Development of Phylogenetic Oligonucleotide Probes for Screening Foodborne Bacteria

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The aim of this study was to develop phylogenetic oligonucleotide probes for screening common foodborne bacteria. Twenty oligonucleotide probes were designed by aligning representative 16S ribosomal DNA (rDNA) sequences from 64 species of common foodborne bacteria and other bacteria. To confirm the specificity of each probe simultaneously, a DNA microarray that the 20 probes were immobilized on a glass slide was prepared. RNAs extracted from 13 species of foodborne bacteria were fluorescently labeled and hybridized on the DNA microarray. The 16S rRNAs showed a unique hybridization pattern in combination with the designed oligonucleotide probes, leading to 16S rDNA phylogenetic analysis of their sequences. Probe EC001 for *Escherichia coli* spp. and *Shigella* spp., probe SSP003-L for *Salmonella enterica* subsp. *enterica* serovar Enteritidis and *Salmonella enterica* subsp. *enterica* serovar Typhimurium, probe YE002 for *Yersinia enterocolitica* and probe BC001 for *Bacillus cereus* group were particularly useful for screening these foodborne bacteria. DNA microarray is useful as a procedure for evaluating the multiple designed probes. The selected probes can be readily applied to screening of foodborne bacteria using hybridization methods, such as fluorescent *in situ* hybridization (FISH), bead array hybridization and so on.

Key words —— foodborne bacteria, oligonucleotide probe, 16S rRNA, phylogenetic analysis, DNA microarray

INTRODUCTION

In industrialized countries, the percentage of people suffering from foodborne diseases each year has been reported to be up to 30%. In the United States of America (U.S.A.), for example, around 76 million cases of foodborne diseases, resulting in 325000 hospitalizations and 5000 deaths, are estimated to occur each year.[1] In Japan, 699–1850 cases of food poisoning occurred from 1995 through 2002 and about 80% of those were caused by bacteria.[2] Simplifying techniques to examine microbiological quality of food will improve food safety and reduce health costs.

Bacterial genomes, plasmids, and rRNAs are known as common surrogate markers for detection of bacteria.[3,4] Specifically, rRNAs are useful surrogate markers. 16S ribosomal DNA (rDNA) sequences have highly conserved regions in identical bacterial species. The design of specific probes for the screening of target bacteria is achieved by the alignment of the 16S rDNA sequences and the identification of sequence idiosyncrasies.[5] Additionally, up to 80% of bacterial RNA is rRNA, and one cell of *Escherichia coli* (*E. coli*) can contain about 20000 copies of rRNA.[6] Therefore, rRNA provides greater sensitivity, eliminating the need for amplification in many applications. Small J. *et al*. have reported a simple microarray method without using PCR for the direct detection of intact 16S rRNA (*Geobacter chapellei* and *Desulfovibrio desulfuricans*) from unpurified soil extracts.[7]

In this study, we aimed to develop the phylogenetic oligonucleotide probes for the screening of foodborne bacteria using hybridization methods such as a fluorescent *in situ* hybridization (FISH), bead array hybridization and so on. We designed 20 oligonucleotide probes by aligning representative 16S rDNA sequences from 64 species of common foodborne bacteria and other bacteria. In recent years, DNA microarray technology has been widely used in research of bacterial gene expression and as a procedure for the identification of environmental bacteria,[8–10] because DNA microarray technology

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can be used to analyze the characteristics of various genes simultaneously. DNA microarray technology is a powerful tool that can be used for detection of multiple genes or target sequences on one glass slide. Thus, the specificity of designed probes was confirmed based on hybridization results by using a DNA microarray.

MATERIALS AND METHODS

Bacterial Strains, Culture Medium, and Growth Conditions —— The 13 species of bacterial strains used in this study are underlined bacteria in Fig. 1. Briefly, cells of each standard foodborne bacterial strain were inoculated into 10 ml of Soybean-Casein Digest (SCD) broth (SCDB; 17 g of peptone for casein, 3 g of peptone for soybean, 5 g of sodium chloride, 2.5 g of dipotassium hydrogen phosphate, and 2.5 g of glucose per 1 l of laboratory-quality water) and incubated on a shaker platform at 30°C overnight. For culture of *Vibrio* spp., the modified SCDB (17 g of peptone for casein, 3 g of peptone for soybean, 30 g of sodium chloride, 2.5 g of dipotassium hydrogen phosphate, per 1 l of laboratory-quality water) were used. Cells of *Campylobacter jejuni* (*C. jejuni*) were only inoculated on SCD agar (SCDA; 17 g of peptone for casein, 3 g of peptone for soybean, 5 g of sodium chloride, 2.5 g of dipotassium hydrogen phosphate, 2.5 g of glucose, and 15 g of agar per 1 l of laboratory-quality water), and cultured on an incubator at 37°C for 3 days in microaerophilic conditions with Campi-Pouch (Becton, Dickinson and Company, NJ, U.S.A.).

RNA Purification —— Total RNA was purified from bacterial cultures with an RNeasy® Mini Kit according to the manufacturer's instructions (QIAGEN Inc., Hilden, Germany). DNA was digested with the RNase-free DNase Set (QIAGEN Inc.) during RNA purification. Extracted RNA was quantified by UV absorbance. The presence of RNA was confirmed by gel electrophoresis in 1.2% formaldehyde agarose gel containing ethidium bromide (1 μg/ml) and formaldehyde gel running buffer. The RNA was stored at –20°C until use.

RNA Cy3 Labeling —— Total RNA was labeled with a Label IT® Cy3 labeling kit according to the manufacturer's instructions (TaKaRa Co. Ltd., Shiga, Japan). Five μg of extracted RNA was used. After the RNA labeled with Cy3 was separated by electrophoresis in 1.2% formaldehyde agarose gel and formaldehyde gel running buffer, the gel image was analyzed using an FMBIO II image analyzer (TaKaRa Co. Ltd.).

Oligonucleotide Probe Design —— The 16S rDNA sequences of the 64 species of foodborne bacteria and other bacteria were retrieved from the SRS sequence database in DDBJ. The GenBank accession numbers of the 16S rDNA sequences of bacteria used in this study were shown in Table 1. For the design of the oligonucleotide probes, the retrieved 16S rDNA sequences were aligned using Clustal W[11] to identify regions with numerous sequence variations. Probe AE001 was designed by modifying EUB338.[12] Probe EUB338 had been used as a positive control for detecting bacteria using FISH. For adjusting theoretical melting temperature (*T*m), two oligonucleotides were added to probe EUB338 (5′-terminus: T, 3′-terminus: C). Probe AE001 was used as a positive control in this research. Twenty oligonucleotide probes including probe AE001 for the identification of foodborne bacterial 16S rRNAs were selected (20–31 mer). The program FASTA[13] was used to determine the uniqueness of the designed probes. A phylogenetic tree was constructed with PhyloDraw[14] based on the alignment results of the 16S rDNA sequences from 13 species of foodborne bacteria and 51 related species using Clustal W.

Oligonucleotide Probe Attachment —— All oligonucleotide probes derivatized with a 5′-terminal thiol group for attachment to a glass slide were purchased from JbioS Co. Ltd. (Saitama, Japan). The oligonucleotide probes dissolved in 50 mM Tris–HCl (pH 8.0) were diluted to 40 μM in the same buffer. An aliquot (25 μl) of each probe and 25 μl of 50 mM Tris–HCl (pH 8.0) containing 20% (w/v) trehalose were mixed. The mixtures were applied in triplicate to a clean, dry amino group-derivatized glass slide (Matsunami Glass, Co. Ltd., Osaka, Japan) treated with *N*-(6-Maleimidocaproyloxy) succinimide (EMCS) using an Affymetrix 417 Arrayer (Affymetrix, CA, U.S.A.). The average size of the spots was 150 μm. The applied droplets were allowed to air dry at room temperature for overnight, and then the slides were washed with 2 × SSC at room temperature for 5 min to remove unbound oligonucleotides. To block unbound EMCS, the slides were soaked in 50 mM phosphate buffer, 1 M NaCl (pH 7.0) containing 2% (w/v) BSA at room temperature for 2 hr, and rinsed 3 times with distilled water, then dried in air before hybridization.

DNA Microarray Hybridization and Detection —— Hybridization was performed with a Gene TAC hybridization station (Genomic Solutions, Inc., MI,

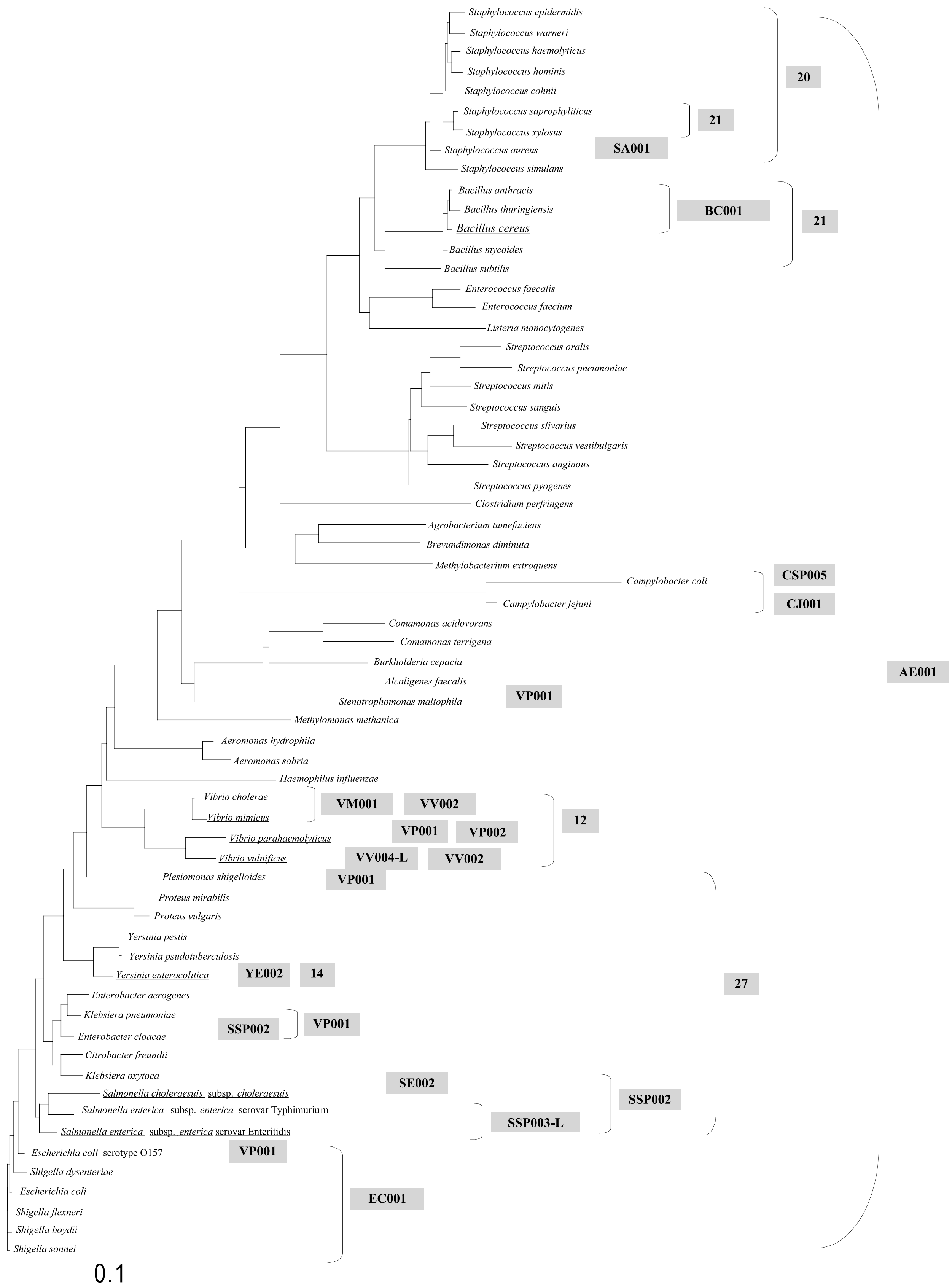

Fig. 1. Phylogenetic Positions of Foodborne Bacteria and Designed Probes

A phylogenetic tree was constructed with PhyloDraw based on the multiple alignment results of the 16S rDNA sequences from 13 species of foodborne bacteria and 51 related species using Clustal W. The scale bar represents a 10% difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting two species.

Table 1. Bacteria Used to Design Phylogenetic Probes in this Study

Bacteria	GenBank accession number
Aeromonas hydrophila	X87271
Aeromonas sobria	X74683
Agrobacterium tumefaciens	M11223
Alcaligenes faecalis	AJ277669
Bacillus anthracis	AB116124
Bacillus cereus	D16266
Bacillus mycoides	AF155957
Bacillus subtilis	AB042061
Bacillus thuringiensis	AB116122
Brevundimonas diminuta	X87274
Burkholderia cepacia	X87275
Campylobacter coli	M59073
Campylobacter jejuni	Z29326
Citrobacyter freundii	AJ233408
Clostrodium perfringens	Y12669
Comamonas acidovorans	AF181575
Comamonas terrigena	AJ420326
Enterobacter aerogenes	AJ251468
Enterobacter cloacae	AJ251469
Enterococcus faecalis	AB036835
Enterococcus faecium	Y18294
Escherichia coli	J01695
Escherichia coli serotype O157	AE005174
Haemophilus influenzae	M35019
Klebsiera oxytoca	AB053117
Klebsiera pneumoniae	X87276
Listeria monocytogenes	X56153
Methylobacterium extorquens	D32224
Methylomonas methanica	AF304196
Plesiomonas shigelloides	M59159
Proteus mirabilis	AJ301682
Proteus vulgaris	J01874
Salmonella choleraesuis subsp. *choleraesuis*	AF276989
Salmonella enterica subsp. *enterica* serovar Enteritidis	U90318
Salmonella enterica subsp. *enterica* serovar Typhimurium	X80681
Shigella boydii	X96965
Shigella dysenteriae	X80680
Shigella flexneri	AE016978
Shigella sonnei	X96964
Staphylococcus aureus	X68417
Staphylococcus cohnii	AB009936
Staphylococcus epidermidis	L37605
Staphylococcus haemolyticus	L37600
Staphylococcus hominis	L37601
Staphylococcus saprophyticus	L20250
Staphylococcus simulans	D83373
Staphylococcus warneri	L37603
Staphylococcus xylosus	D83374
Stenotrophomonas maltophila	AJ011332
Streptococcus anginosus	X58309
Streptococcus mitis	D38482
Streptococcus oralis	X58308

Table 1. Continued

Bacteria	GenBank accession number
Streptococcus pneumoniae	X58312
Streptococcus pyogenes	AB023575
Streptococcus salivarius	M58839
Streptococcus sanguis	AB002524
Streptococcus vestibularis	X58321
Vibrio cholerae	X76337
Vibrio mimicus	X74713
Vibrio parahaemolyticus	X74720
Vibrio vulnificus	X74726
Yershinia enterocolitica	Z75316
Yershinia pestis	AF366383
Yershinia pseudotuberclulosis	AF366375

U.S.A.). The hybridization solution was prepared as follows: 2 μg of Cy3-labelled RNA, 45 μl of 20 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), and 15 μl of 5% (w/v) sodium dodecyl sulfate (SDS) solution were mixed, and adjusted to a total volume of 150 μl with RNase-free water. Afterwards, the hybridization solution was heated at 65°C for 10 min, and 120 μl of solution was poured on the DNA microarray. The hybridization was performed for 15 hr at 45°C. After the hybridization solution was aspirated off, the DNA microarray was rinsed with 2 × SSPE. Then, the DNA microarray was rinsed with distilled water and vacuum dried. After drying, the DNA microarray was scanned with a GenePix 4000 laser scanner (Axon, CA, U.S.A.). A laser light of wavelength at 532 nm was used to excite the Cy3 dye. A fluorescent image was captured in multi-image tagged image file format and analyzed with GenePix Pro 3.0 software (Axon). The hybridization signal generated from probe AE001 was used as a positive signal (AE001 hybridization signal). If the hybridization signal generated from each probe hybridized to 16S rRNA was higher than the AE001 hybridization signal (> 1.0), the reactivity of the probe was considered positive. A hybridization signal of < 1.0 was considered negative.

RESULTS AND DISCUSSION

The 20 oligonucleotide probe sequences were designed to detect common 13 foodborne bacteria (Table 2). The 13 species of common foodborne bacteria were divided broadly into 4 clusters as follows: *Enterobacteriaceae* consisting of *E. coli*, *Shigella* spp., *Salmonella* spp., and *Yersinia* spp. *etc*., *Vibrionaceae* consisting of *Vibrio vulnificus* (*V. vulnificus*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Vibrio cholerae* (*V. cholerae*), and *Vibrio mimicus* (*V. mimicus*), bacilli consisting of *Bacillus* spp. and *Staphylococcus* spp., and *Campylobacter* consisting of *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli)*. The 20 designed oligonucleotide probes could be positioned at informative sites (perfect match to 16S rDNA sequences) in the reconstructed phylogenetic tree (Fig. 1).

Thirteen species of common foodborne bacteria showed unique hybridization results in combination with the oligonucleotide probes leading to phylogenetic analysis of their 16S rDNA sequences (Fig. 2).

Probe AE001 hybridized to all the 16S rRNAs from the 13 species of foodborne bacteria. Therefore, probe AE001 was usable as a positive control for evaluation of all other designed oligonucleotide probes.

E. coli serotype O157 and *Shigella sonnei* (*S. sonnei*) showed positive hybridization results against probes 27, EC001, and VP001. Probe EC001 hybridized only to these bacteria. All the 3 species of *Salmonella* spp. showed positive hybridization results against probes 27 and SSP002. Additionally, *Salmonella choleraesuis* (*S. choleraesuis*) subsp. *choleraesuis* hybridized to probe SE002, and *S.* Typhimurium and *S.* Enteritidis hybridized to probe SSP003-L. *Yersinia enterocolitica* (*Y. enterocolitica*) showed positive hybridization results against probes 27, 14, and YE002. Probes 14 and YE002 hybridized only to *Y. enterocolitica*. *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. mimicus* showed hybridization positive results against probe 12. *V.*

Table 2. Designed Oligonucleotide Probe Sequences and their Specificities against Common 13 Foodborne Bacterial 16S rDNA Sequences

Probe	Probe sequence (5′ to 3′)	Length (nt)	GC content (%)	Position[a)]	Species[b)] EC	SS
AE001	TGCTGCCTCCCGTAGGAGTC	20	65	337–356	PM[c)]	PM
VM001	TGCTTTGCTCTTGCGAGGTT	20	50	181–200	> 4	> 4
VV004-L	ATAGTGCTATTAACACTACCACCTTCCTCAC	31	42	445–475	> 4	> 4
VV002	CACTCCAGCGTCTCCGCTAGA	21	62	1006–1026	> 4	> 4
VP001	GCACCAATCCATCTCTGGAAAG	22	50	1006–1027	PM	4
VP002	ATGCAGCTATTAACTACACTACCTTCCTCAC	31	42	445–475	> 4	> 4
EC001	GAGCAAAGGTATTAACTTTACTCCCTTCCT	30	40	448–477	PM	PM
SSP003-L	ATCTCTGGATTCTTCTGTGGATGTC	25	44	993–1017	3	3
SE002	TCTCATCTCTGAAAACTTCCCGTG	24	46	999–1021	> 4	> 4
SSP002	TGCTGCGGTTATTAACCACAACA	23	43	455–471	> 4	> 4
BC001	AACTTCATAAGAGCAAGCTCTTAATCCATT	30	33	69–101	> 4	> 4
YE002	AATCACAAAGGTTATTAACCTTTATGCCTT	30	30	451–480	> 4	> 4
SA001	AGAGAAGCAAGCTTCTCGTCCG	22	55	71–93	> 4	> 4
CSP005	TCTAAGTTCTAGCAAGCTAGCACCCTCT	28	46	1019–1044	> 4	> 4
CJ001	CCCTACTCAACTTGTGTTAAGCAGGAG	27	48	182–209	> 4	> 4
12	GGTGAGCCCTTACCTCACCAACTA	24	54	249–272	3	3
14	CGAAGGCACTAAAGCATCTCTGCTAAA	27	44	1006–1032	> 4	> 4
20	ACCGTAGCATGCTGATCTACGATT	24	46	1349–1372	3	3
21	TTTGTCACCGGCAGTCACCTTAGA	24	50	1148–1171	4	4
27	GCACCTGAGCGTCAGTCTTTGTC	23	57	742–764	1 (20)	1 (20)

Probe	Species[b)] SEC	SET	STY	YE	VV	VP	VC	VM	SA	BC	CJ
AE001	PM	PM	PM	PM	PM	PM	PM	PM	PM	PM	PM
VM001	> 4	> 4	> 4	> 4	> 4	> 4	PM	PM	> 4	> 4	> 4
VV004-L	> 4	> 4	> 4	> 4	PM	> 4	> 4	> 4	> 4	> 4	> 4
VV002	> 4	> 4	> 4	> 4	PM	> 4	PM	PM	> 4	> 4	> 4
VP001	> 4	> 4	3	> 4	> 4	PM	> 4	> 4	> 4	> 4	> 4
VP002	> 4	> 4	> 4	> 4	> 4	PM	> 4	> 4	> 4	> 4	> 4
EC001	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
SSP003-L	> 4	PM	PM	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
SE002	PM	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
SSP002	PM	PM	PM	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
BC001	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	PM	> 4
YE002	> 4	> 4	> 4	PM	> 4	> 4	> 4	> 4	> 4	> 4	> 4
SA001	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	PM	> 4	> 4
CSP005	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	PM
CJ001	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4	PM
12	2 (9,23)	3	1 (9)	3	PM	PM	PM	PM	3	1 (9)	3
14	> 4	> 4	> 4	PM	> 4	> 4	> 4	> 4	> 4	> 4	> 4
20	4	3	3	> 4	3	3	> 4	> 4	PM	4	1 (11)
21	4	4	4	3	3	4	4	4	1 (7)	PM	> 4
27	PM	1 (20)	PM	PM	4	4	4	4	> 4	> 4	> 4

a) *E. coli* (J01695) numbering. *b)* Species: EC, *E. coli* serotype O157; SS, *S. Sonnei*; SEC, *S. choleraesuis* subsp. *choleraesuis*; SET, *S.* Enteritidis; STY, *S.* Typhimurium; YE, *Y. enterocolitica*; VV, *V. vulnificus*; VP, *V. parahaemolyticus*; VC, *V. cholerae*; VM, *V. mimicus*; SA, *S. aureus*; BC, *B. cereus*; CJ, *C. jejuni*. *c)* Number of mismatch to 16S rDNA sequences of targeted species. For up to two mismatches, the position of the mismatches is also stated (numbers in parentheses counted from the 3′ end). PM, perfect match.

	Oligonucleotide probes															
Species	AE001	27	12	21	CSP005	EC001	SE002	VP001	SSP003-L	SSP002	14	YE002	VV002	20	BC001	CJ001
Escherichia coli serotype O157	[a]	3.0[b]	[c]			3.0		1.8								
Shigella sonnei		5.1				10.3		1.5								
Salmonella choleraesuis subsp. *choleraesuis*		7.1					1.3	1.0		1.7						
Salmnonella enterica subsp. *enterica* serovar Enteritidis		7.1							7.6	1.7						
Salmnonella enterica subsp. *enterica* serovar Typhimurium		5.0						1.9	3.5	1.0						
Yersinia enterocolitica		5.4									3.7	1.2				
Vibrio parahaemolyticus			1.0					1.8								
Vibrio vulnificus			1.0										1.0			
Vibrio cholerae			1.6										1.6			
Vibrio mimicus			1.0										1.0			
Staphylococcus aureus				1.0										1.5		
Bacillus cereus				1.0											2.0	
Campylobacter jejuni					1.4											1.7

Fig. 2. Reactivity of Oligonucleotide Probes for Screening Foodborne Bacteria Selected in this Study

a) Gray columns represent that 16S rRNA from individual microorganism showed positive result against each probe. b) Scores represent the ratio between each probe signal and probe AE001 signal generated from individual organism. c) White columns represent that the ratio between each probe signal and probe AE001 signal generated from individual microorganism was < 1.0.

parahaemolyticus hybridized to probe VP001. On the other hand, *V. vulnificus*, *V. cholerae*, and *V. mimicus* hybridized to probe VV002. *Staphylococcus aureus* (*S. aureus*) showed positive hybridization results against probes 21 and 20. *Bacillus cereus* (*B. cereus*) hybridized to probes 21 and BC001. *C. jejuni* showed positive hybridization results against probes CSP005 and CJ001.

According to determination of the specificity of the designed probes using the program FASTA and their hybridization results using the DNA microarray, the several probes for screening common foodborne bacteria were selected. Probe EC001 for *E. coli* spp. and *Shigella* spp., probe SSP003-L for *S.* Enteritidis and *S.* Typhimurium, probe YE002 for *Y. enterocolitica*, and probe BC001 for *B. cereus* group were particularly useful for screening these foodborne bacteria.

There were important considerations while designing oligonucleotide probes. Firstly, it was shown that the probes possessing one mismatch within the target sequence resulted in positive hybridization. Probes 12 and 20 possessed one central mismatch to the target sequence (probe 12 to *S.* Typhimurium and to *B. cereus*; probe 20 to *C. jejuni*) did not cause positive results, but probes 21 and 27 possessed one base mismatch near the 5′ or 3′ terminus of the target sequence (probe 21 to *S. aureus*; probe 27 to *E. coli* O-157, *S. sonnei* and *S.* Enteritidis) and showed positive results. Thus, mismatch position of the oligonucleotide probe to the target sequence is important for the discrimination of one base mismatch in hybridization. Urakawa H. *et al.*[15] and Peplies J. *et al.*[16] also reported that the discrimination of a mismatch near the terminus of a short duplex is hard to achieve. Secondly, the lack of a detectable hybridization signal for probes possessing a perfect match within the target sequence may be caused by the effect of the secondary structure of 16S rRNA or steric hindrance.[16] Probes VM001 (for *V. cholerae* and *V. mimicus*), VV004-L (for *V. vulnificus*), VP002 (for *V. parahaemolyticus*) and SA001 (for *S. aureus*) had no mismatch to the target sequence, but their hybridization signals were remarkably low. For improving nonconcurring signals caused by signal suppressing parameters such as secondary structure of the target molecules, the modification of the probe sequence or the addition of a spacer to the 5′ terminus of the probe must be considered.

In conclusion, phylogenetic oligonucleotide probes for screening of foodborne bacteria was developed: Probe EC001 for *E. coli* spp. and *Shigella* spp., probe SSP003-L for *S.* Enteritidis and *S.* Typhimurium, probe YE002 for *Y. enterocolitica* and probe BC001 for *B. cereus* group. The selected probes can be readily applied to screening of foodborne bacteria using hybridization methods, such as FISH, DNA microarray hybridization, and bead array hybridization.

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