Simultaneous Analysis of Multiple Staphylococcal Enterotoxin Genes by an Oligonucleotide Microarray Assay

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Staphylococcal enterotoxins (SEs) are a family of 17 major serological types of heat-stable enterotoxins that are one of the leading causes of gastroenteritis resulting from consumption of contaminated food. SEs are considered potential bioweapons. Many *Staphylococcus aureus* isolates contain multiple SEs. Because of the large number of SEs, serological typing and PCR typing are laborious and time-consuming. Furthermore, serological typing may not always be practical because of antigenic similarities among enterotoxins. We report on a microarray-based one-tube assay for the simultaneous detection and identification (genetic typing) of multiple enterotoxin (*ent*) genes. The proposed typing method is based on PCR amplification of the target region of the *ent* genes with degenerate primers, followed by characterization of the PCR products by microchip hybridization with oligonucleotide probes specific for each *ent* gene. We verified the performance of this method by using several other techniques, including PCR amplification with gene-specific primers, followed by gel electrophoresis or microarray hybridization, and sequencing of the enterotoxin genes. The assay was evaluated by analysis of previously characterized staphylococcal isolates containing 16 *ent* genes. The microarray assay revealed that some of these isolates contained additional previously undetected *ent* genes. The use of degenerate primers allows the simultaneous amplification and identification of as many as nine different *ent* genes in one *S. aureus* strain. The results of this study demonstrate the usefulness of the oligonucleotide microarray assay for the analysis of multitoxigenic strains, which are common among *S. aureus* strains, and for the analysis of microbial pathogens in general.

Staphylococcal food-borne diseases resulting from the consumption of food contaminated with staphylococcal enterotoxins (SEs) are one of the most common food-borne illnesses (1, 5, 11, 14, 31). SEs are also involved in rheumatoid arthritis (17, 38), atopic eczema (9, 10, 27), and toxic shock syndrome (16). SEs are considered potential bioweapons.

SEs belong to a protein family called superantigens, which induce a polyclonal immune response by direct binding to class II major histocompatibility complex proteins and T-cell receptors on the surfaces of B and T cells without being internalized and processed like a normal antigen (3, 15, 28). These toxins may be involved in modulating the host immune response and may contribute to evasion of host defenses and bacterial persistence (12). Expression of specific enterotoxin (*ent*) genes by *Staphylococcus aureus* depends on the host tissue source and may play a role in the adaptation of *S. aureus* to the host environment (4).

There are 17 known major types of SEs (SEA to SER, respectively, with no SEF), and multiple SEs are commonly found among *S. aureus* strains (19, 20, 32). Many of the known staphylococcal enterotoxins (SEK to SER) were discovered recently.

The traditional method of identifying SEs by serological typing is relatively complex and time-consuming and is impractical for the detection and identification of a large group of related toxins with significant antigenic similarities (23, 24). Furthermore, the concentrations of toxins produced by *S. au-*

reus strains differ when the strains are grown on various natural substrates and laboratory media (7, 33). Other techniques have been used to identify toxin genotypes, including DNA-DNA hybridization and PCR, but these protocols were designed to detect only one or a few toxin genes (21, 35). Multiplex PCR for detection of several *ent* genes has been reported (6, 26, 29, 30, 36), but additional restriction endonuclease assays or other steps are required to ensure unambiguous identification of *ent*-specific amplicons. Therefore, there is still a need for a rapid and specific method for simultaneous detection and identification of SEs for diagnostic and epidemiological purposes.

Here we describe a rapid and reliable one-tube microarray-based assay for simultaneous detection and identification (genetic typing) of almost all known *ent* genes. The method includes PCR amplification of part of the *ent* genes with universal primers, followed by analysis of amplicons by hybridization with *ent*-specific oligonucleotide probes immobilized on the microchip.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study were obtained from the *S. aureus* collection of the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA; Focus Technologies, Inc., Herndon, Va.) and from the bacterial collection of Farukh Khambaty, Center of Food Safety and Applied Nutrition, Food and Drug Administration (FDA).

Total DNA preparation. DNA was extracted from freshly grown cells by phenol-chloroform extraction (34). The presence, concentration, and purity of genomic DNA in the prepared samples were detected by measuring the absorbances at 260 and 280 nm with an Ultraspec 3000 spectrophotometer (Pharmacia, Peapack, N.J.).

PCR amplification. Table 1 lists the primers used to amplify different *S. aureus* enterotoxin genes. Specific primers were used for amplification of individual enterotoxin genes. The standard PCR mixture (30 μ l) contained 1.5 U of Hot-

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TABLE 1. Primers used for amplification of *S. aureus* *ent* genes

Gene	GenBank accession no(s).	Primers	Nucleotide sequence(5'→3' end)	T_m (°C) ^a	Length (nt) ^b
<i>sea</i>	M18970	setA-F	GGATATTGTTGATAAAATATAAAGGGAAAAAAG	51	439
		setA-R	GTTAATCGTTTTATTATCTCTATATATTCTTAATAGT	54	
<i>seb</i>	M11118	setB-F	AGATTTAGCTGATAAATACAAAAGATAAATACG	54	494
		setB-R	TCGTAAGATAAACTTCAATCTTCACATCT	54	
<i>sec</i>	M28364, X05815, AB084256	setC-F	AGATTTAGCAAAGAAGTACAAAGATG	52	490
		setC-R	AAGGTGGACTTCTATCTTCACACTT	54	
<i>sed</i>	M28521	setD-F	AGATTTAGCAAAGAAGTACAAAGATG	55	481
		setD-R	CTACTTTTCATATAAATAGATGTCAATATG	52	
<i>see</i>	M21319	setE-F	AGATTTAGCAAAGAAGTACAAAGATG	54	473
		setE-R	TGTATAAATACAAATCAATATGGAGGTTCTCT	55	
<i>seg</i>	AF064773, AB016487	setG-F	AGAATTAGCTAACAATTATAAAGATAAAAAAAG	52	496
		setG-R	TCAGTGAGTATTAAGAAATACTTCCAT	52	
<i>seh</i>	U11702	setH-F	TGATTTAGCTCAGAAGTTTAAAAATAAAAAATG	52	466
		setH-R	TTTCTTAGTATATAGATTTACATCAATATG	51	
<i>sei</i>	AF064774	setI-F	TGATTTAGCTCAGAAGTTTAAAAATAAAAAATG	52	505
		setI-R	TTAGTTACTATCTACATATGATATTTCTGA	52	
<i>sej</i>	AF053140	setJ-F	ATGAAAAAACAATATTTTACTGATTTTCTCCC	55	807
		setJ-R	TCTACAGAACCAAGGTAGACTTATTAATAC	57	
<i>sek</i>	U93688, AF410775, AP004828	setK-F	ATGAATCTTATGATTTAATTTTCAAGATCAA	51	545
		setK-R	ATTTATATCGTTTCTTTATAAGAAATATCG	51	
<i>sel</i>	AF217235	setL-F	ATGAAAAAAGATTATTATTTGTAATTGTTATTAC	52	723
		setL-R	ATCATCTTTTTGAAATTTGACATCTAG	53	
<i>sem</i>	AF285760, AP003363	setM-F	ATGAAAAGAATACTTATCATTGTTGTTTTATTG	53	720
		setM-R	CTTCAACTTTCGTCCTTATAAGATATTTTC	54	
<i>sen</i>	AF285760	setN-F	ATAAAAAATATTTAAAAAGCTTATGAGATTGTTTC	52	777
		setN-R	ACTTAATCTTTATATAAAAAATACATCAATATG	50	
<i>seo</i>	AF285760	setO-F	TATGTAGTGTAACAATGCATATGCA	53	685
		setO-R	TCTATTGTTTTATTATCATTATAAATTTGCAAAT	53	
<i>sep</i>	AP003135	setP-F	TTAGACAAACCTATTATCATAATGGAAGT	52	618
		setP-R	TATATAAATATATATCAATATGCATATTTTAGACT	53	
<i>seq</i>	U93688, AF410775	setQ-F	GGAAAATACACTTTATATTACAGTTTCA	53	539
		setQ-R	ATTTATTACAGTTTTCTCATATGAAATCTC	52	
		Uni1 P1F	TRYAYRTAYGGIGGDVTHAC	40–53	
		Uni2 P2R	AHHRTTYTATTRTCWYYRTAHA	36–51	

^a The basic melting temperature (T_m) was calculated with the Oligonucleotide Properties calculator (<http://www.basic.nwu.edu/biotools/oligocalc.html>).

^b Amplicon size (in nucleotides [nt]).

Star *Taq* DNA polymerase, 1× buffer supplemented with 2.0 mM MgCl₂ (Qiagen, Valencia, Calif.), 200 nM each forward and reverse primers, 200 μM each deoxynucleoside triphosphate (dNTP; dATP, dGTP, dCTP, and dTTP), and 100 to 300 ng of DNA template. PCR was performed with a Gene AMP PCR system 9600 thermocycler (Applied Biosystems, Foster City, Calif.) with the following cycle conditions: initial activation at 95°C for 15 min; 40 cycles at 94°C for 30 s, 55°C for 40 s, and 72°C for 60 s; and a final extension at 72°C for 7 min. The presence of amplified PCR products was detected by 2% agarose gel electrophoresis in 1× Tris-acetate-EDTA or Tris-borate-EDTA buffer. The gels were stained with ethidium bromide and photographed under UV light with a digital camera (EDAS 290; Kodak, Rochester, N.Y.).

For simultaneous amplification of multiple enterotoxin genes by PCR with universal primers, the standard PCR mixture (50 μl) contained 5 U of HotStart *Taq* DNA polymerase (Qiagen), 1× buffer supplemented with 3.5 mM MgCl₂ (Qiagen), the universal primer mixture (700 nM forward universal primer, 1,400 nM reverse universal primer, and the *seh*-specific reverse primer at a

concentration of 50 nM), 200 μM each dNTP, and 600 to 800 ng of template (total DNA). PCR was performed with a Gene AMP PCR system 9600 thermocycler (Applied Biosystems) with the following conditions: initial activation of the enzyme at 95°C for 15 min; 7 cycles at 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min; 35 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR products were purified with a Qiaquick PCR purification kit (Qiagen). The concentrations of the PCR products were estimated by measuring the absorbance at 260 nm.

Synthesis of ssDNA. Single-stranded DNA (ssDNA) samples were synthesized by use of a primer extension (PE) reaction in the presence of only the reverse primer. The standard mixture (50 μl) for PE with enterotoxin gene-specific primers contained 3 U of *Taq* DNA polymerase (Sigma, St. Louis, Mo.), 1× PCR buffer, 200 nM the corresponding reverse primer, 200 μM each dNTP, and 300 to 500 ng of the amplicon obtained during the previous PCR step. PE reactions were performed with a Gene AMP PCR system 9600 thermocycler (Applied Biosystems) with the following temperature conditions: initial denaturing of

TABLE 2. Oligonucleotide probes for detection and discrimination among *ent* genes

Probe	Sequence	Length (nt) ^a	T _m ^b (°C)	Probe	Sequence	Length (nt) ^a	T _m ^b (°C)
A-1	CGATCAATTTATGGCTAGACG	21	50	J-1	CTGCATGAAAACAATCAACTTTAT	24	49
A-2	ACTCTGATGTTTTTGATGGGAAG	23	52	J-2	ATAGCATCAGAACTGTTGTTC	21	49
A-3	GTTTCATACTTCTACAGAACCTTC	24	52	J-3	TATACAACCCCTAGTACCTTTGATG	24	52
A-4	ATTCAAATACACTATTAAGAATATATAGAG	30	51	J-4	ACCTCAAAGAACCCTTGGTTA	22	51
B-1	AATATAGAAGTATTACTGTTCCGGGT	25	51	K-1	GCTACTAACGAATATCTAGATAAAT	25	49
B-2	AGAAGTATTACTGTTCCGGGTATTT	24	51	K-2	CATAACGGCACTAAAAAGGAG	22	51
B-3	AAACTCTATGAATTTAACTACG	24	49	K-3	ATAATGACACTTTTTCATATGATTATTCT	30	51
B-4	ATGAGAATAGCTTTTGGTATGACA	24	51	K-4	TATTCTACACAGGAGATGATGG	22	51
C-1	AACCACCTTGATAATGGGAACCTTA	24	51	L-1	ACAATAAATTAGATTCCGCAAGAAT	25	49
C-2	TGTACTTATAAGAGTTTATGAAAAATAAAG	30	51	L-2	GCAAGCATCAAAACAGTTACAAC	22	51
C-3	AGTTTAAACAGTTCACCATATGAAACA	26	52	L-3	ACGGCACTTCTTCAAAATTTTATAG	22	51
C-4	ATTGAAAAAATACGGCAATACTTTTTG	26	50	L-4	ATGATGATGGTTCTAATTCTCTCG	24	51
D-1	TCACTCCACACGAAGGTAATA	21	50	M-1	TTAGCAGGTGATTATTTAGAGAAAT	25	49
D-2	TCAATTTGTGGATAAATGGTGTAC	24	51	M-2	TATGGCTTTAATGATACAAATAAAG	25	48
D-3	ATTTAAAATTGTATAAATGATACTCTCG	30	51	M-3	ATGATGGTTCATCATTTTCTTATG	24	49
D-4	TTCTGATGGGTCTAAAGTCTCT	22	51	M-4	AACAGGACAAGCTGAAAGTTTC	22	51
E-1	ACGGAAAAATTGGTTTATATAACTC	25	49	N-1	AGATGAAGAGAAAGTTATAGGC	22	49
E-2	TGGTTTATATAACTCAGACAGCTTT	25	51	N-2	ATGGTGTCCAACAAGAAGGTT	21	50
E-3	TAAGGTGCAAAAGAGGCTTGATT	23	52	N-3	ATACAATAAAGATACCGGTACAT	24	49
E-4	ATTCTTCTGAAGGGTCCACG	20	52	N-4	CTTTCATTCTCATAATCAATCAAGAT	25	49
G-1	TTAATAGTTCAGAAAAATGAAAGAGAT	26	49	O-1	ATACATTTATCGTTACTACAGATAA	25	48
G-2	AACAATCGACAATAGACAATCACT	24	51	O-2	ATGACAGAATGACTAGTGATGTA	23	50
G-3	ATCACTTGGATTTACAATAACTAC	24	49	O-3	ACTAGTGATGTACAAAAAGGTTAT	24	49
G-4	ATGGTTCTGCATTGAATCTGG	22	51	O-4	AGGAAATTTACCAGATCAATATTTG	25	49
H-1	AAAGGGTGATTGGTGCTAATGT	22	51	P-1	AGACCTTCAGTCAAGACATTATT	23	50
H-2	ATGTTTGGGTAGATGGTATTCAAA	24	51	P-2	ACACAGATGCATTTAATGGAAAAATA	26	50
H-3	ATAAAGACAGCGAAATAAGTAAAG	24	49	P-3	ATTGAGTTTCACCCCTTCTTCTG	22	51
H-4	AACTCCTAGAGATTACTCATTC	22	49	P-4	ATCCAGATACACAGTTGAGGA	21	50
I-1	TTATCAGGACAATACTTAAATTCTG	25	49	Q-1	TTGGCGAATCAAAATTTAGATAAAC	25	49
I-2	AGGCAAAGAATATGGATATAAATCT	25	49	Q-2	ACTATATCTACAGACAAAGTTTCT	24	49
I-3	ATTCAGGTTTAAATAATGGGAAAGT	25	49	Q-3	GGATATCAGTCAAAATTTAATTCTG	25	49
I-4	GCCTGTAAGTTTTTGAATAATTATGAA	28	51	Q-4	TACATACGATTGTTTTACACCG	23	50
				QCprb	TGGCAGAAGCTATGAAACGATATGGG	27	58
				Cy3-QC	CCCATATCGTTTCATAGCTTCTGCCA	26	58

^a Amplicon size (in nucleotides [nt]).^b The basic melting temperature (T_m) was calculated with the Oligonucleotide Properties Calculator (<http://www.basic.nwu.edu/biotools/oligocalc.html>). The references for the oligonucleotide probe are cited in Materials and Methods.

DNA at 94°C for 2 min, followed by 40 cycles each of 94°C for 30 s, 52°C for 40 s, and 72°C for 1 min and a final extension at 72°C for 7 min.

The PE reaction mixture for multiple *ent* genes with a universal reverse primer (700 nM) and *seh* reverse primer (50 nM) was the same as described above, except that the amount of DNA template (PCR amplicons) was increased to 800 ng to 1 µg. The cycling conditions were the following: initial activation of the enzyme at 95°C for 15 min; 40 cycles at 94°C for 30 s, 45°C for 40 s, and 72°C for 60 s; and a final extension at 72°C for 7 min. The ssDNA was purified with a Qiaquick PCR purification kit (Qiagen) and dried under vacuum.

Chemical labeling of ssDNA. The dry ssDNA was reconstituted in 20 µl of water and chemically labeled with a fluorescent dye (cyanine 5 [Cy5]) with a MicroMax labeling kit (Perkin-Elmer, Boston, Mass.), according to the protocol of the manufacturer. Nonincorporated dye was removed from the DNA by purification through Centriscap columns (Princeton Separations, Adelphia, N.J.). The amount of the Cy5 dye incorporated into ssDNA was monitored by measuring the ratio of the absorbance at 649 to the absorbance at 260 nm. The typical ratio of λ₆₄₉/λ₂₆₀ was about 0.15 to 0.25, which corresponds to 1.5 to 3 dye moieties per 100 nucleotides of ssDNA.

Design of PCR primers and enterotoxin gene-specific microarray oligonucleotide probes. Searches with the BLAST program were used to find and retrieve the sequences of the available *ent* genes. The retrieved sequences were aligned by using ClustalX software (37). Sequences of highly conserved regions among all alleles of each *ent* gene were selected to design toxin-specific primers for accurate detection and identification of each target toxin gene. Toxin-specific

oligonucleotide probes were designed by using highly conserved regions for alleles of each *ent* gene within the region flanked by primers. The oligonucleotides selected are summarized in Table 2. The 5' end of the amino acid sequence of each oligonucleotide probe was modified during the synthesis (Qiagen) to enable the immobilization of the oligonucleotide to silylated (aldehyde) slides (ArrayIt, Sunnyvale, Calif.).

Microchip design and fabrication. To increase the confidence in the results of the microarray analysis, four individual oligonucleotide probes were selected for each *ent* gene. To facilitate interpretation of the microarray data, all oligonucleotide probes specific for one gene were placed on a separate row of the array.

Microchips were printed by use of a contact microspotting robotic system (PIXSYS 5500; Cartesian Technologies, Inc., Irvine, Calif.). The average size of the spots was 250 µm. The concentrations of the oligonucleotide probes were adjusted to 100 µM in 50% dimethyl sulfoxide before they were printed on the slides. A quality control oligonucleotide probe (39) of nonbacterial origin was added to each oligonucleotide probe at a concentration of 10 µM to enable monitoring of the spotting and hybridization steps of the microarray assay. Printed slides were incubated for at least 10 min at 85°C to evaporate the dimethyl sulfoxide completely, followed by 15 min of incubation in a freshly prepared 0.25% NaBH₄ solution in water. The slides were washed once for 5 min with 0.1% sodium dodecyl sulfate in water and five times for 1 min each time with distilled water to remove unbound oligonucleotides. Control spots used to mark the array position on the slide were generated by using 1× Spotting Solution (ArrayIt) in 0.25 M acetic acid.

Hybridization conditions. Hybridization of the fluorescently labeled DNA samples to the microarray was performed in 1× hybridization buffer (5× Denhardt's solution, 6× SSC buffer [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% Tween 20) at 45°C for 45 min. Before hybridization, 2 to 3 µl of Cy5-labeled DNA sample was mixed with an equal volume of 2× hybridization buffer containing 0.1 µM Cy3 quality control probe, followed by denaturation at 95°C for 3 min and chilling on ice. Each sample was placed on the microchip and covered with a glass coverslip (6 by 15 mm) to prevent evaporation of the probe during incubation. After the hybridization, the coverslips were washed away with 6× SSC containing 0.2% Tween 20 at room temperature. The slides were washed in a stepwise manner with 6× SSC buffer, 2× SSC buffer, and 1× SSC buffer for 2 min each and dried by airflow.

Microarray scanning. Fluorescent images of the microarrays were taken by scanning the slides with a ScanArray 5000 instrument (Perkin-Elmer). The fluorescent signals from each spot were measured and compared by using QuantArray software (Perkin-Elmer).

Sequencing. We sequenced some enterotoxin genes, including *seb*, *sed*, *see*, and *seq*. The PCR-amplified DNA fragments were purified by agarose gel electrophoresis, extracted with a QIAquick gel extraction kit (Qiagen) according to the protocol of the manufacturer, and sequenced with an ABI Prism 310 Genetic Analyzer System (PE Applied Biosystems, Foster City, Calif.).

Nucleotide sequence accession numbers. The accession numbers of the sequences deposited in GenBank are AY518386 for *seb* of strain ATCC 14458, AY518387 for *sed* of strain NTCC10656, AY518772 for *see* of strain ATCC 19095, and AY518388 and AY518389 for *see* and *seq* of strain ATCC 27664, respectively.

RESULTS

Enterotoxin gene-specific PCR primers and microarray oligonucleotide probe designs. For our genotyping scheme for simultaneous detection and identification (genetic typing) of multiple SE (*ent*) genes, we developed PCR amplification assays for 16 of the 17 known enterotoxin genes (*sea* to *see* and *seq* to *sej*) and an oligonucleotide microarray for the identification of the PCR amplicons.

To develop toxin-specific PCR primers and microarray oligonucleotide probes, we performed multiple-sequence alignment analysis of the *ent* genes using sequence data from GenBank. As shown in Fig. 1, the analysis identified conserved regions flanking variable regions. The conserved regions were used to design universal primers for simultaneous amplification of multiple *ent* genes. The genetically divergent regions were used to design individual PCR primers specific for each *ent* gene and to design gene-specific oligonucleotide probes to discriminate among the 16 *ent* genes (Fig. 1).

Our sequence data analysis revealed discrepancies in the nomenclatures of several *ent* genes from the published sequences of strains MW2 and MU50. For example, the sequence named *seg* in strain MW2 has 98% similarity to *seq* (GenBank accession number AF410775) and a lower degree of homology to another sequence of *seg* (GenBank accession number AF064773). Similarly, *sep* from strain MU50 has only 83% similarity to a different reported *sep* gene (GenBank accession number NC002745), whereas it has 98% similarity to the *sea* enterotoxin gene (GenBank accession number M18970).

For the amplification of each *ent* gene, gene-specific primers were selected on the basis of unique sequences common to all alleles of each toxin gene determined by the multiple-sequence alignment (Fig. 1). To minimize cross-amplification between different toxin genes, the primers selected contained five and more mismatches with homologous toxins. However, in the case of *s*, we decided not to develop allele-specific oligonucle-

FIG. 1. Multiple-sequence alignment analysis of *ent* genes. The DNA sequences of 16 major SEs were retrieved from GenBank and aligned by using ClustalX software. The alignment results were presented by using GeneDoc software. Relatively conserved regions that were used for universal primer design are marked with arrows. The sequences of the oligonucleotide probes used for discrimination of the *ent* genes were selected from within the variable region flanked by the conserved regions. Gray background indicates similar sequences, and black background indicates conserved sequences.

otide probes for the three described alleles, *s*, *sec1*, and *sec2*, because of their sequence similarities. In the assay, all three are treated as a single gene, the *s* gene.

To design the oligonucleotide probes for the microarray, toxin-specific sequences were selected from the variable region identified by the multiple-sequence alignment (Fig. 1). Four individual oligonucleotide probes (21 to 30 nucleotides in length, with an average melting temperature of 50°C) were designed to represent the sequence of each target *ent* gene (Table 2). To minimize cross-hybridization with other *ent* genes, oligonucleotide probes whose sequences had at least three mismatches with the sequence of the genetically closest *ent* genes were selected.

Microarray analysis of *ent* genes in reference strains. For validation of the selected gene-specific primers and oligonucleotide probes, we used three well-characterized *S. aureus* sequencing strains, N315, MU50 (22), and MW2 (2), which contain most of the known staphylococcal toxin genes (*sea*, *s*, *seg*, *seh*, *sei*, *sek*, *sel*, *sem*, *sen*, *seo*, and *sep*). For the four toxins not coded for by these strains, we used three additional reference strains: ATCC 14458 for *seb*, NTCC10656 for *sed* and *sej*, and ATCC 27664 for *see* and *seq*.

Genomic DNA from the five reference strains (N315, MW2, ATCC 14458, NTCC10656, and ATCC 27664) was amplified with the *ent*-specific primers. The sizes of the PCR products generated with *ent*-specific primers varied from 466 to 807 bp, depending on the *ent* gene (Fig. 2A to C), and there was good agreement between the observed and the predicted sizes of the amplicons. We unambiguously identified the presence of all 16 toxin genes previously shown in these strains: the toxin genes *s*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, and *sep* were found in strain N315 (Fig. 2A); the toxin genes *sea*, *s*, *seh*, *sek*, *sel*, and *seq* genes were found in strain MW2 (Fig. 2B); and the toxin gene *seb* was amplified from ATCC 14458, the toxin genes *sed* and *sej* were amplified from NTCC10656, and the toxin gene *see* was amplified from ATCC 27664 (Fig. 2C). The toxin genes *sea*, *s*, *seg*, *sei*, *sel*, *sem*, *sen*, and *seo* were found in strain MU50 (data not shown). We confirmed the identities of the *seb*, *sed*, *see*, and *seq* amplicons by sequencing using the corresponding toxin-specific primers. The GenBank accession numbers of the deposited sequences are presented above, in Materials and Methods.

The *ent* microarray was prepared by immobilizing the four oligonucleotides specific to each of the 16 *ent* genes in separate rows (shown schematically in Fig. 3I). The left-hand rows contain probes specific for *sea* to *sei*, and the right-hand rows contain probes specific for *sej* to *seq*. The quality control scan of the array (Fig. 3II) shows the actual scan of such an array. In our quality control procedure (40), each spot was printed with a quality control oligonucleotide, in addition to the specific oligonucleotide probe. Each Cy5-labeled target ssDNA was spiked with quality control Cy3-labeled reference material complementary to the quality control oligonucleotide. The Cy3 quality control scan provides a control image of the entire microarray, which allows validation of the fabrication and hybridization steps.

The chip contains 10 identical microarrays for simultaneous analysis of five different microbial samples, each with two duplicates (data not shown).

For microarray analysis of the *ent*-specific primers amplicons, DNAs from *S. aureus* reference strains (strains N315,

FIG. 2. PCR amplification of staphylococcal *ent* genes with gene-specific primers. Total DNA from reference strains N315, MW2, ATCC 14458, NTCC10656, and ATCC 27664 was amplified with gene-specific primers, and the resulting products were separated by electrophoresis on a 2% agarose gel. Lanes: Mr, molecular size markers (indicated on the left in nucleotides); A, *sea*; B, *seb*; C, *s*; D, *sed*; E, *see*; G, *seg*; H, *seh*; I, *sei*; J, *sej*; K, *sek*; L, *sel*; M, *sem*; N, *sen*; O, *seo*; P, *sep*; Q, *seq*. (A) Strain N315 DNA amplification with all 16 gene-specific primers; (B) strain MW2 DNA amplification with all 16 gene-specific primers; (C) amplification of *seb* for strain ATCC 14458, *sed* and *sej* for NTCC10656, and *see* for ATCC 27664.

MU50, MW2 ATCC 14458, NTCC10656, and ATCC 27664) were amplified by using the *ent* gene-specific primers, and fluorescently labeled ssDNA was synthesized from each of the *ent* amplicons by PE of the PCR products (see Materials and Methods).

The microarray accurately detected each toxin gene. For example, the detection of *sep* is shown schematically in Fig. 3III, and the actual results from microarray hybridization are shown in Fig. 3IV. It is noteworthy that occasional cross-reacting spots are observed, such as a *sel* spot (Fig. 3IV, spot L2), which cross-reacted with *sep* amplicons because of sequence similarity. However, because four oligonucleotide probes were used to detect each *ent* gene, a small number of cross-reacting spots did not interfere with toxin identification. Figure 4 shows the results of microarray analysis of all 16 *ent* genes (*sea* to *seq*) amplified by specific primers.

Overall, the results demonstrate the ability of the microarray to amplify each *ent* gene with the gene-specific primers and to unambiguously discriminate among the *ent* genes.

Development of one-tube assay for typing *ent* genes. Our attempts to combine all 16 primer pairs in one tube for a

FIG. 3. Layout of staphylococcal *ent* genes on the microarray. Each microarray is composed of four sections, and each section contains four different oligonucleotide probes specific for each of four different *ent* genes in the section. (I) Schematic illustration of the *ent* gene microarray. Genes *sea* to *sei* are in the rows on the left, and genes *sej* to *seq* are in the rows on the right, with each gene represented by four spots (columns). The first columns of each array are printed with spotting solution (U) to assist with array orientation. (II) Quality control scan of an array. Each spot of the array is visualized because each spot contains a small amount of quality control oligonucleotide probe QCprb, which hybridizes with a Cy3-labeled oligonucleotide, Cy3-QC, which is used to spike each target mixture. The spot is visualized with a 543-nm laser. (III) Schematic showing microarray detection of *sep*. (IV) Microarray analysis of *sep* genomic DNA from strain N315, which was amplified with *sep*-specific primers. The resulting PCR product was fluorescently labeled with Cy5 and hybridized to the *ent* gene array, which was then scanned with a 632-nm laser.

multiplex PCR assay did not succeed in amplifying all target *ent* genes. Several amplicons were not represented; and the yields of *seo*, *sen*, *seh*, and *sej* were too low to be detected by microarray hybridization (data not shown). This is likely due to interference between primers that significantly reduces the levels of amplification of particular toxin genes (data not shown).

We overcame this problem by developing degenerate primers (universal primers) corresponding to the highly conserved regions of *ent* (Table 1) and by adding a primer specific for the underrepresented *seh* gene to the universal primer set. This combination significantly improved the representation of all 16 *ent* genes. Control DNA and the five individual strains used in the mixture are presented in three-by-two matrix composite image (Fig. 5). All 16 *ent* genes in the mixture were detected in this analysis (Fig. 5AI). Interestingly, ATCC 14458 (Fig. 5BIII), which is known to code for *seb*, was found to contain the

recently discovered *sek* and *seq* genes. Similarly, NTCC10656 was found to encode the *seg*, *sei*, *sej*, *sem*, *sen*, and *seo* genes, in addition to the *sed* gene, the presence of which in NTCC10656 was already known (Fig. 5BI); and the *seq* gene was unexpectedly found in ATCC 27664 (Fig. 5BII).

Presence of newly discovered *ent* genes in staphylococcal isolates analyzed previously. As shown above, our microarray assay detects multiple *ent* genes simultaneously and detects additional *ent* genes in isolates analyzed previously. We used the microarray to analyze additional *S. aureus* isolates analyzed previously and found that other isolates contained multiple genes in several different combinations (Fig. 6). Strain FRI109, listed in the NARSA collection as coding for *sec2*, also contains *sed*, *seg*, *sei*, *sej*, *sel*, *sem*, *sen*, and *seo* (Fig. 6AII). Strain A900322, which was thought to contain only the enterotoxin gene cluster with *seg*, *sei*, *sen*, *seo*, and *sem* (20), also contains

FIG. 4. Microarray-based detection of 16 staphylococcal *ent* genes with gene-specific primers. Total DNAs from five *S. aureus* reference strains (strains N315, MW2, ATCC 14458, NTCC10656, and ATCC 27664) were amplified with the *ent* gene-specific primers, with the panel labels A to E and G to Q corresponding to the lanes described in the legend to Fig. 2. The ssDNAs derived from the PCR products were labeled with Cy5 and hybridized to the *ent* gene microarray (Fig. 3), which was then scanned with a 632-nm laser.

the *sep* gene (Fig. 6BI). Strain MNDON, which was reported in the NARSA catalogue to code for *secI*, *seg*, *seh*, and *sei*, is shown here to also encode *sel*, *sem*, *sen*, and *seo* (Fig. 6BII).

DISCUSSION

The traditional method of identifying SEs is serological typing with antibodies. In general, serological typing is less sensitive to small variations among SEs than DNA-based methods. Several PCR-based methods are available for *S. aureus* toxin typing (21, 35). Most require several separate reactions to distinguish among several *ent* genes. More recently, methods for *S. aureus* toxin typing by multiplex PCR have been reported (6, 26, 29). These PCR methods are based on combinations of *ent* gene-specific primers or a combination of universal forward primers and specific reverse primers (36).

One problem with all present PCR-based methods is that

novel or unexpected toxin genes can lead to false-positive or -negative results. For example, we observed that all *sea* gene-specific primers described in the literature can be used for successful amplification of *sep* as well. This might lead to the mistaken conclusion that a strain encodes *sea* and incorrect data about the distributions of *ent* genes and their roles in food poisoning. Since the relationship between the presence of a specific enterotoxin (or a combination of enterotoxins) and human food poisoning is not clearly understood, there is a need for a reliable and universal method for unambiguous identification of known *ent* genes and for detection of novel *ent* genes.

We describe here a combination PCR-microarray assay for detection and identification of *ent* genes. The analysis is based on PCR amplification of a variable region of almost all known *ent* genes with a single set of degenerate primers whose sequences correspond to those of the flanking highly conserved

FIG. 5. One-tube microarray-based detection of 16 staphylococcal *ent* genes with universal primers. Genomic DNAs from five *S. aureus* reference strains (strains N315, MW2 ATCC 14458, NTCC10656, and ATCC 27664) were amplified with a mixture of *ent* gene-specific universal primers supplemented with the primers specific for *seh*. The resulting PCR amplicons were subjected to PE with a mixture of the reverse *ent* gene-specific universal primers supplemented with the reverse primers specific for *seh*, followed by Cy5 chemical labeling. The labeled targets were hybridized to the *ent* gene microarray, which was then scanned with a 632-nm laser. A control array which contains a mixture of DNA representing all genes (from strains N315, MW2 ATCC 14458, NTCC10656, and ATCC 27664) was included for demonstration of the ability of the assay to detect 16 *ent* genes in a single sample. AI, strain control array; AII, strain N315; AIII, MW2; BI, NTCC10656; BII, ATCC 27664; BIII, ATCC 14458.

regions. The amplicons are then identified by analysis on the oligonucleotide microarray. This combined method takes advantage of the strengths of each technique. PCR amplification is highly sensitive, detecting target genes from genomic DNA even when they are present at low concentrations. DNA-DNA hybridization on the microarray increases the specificity of the assay and allows parallel analysis of multiple sequences simultaneously. In addition, the nonspecific amplicons often seen in PCRs have no effect on the hybridization of the targets with specific oligonucleotide probes.

Microarrays are not in common use in average laboratories today. However, like any new technology, as more applications are developed for the microarray technology, it will become more practical and may well become widely used. In the work described here, the presence of genes for each of 16 *ent* genes was analyzed by four methods: PCR amplification with primers specific for each of the 16 enterotoxin genes, followed by analysis by gel electrophoresis (Fig. 2); PCR amplification with specific primers (Fig. 4), followed by analysis by the microarray assay; amplification of the 16 genes with universal primers, followed by microarray analysis (Fig. 5); and sequencing of the enterotoxin genes to verify their identities. Thus, using two amplification methods (methods with specific primers and universal primers) as well as three DNA analysis methods (gel electrophoresis, DNA sequencing, and DNA microarray analysis), we verified the performance of the method. Using this array, we have shown that some strains previously analyzed by immunological methods contain additional *ent* genes not detected by the original assays (Fig. 5 and 6).

In our microarray system, we used relatively short oligonucleotides (21 to 30 nucleotides) for three reasons. First, shorter

FIG. 6. One-tube microarray-based analysis of previously analyzed *S. aureus* strains. Genomic DNA from four strains analyzed previously (strains Mu50, FRI361, A90322, and MNDON) was amplified by using a mixture of *ent* gene-specific universal primers supplemented with the primer specific for *seh*. The resulting PCR amplicons were subjected to PE with a mixture of the reverse *ent* gene-specific universal primers supplemented with the reverse primer specific for *seh*, followed by Cy5 chemical labeling, and were then hybridized to the *ent* gene microarray. The resulting image was scanned with a 632-nm laser. The analysis of the four strains is presented in a two-by-two matrix composite image. AI, strain Mu50; AII, strain FRI361; BI, A90322; BII, MNDON.

oligonucleotide probe sequences (<25 bp) are often capable of detecting a single-nucleotide mismatch between the target ssDNA and the oligonucleotide probe, which allows detection of minor genetic variants in target genes in a bacterial population. Second, the shorter oligonucleotide probes allow independent testing of several species-specific regions of each gene, enabling effective coverage of the target sequence with more (but shorter) oligonucleotide probes. This reduces the probability of misidentification. Third, short oligonucleotides reduce the cost of chip production.

The redundancy of the testing (the number of spots representing each gene) is one way to reduce the risk of SE misidentification. While only one portion of each SE gene (the variable region shown in Fig. 1) is used for the analysis, leaving open the possibility of significant sequence variation in other parts of the genes, such variation is not common.

The main disadvantage of simultaneous PCR amplification of multiple targets is that different copy numbers of the genes in a cell result in different signal intensities on the array. This might be overcome by use of supplemental specific primers to improve the detection of underrepresented amplicons (Fig. 5 and 6).

Many of the known SEs have been discovered only in the last few years, including some, such as *sep*, that were discovered only through the sequencing of the *S. aureus* N315 genome (22). Given the genetic variability and the spread of the *ent* gene family, it is possible that there are other, as yet unknown, *ent* genes. However, the similarity in the conserved regions of these genes (Fig. 1) suggests that any additional gene family members will share those conserved sequences. Thus, it is possible that this assay might lead to the discovery of additional *ent* genes in new strains. The amplicons of the novel *ent* genes can be discernible as amplicons that hybridize to common spots but not to any *ent* gene-specific spots.

Multiple SE genes are commonly found in *S. aureus* strains (19, 20, 32). Among 198 *S. aureus* isolates implicated in *S. aureus* infections in France, 85.4% expressed multiple SEs (19). Our analysis of these data suggests that the majority (92%) contain multiple SEs, especially the *egc* cluster (*seg*, *sei*, *sen*, *seo*, and *sem*). Among the *S. aureus* isolates implicated in food-poisoning episodes in Japan, 93% expressed SEs, while only 72.2% of isolates from healthy people expressed SEs (32).

One explanation for the presence of multiple toxins in most strains is that these genes are often structurally linked. Several pathogenicity islands have been reported in *S. aureus*, including one encoding the toxic shock syndrome toxin (*tst*) and *s*- and *sel*-like proteins (13) and another encoding SE serotypes B, K, and Q (41). Others have reported pathogenicity islands containing the *tst* gene and an open reading frame with sequence similarity to those encoding SEs (25) and a region contains enterotoxins D and J (42). In addition, as noted above, a group of five toxin genes (*seg*, *sei*, *sen*, *seo*, and *sem*) is encoded by the enterotoxin gene cluster, *egc* (20). Interestingly, in the study of 198 clinical isolates by Jarraud et al. (19), half of the 14 strains that carried only a single enterotoxin gene had the *sea* gene, perhaps because it has been shown to be associated with a structurally unstable, possibly mobile, discrete genetic element (8) that is not part of the *egc* cluster.

In terms of the functionalities of SEs, multiple toxins with diverse spectra of activities may offer the pathogen versatility

in terms of the host range. For example, it has been shown that different types of SEs have different emetic response activities in house musk shrews, although it was thought that there are no differences in the emetic response activities of SEA, SEB, SEC, SED, and SEE in humans and primates (18). In addition, *S. aureus* expression of specific *ent* genes may depend on the host tissue and may play a role in the adaptation of *S. aureus* to the host environment (4). Some have speculated that some cases of food poisoning result from the simultaneous expression of several enterotoxins in a single pathogenic *S. aureus* strain rather than from the expression of a single toxin.

However, it is unclear whether all the toxins are actually expressed and what the biological and clinical effects of multiple toxins might be. The method presented here can detect *ent* genes but does not determine whether the gene is expressed or whether the encoded protein is functional. The levels of correlation between the presence of genes that code for the production of SE (as determined by PCR) and the expression of these genes (as determined by enzyme-linked immunosorbent assay) were 100% for SEA and SEE, 86% for SEC, 89% for SED, and 47% for SEB (30). Thus, the actual presence of the toxin needs to be assessed by an immunological or activity assay.

In summary, the PCR-microarray method described here is a potentially powerful tool for the analysis of *S. aureus* strains. We used this method to test clinical isolates analyzed previously and found that these isolates frequently carry the genes for numerous toxins, including some of the newly discovered SEs. More studies need to be done to understand the biological regulation and the biological and clinical effects of multiple enterotoxins. Our method has great potential for application in high-throughput screening and accurate genotyping of *ent* genes, which are especially important in epidemiological studies.

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