencing the degree of sequence similarity or divergence within the HSP60 genes among different microbial species. Such information will be invaluable in ascertaining whether HSP60 gene sequences might be useful for species-specific microbial identification or phylogenetic investigation of certain microorganisms. For this reason, we are currently in the process of obtaining the DNA sequence data of the entire set of the 600-bp HSP60 products generated with our degenerate HSP60 primers from the genus *Staphylococcus.*

If these HSP60 genes are ultimately proven to contain internal sequences that are species specific, several approaches can be utilized to exploit these universal targets for microbial identification and identification to the species level both from pure cultures and directly from clinical specimens. The latter approach may be particularly useful for the rapid diagnosis of infections due to slowly growing, fastidious, or nonculturable microorganisms. Either species-specific HSP60 DNA probes can be developed, or PCR-amplified HSP60 sequences obtained from infected clinical specimens can be used for nucleotide homology searches from expanded databases, much in the same way that 16S rRNA sequences have been utilized to identify uncultured microbial pathogens (23).

In summary, our preliminary data from staphylococci do suggest the presence of species-specific sequence variation within the highly conserved HSP60 genes of this genus. Further work is clearly warranted to determine whether these degenerate HSP60 primers may be exploited for species-specific microbic identification and phylogenetic investigation of all staphylococci and perhaps other microorganisms in general.

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