AEM
Journals.ASM.org

Specific Discrimination of Three Pathogenic *Salmonella enterica* subsp. *enterica* Serotypes by *carB*-Based Oligonucleotide Microarray

Hwa Hui Shin, Byeong Hee Hwang, Jeong Hyun Seo, Hyung Joon Cha

Department of Chemical Engineering, Pohang University of Science and Technology, Pohang, South Korea

It is important to rapidly and selectively detect and analyze pathogenic *Salmonella enterica* subsp. *enterica* in contaminated food to reduce the morbidity and mortality of *Salmonella* infection and to guarantee food safety. In the present work, we developed an oligonucleotide microarray containing duplicate specific capture probes based on the *carB* gene, which encodes the carbamoyl phosphate synthetase large subunit, as a competent biomarker evaluated by genetic analysis to selectively and efficiently detect and discriminate three *S. enterica* subsp. *enterica* serotypes: Choleraesuis, Enteritidis, and Typhimurium. Using the developed microarray system, three serotype targets were successfully analyzed in a range as low as 1.6 to 3.1 nM and were specifically discriminated from each other without nonspecific signals. In addition, the constructed microarray did not have cross-reactivity with other common pathogenic bacteria and even enabled the clear discrimination of the target *Salmonella* serotype from a bacterial mixture. Therefore, these results demonstrated that our novel *carB*-based oligonucleotide microarray can be used as an effective and specific detection system for *S. enterica* subsp. *enterica* serotypes.

Pathogenic bacteria are common reasons for food-borne diseases, which are widespread and growing public health and hygiene problems in most countries. Representatively, *Salmonella enterica* subsp. *enterica* strains are pathogenic bacteria frequently occurring worldwide (1). In the United States, annual statistics estimate there are 15,000 hospitalizations and 400 deaths from 1.4 million nontyphoidal *Salmonella* infections (2). In addition, among the many serotypes of *S. enterica* subsp. *enterica*, the Food and Drug Administration has characterized 51 serotypes that can infect humans (3) and have diverse host specificity, pathogenicity, disease severity, and antimicrobial resistance (4, 5). To reduce the morbidity and mortality from *Salmonella* infections and to guarantee food safety, it is important to rapidly and sensitively detect and analyze pathogenic *S. enterica* subsp. *enterica* from contaminated food. Additionally, discrimination at the serotype level is also critical to epidemiologically investigate *Salmonella* outbreaks and trace the outbreak sources in addition to helping to choose an adequate therapy (6–8).

Technically, the culture method is the standard protocol used and can discriminate at the *S. enterica* subsp. *enterica* serotype level. However, this method is unsuitable for rapid detection due to the long time required for culture (usually 2 to 7 days) (9, 10). For rapid detection and analysis, biosensors based on nucleic acids and antibodies as the detection molecules have been developed (11–15). Oligonucleotide microarrays have been suggested as potentially effective biosensors and have been developed for rapid and high-throughput analysis (16–19). However, effective specific capture probes in an oligonucleotide microarray are requisite for the successful discrimination of subtle sequence differences, and these are controlled by particular factors, such as the genetic dissimilarity, position of mismatch, and secondary structure of the nucleotides (20). Therefore, the selection of an appropriate detection biomarker (DNA sequence) is an important first step in designing effective and specific capture probes.

Many studies have employed 16S rRNA gene sequences as a universal detection biomarker due to several advantages, including usefulness in phylogenetic discrimination, the abundance of complete gene sequence information, and the availability of universal primers (12–14, 17, 21–24). In spite of its advantages, however, 16S rRNA-based microarray systems, including those in our previous studies, show the limitation of difficulty in the definitive discrimination of serotype or phylogenetically close species due to the low sequence diversity (13). In particular, the 16S rRNA of *S. enterica* subsp. *enterica* exhibits very low sequence variability between serotypes. Therefore, a new unified detection biomarker that can specifically discriminate serotypes is needed.

In the present work, we introduced a new potential unified detection biomarker for the specific and reliable serotype detection of *S. enterica* subsp. *enterica*. We employed the carbamoyl-phosphate synthetase (CPS) large subunit (*carB*) gene as a detection biomarker after ascertaining its competence through genetic analysis. We surmised that the *carB* gene would be suitable because this encodes an enzyme vital to bacterial survival that contains both conserved and variable regions and has been already used for phylogenetic studies (29–32, 47). We designed specific capture probes based on the *carB* gene and constructed an oligonucleotide microarray for the effective and specific discrimination of three *Salmonella* serotypes, Choleraesuis, Enteritidis, and Typhimurium, which are the major serotypes in South Korea (25, 26).

MATERIALS AND METHODS

Pathogenic strains and genomic DNA isolation. Most of the bacterial strains used in this study, including *S. enterica* subsp. *enterica* serotype Choleraesuis (American Type Culture Collection [ATCC], Manassas,

Received 3 September 2013 Accepted 23 October 2013
Published ahead of print 1 November 2013
Address correspondence to Hyung Joon Cha, hjcha@postech.ac.kr.
H.H.S. and B.H.H. contributed equally to this article.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02978-13.
Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.02978-13

TABLE 1 Probes used in this study and their thermodynamic properties[a]

Probe type	Target serotype	Probe name	Antisense sequence (5′→3′, 5′-amine-spacer, C_6 spacer)	Length (bp)	T_m (°C)	Rating[b]
Positive control	All *S. enterica* subsp. *enterica*	Sal_POCO	GATATCGAAGCGACGACGGTCT	22	63.2	88
Serotype-specific capture	*S. enterica* subsp. *enterica* serotype Choleraesuis	SC-1	CAGAGTCTGCGCGGGCG	17	64.1	82
		SC-2	AAGGCTGACTTTCGGGTCGAAG	22	64.6	82
	S. enterica subsp. *enterica* serotype Enteritidis	SE-1	TCTTCAAGTACGCCCTGACGCT	22	63.8	94
		SE-2	CTGTACCAAGAACCAGCGGTCAA	23	64	89
	S. enterica subsp. *enterica* serotype Typhimurium	ST-1	ATGGTAAAGCTAGGCCGGATGAT	23	63.4	83
		ST-2	AAAGACGCCATCGACGGAGAG	21	64	89

[a] All of the thermodynamic properties were calculated using Primer Premier 5.
[b] Parameter based on ΔG to set weights for secondary structures. The more stable the secondary structure is, the lower the rating would be.

VA), *S. enterica* subsp. *enterica* serotype Enteritidis (Institute for Fermentation [IFO], Osaka, Japan), *S. enterica* subsp. *enterica* serotype Typhimurium (IFO 12529), *S. enterica* subsp. *enterica* serotype Newport (ATCC 6962), *Bacillus cereus* (ATCC 13061), *Escherichia coli* K-12 (ATCC 29425), *Shigella boydii* (ATCC 8700), *Shigella dysenteriae* (ATCC 13313), *Shigella sonnei* (ATCC 29930), *Staphylococcus aureus* (ATCC 6538), and *Yersinia enterocolitica* (ATCC 23751) were cultured in nutrient broth (NB) medium (0.5% [wt/vol] peptone and 0.3% [wt/vol] beef extract) at 37°C. *Vibrio cholerae* (ATCC 14035), *Vibrio vulnificus* (ATCC 27562), and *Vibrio parahaemolyticus* (ATCC 17802) were grown in Trypticase soy broth (TSB) medium (1.7% (wt/vol) tryptone, 0.3% (wt/vol) peptic digest of soybean meal, 0.5% (wt/vol) NaCl, 0.25% (wt/vol) K_2HPO_4, and 0.25% (wt/vol) dextrose) with 2% (wt/vol) NaCl at 37°C. *Clostridium perfringens* (ATCC 13124) was grown in reinforced clostridial medium (RCM) (1% [wt/vol] peptone, 1% [wt/vol] beef extract, 0.3% [wt/vol] yeast extract, 0.5% [wt/vol] dextrose, 0.5% [wt/vol] NaCl, 0.1% [wt/vol] soluble starch, 0.05% [wt/vol] cysteine-HCl, 0.3% [wt/vol] sodium acetate, and 0.05% [wt/vol] agarose) at 37°C in an anaerobic environment. The isolated chromosome of *Campylobacter jejuni* (ATCC 33291) was acquired from the Korean Collection for Type Culture (KCTC), Daejeon, South Korea. Genomic DNA was extracted and purified by the same procedure as in our previous studies (12, 13, 27). The purified genomic DNA was used as the template for the PCR to prepare amplified target DNAs.

Genetic analysis for evaluation of *carB* biomarker. All available *carB* sequences of 27 *S. enterica* subsp. *enterica* serotypes were downloaded from the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/gene). The *carB* sequence of each serotype was searched in the NCBI BLAST site (http://blast.ncbi.nlm.nih.gov). To evaluate the distinctive ability of the *carB* biomarker gene, mutations that are the numbers of sequence mismatches and numbers of candidate regions for specific probe design were counted from 16 representative serotypes out of 27 *Salmonella* serotypes (see Tables S1, S2, and S3 in the supplemental material). The criterion for the candidate region was the dissimilarity over 10 to 15% within 20 to 30 bp, which was also major criterion for design of serotype-specific capture probes. Mutations and numbers of capture probe candidate regions for 16S rRNA gene were also analyzed from 15 representative serotypes out of 27 *Salmonella* serotypes for the comparison (see Tables S4 and S5 in the supplemental material).

Probe design and synthesis. The *carB* sequences of the *S. enterica* subsp. *enterica* serotypes and other bacteria were downloaded from NCBI GenBank. The oligonucleotide probes were designed using the BioEdit software (Ibis Biosciences, Carlsbad, CA) and Primer Premier 5 (Premier Biosoft International, Palo Alto, CA). First, we compared the aligned sequences of the target *S. enterica* subsp. *enterica* serotypes and target-related bacteria to identify specific regions (see Fig. S1 in the supplemental material). Based on these regions, capture probe candidates were designed with the criteria of a length of 15 to 25 bp, sequence dissimilarities of over 10 to 15%, and similar melting temperatures (T_m). Finally, the six serotype-specific capture probes and one *Salmonella*-positive-control probe were determined through NCBI BLAST searches (Table 1 and Fig. 1A). The selected probes were chemically synthesized with 5′-end modification (Integrated DNA Technology, Coralville, IA).

Design and construction of DNA microarray. We designed the format of the DNA microarray in a manner similar to that of our previous reports (13, 14). Each serotype-specific capture probe was horizontally repeated as four spots and rectangularly surrounded by five replicate spots of an artificial standard capture probe, which was used to correlate chip-to-chip or regional variations (13, 14, 27). Consequently, 1×4 spots of the *Salmonella* positive-control probe in the first row, 1×4 spots of each of the 6 serotype-specific capture probes, and 7×5 spots of the artificial standard capture probe (27) are contained on each oligonucleotide microarray (Fig. 1B). The DNA microarray was prepared by the same method described in our previous reports (12–14, 27).

PCR amplification of the *carB* gene. We designed and synthesized universal primers (Sal_220f, 5′-GGAAGTGGTGCGCAAAATC-3′; Sal_1596r, 5′-CGAATTCCGCCGCGC-3′) (Genotech, Daejeon, South Korea) to amplify the *carB* genes from *S. enterica* subsp. *enterica* chromosomes as the target DNA. PCRs were performed using each DNA from 17 bacteria, including three *S. enterica* subsp. *enterica* serotype targets. The PCR mixtures consisted of 1 U *Taq* polymerase (TaKaRa, Otsu, Japan), 2 μM Sal_220f universal primer, 2 μM Sal_1596r universal primer, 1 to 50 ng/ml chromosomal DNA, 250 nM dUTPs, and 1× *Taq* buffer (TaKaRa). The PCR conditions were as follows: 95°C for 5 min and 30 cycles of 95°C for 1 min, 61°C for 45 s, and 72°C for 2 min, followed by 72°C for 5 min. The PCR amplicons were purified with the Wizard SV gel and PCR cleanup system (Promega, Madison, WI) and analyzed by 1% (wt/vol) agarose gel electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer containing RedSafe nucleic acid staining solution (iNtRON Biotechnology, Sungnam, South Korea).

Target DNA preparation. The *carB* DNA targets of three *S. enterica* subsp. *enterica* serotypes were amplified by PCR using mixtures that contained 2 U *Taq* polymerase, 2 μM Sal_220f universal primer, 2 μM Sal_1596r universal primer, 1 to 50 ng/ml chromosomal DNA, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.3 mM dTTP, 0.15 mM amine-modified dUTP, and 1× *Taq* buffer. The PCR was performed under the same conditions as stated above. Next, the amine-modified amplicons were purified using the Wizard SV gel and PCR cleanup system and precipitated with ethanol. The target DNA was then denatured by heating at 95°C for 5 min, immediately cooled on ice, and labeled with the ARES Alexa Fluor 647 DNA labeling kit (Molecular Probes, Eugene, OR). For the target DNA of the mock sample, we used a PCR mixture that contained the genomic DNA of *Salmonella* serotype Typhimurium and 8

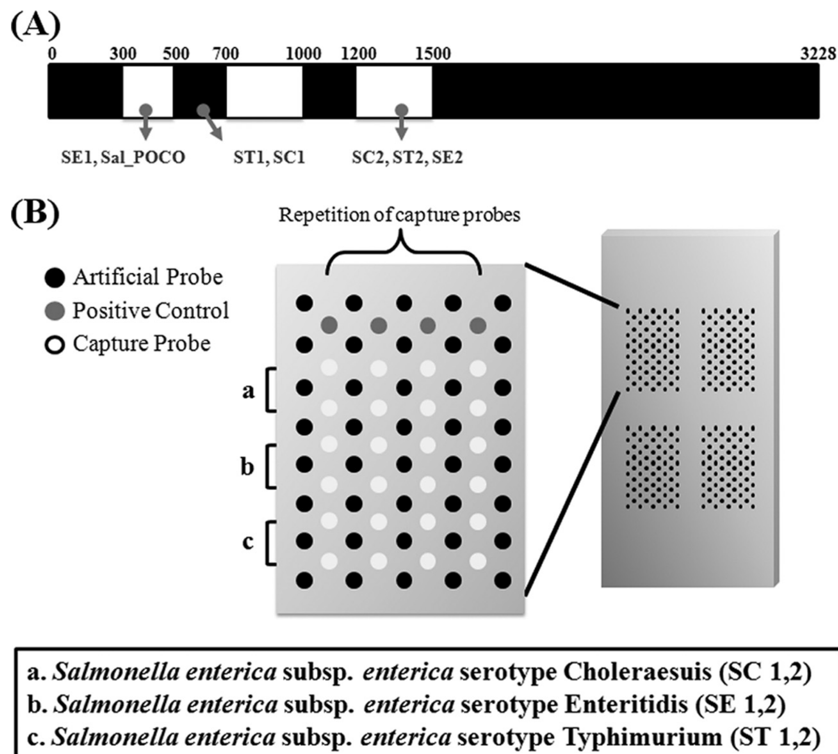

FIG 1 Schematic diagrams of (A) the regions of the *carB* gene used in the design of the *Salmonella* positive-control and serotype-specific capture probes and (B) the repeated array format for the *carB*-based oligonucleotide microarray.

other bacteria: *E. coli*, *S. boydii*, *S. dysenteriae*, *S. sonnei*, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *Y. enterocolitica*.

Hybridization and fluorescence intensity scanning. Prehybridization for the constructed oligonucleotide microarray was performed in buffer containing 3× SSC solution (450 mM NaCl and 3 mM trisodium citrate [pH 7.0]) with 1% (wt/vol) bovine serum albumin (BSA) (Sigma, St. Louis, MO) and 0.1% (wt/vol) sodium dodecyl sulfate (SDS) for 30 min at 50°C. The array was washed three times (twice with distilled water and once with ethanol) and dried by centrifugation at 1,500 rpm for 3 min. Hybridization was accomplished by exposing the array to hybridization solution containing 50 to 150 μg/ml PCR-amplified target DNA, 1 μM artificial standard target DNA (27), and 1× hybridization solution (3× SSC, 0.1% [wt/vol] SDS, and 0.2% [wt/vol] BSA) at 50°C for 1 h. Next, the array was washed four times: first with buffer I (1× SSC and 0.2% [wt/vol] SDS) for 1 min, second with buffer II (0.1× SSC and 0.2% [wt/vol] SDS) for 1 min, and third and fourth with buffer III (0.1× SSC) for 1 min at room temperature. After the microarray was dried, the fluorescence intensity was scanned with a commercial confocal laser scanner (ScanArray Lite; GSI Lumonics, Wilmington, MA), and the data were analyzed using quantitative microarray analysis software (QuantArray; GSI Lumonics). The obtained raw fluorescence intensity data were transformed into two-dimensional (2D) visualization data for the specific spots by our previously developed method (12).

RESULTS AND DISCUSSION

Selection and verification of *carB* gene as biomarker. We selected *carB* as a detection biomarker to design the specific capture probes for the serotype-level analysis of *S. enterica* subsp. *enterica*. The *carB* gene encodes the large subunit (~118 kDa) of CPS, which catalyzes the formation of carbamoyl-phosphate required for the biosynthesis of pyrimidine nucleotides and arginine or the urea cycle (28–30). In addition, CPS has occasionally been used in phylogenetic analyses to examine the evolutionary relationships among *Bacteria*, *Archaea*, and *Eukarya* (31, 32). The CPS large subunit harbors conserved domains that have internal sequence similarity and have been suggested to be evidence of ancestral gene duplications during evolution (29, 30, 47). For example, the CPS large subunit from *E. coli* contains two homologous domains (residues 1 to 400 and 553 to 933), with ~40% of the amino acid sequence being identical (29). All bacteria, including *S. enterica* subsp. *enterica*, possess a *carB* gene, which encodes an enzyme vital to their survival, and it is actually expedient to design universal primers and selective serotype-specific capture probes based on this gene because it contains both conserved and variable regions (see Fig. S1 in the supplemental material).

To evaluate the potential of the *carB* gene as a biomarker for distinction of *S. enterica* subsp. *enterica* serotypes, we performed the genetic analysis of representative 16 serotypes (see Table S1 in the supplemental material). Through the analysis using NCBI BLAST search, we found that 14 (87.5%) out of 16 *Salmonella* serotypes can be clearly distinguished based on the *carB* gene (see Tables S2 and S3 in the supplemental material), except for distinctions between *S.* Choleraseuis and *S.* Paratyphi C and between *S.* Enteritidis and *S.* Gallinarum. Interestingly, *carB* genes had identical sequence within strains of the same serotype (2 strains of *S.* Heidelberg, 2 of *S.* Paratyphi A, 3 of *S.* Typhi, and 8 of *S.* Typhimurim) (see Table S1), and this tendency toward sequence similarity coincided with that of the concatenated seven-housekeeping-gene-based phylogenetic tree (33). This indicates that the distinctive resolution of *carB* biomarker can correspond to the serotype discrimination. Excellent distinctive ability among dif-

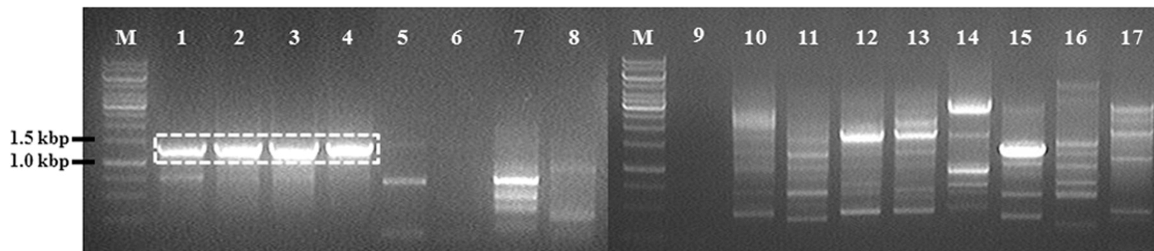

FIG 2 Gel electrophoresis analyses of 18 pathogenic bacteria for PCR amplification of the partial *carB* gene. Lanes: M, 1-kb DNA size maker; 1, *S.* Choleraesuis; 2, *S.* Enteritidis; 3, *S.* Typhimurium; 4, *S.* Newport; 5, *B. cereus*; 6, *C. perfringens*; 7, *L. monocytogenes*; 8, *S. aureus*; 9, *C. jejuni*; 10, *E. coli*; 11, *S. boydii*; 12, *S. dysenteriae*; 13, *S. sonnei*; 14, *V. cholerae*; 15, *V. parahaemolyticus*; 16, *V. vulnificus*; 17, *Y. enterocolitica*.

ferent serotypes can be supported by an average of 102 mutations and 15 candidate regions for the specific probes. Especially, the numbers of mutations and candidate regions among three target serotypes focused in the present work were enough to discriminate each other (70 and 11, respectively, between *S.* Choleraesuis and *S.* Typhimurium LT2, 107 and 16 between *S.* Choleraesuis and *S.* Enteritidis, and 120 and 17 between *S.* Enteritidis and *S.* Typhimurium LT2). Consequently, the genetic analysis of the *carB* biomarker showed great competence for discriminating *S. enterica* subsp. *enterica* serotypes as well as representing the phylogenetic relationship.

Development of serotype-specific capture probes. We designed 6 serotype-specific capture probes and 1 *Salmonella* positive-control probe to detect and distinguish *S. enterica* subsp. *enterica* serotypes Choleraesuis, Enteritidis, and Typhimurium, using their *carB* gene sequences, which are sufficiently diverse for specific discrimination (Table 1). For the design of the serotype-specific capture probes, we first considered the sequence dissimilarity to be more than 10 to 15%. Thus, the full-length *carB* sequences of 13 serotypes (not shown) were aligned and compared to identify 15- to 25-bp sequence regions having two or more mismatches. After these specific sequence regions were obtained, their thermodynamic properties were calculated. A similar melting temperature among the calculated thermodynamic properties was regarded as the most important factor to ensure similar stringencies during hybridization. Another thermodynamic property rating that represents the ΔG values of secondary structures, such as hairpins and oligonucleotide dimers, was also considered to reduce structural hindrance, which can significantly impair the hybridization affinity (34, 35). Finally, the serotype-specific capture probes with high selectivity and optimal thermodynamic properties were selected by match analyses using an NCBI BLAST search. We selected two types of serotype-specific capture probes for each *S. enterica* subsp. *enterica* serotype to increase the possibility and accuracy of detection. The *Salmonella* positive-control probe for the general analysis of *S. enterica* subsp. *enterica* was designed based on conserved *carB* gene sequence regions. A previously developed artificial standard capture probe was used to normalize the chip-to-chip or regional variations and indicate the probe positions (13, 14, 27).

Amplification of *carB* detection biomarker. We designed and synthesized universal PCR primers (Sal_220f and Sal_1596r) that enable the specific amplification of partial *carB* sequences from *S. enterica* subsp. *enterica*. These partial *carB* sequences (~1,400 bp) were used as targets to reduce the PCR cycling duration and to conserve consumable materials. More importantly, the use of the fragment caused no issue of hybridization with the serotype-specific capture probes because all of the designed capture probe sequences are in the nucleotide segment from residues 300 to 1,500 of each *carB* gene (Fig. 1A).

To validate the synthesized universal primers, we performed PCR amplifications of the partial *carB* genes for a total of 17 common food-borne pathogenic bacteria, including 3 of the targets (*S.* Choleraesuis, *S.* Enteritidis, and *S.* Typhimurium) and 14 nontarget bacteria: *S.* Newport, *B. cereus*, *C. perfringens*, *Listeria monocytogenes*, *S. aureus*, *E. coli*, *S. boydii*, *S. dysenteriae*, *S. sonnei*, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *Y. enterocolitica* (Fig. 2). Based on gel electrophoresis analyses, ~1,400-bp-long PCR fragments were successfully obtained from the DNA of 4 *S. enterica* subsp. *enterica* serotypes (lanes 1 to 4). In contrast, there were hardly any amplified DNA bands (lanes 5, 6, 8, and 9) or nonspecific amplified bands (lanes 7 and 10 to 17) from the non-*Salmonella* pathogenic bacteria. In the case of Gram-positive bacteria, the amplified band intensities were generally lower than for Gram-negative bacteria. *S. dysenteriae* and *S. sonnei* showed nonspecifically amplified bands having similar sizes to *S. enterica* subsp. *enterica* (lanes 12 and 13); these nonspecific bands might be explained from the 90% identical *carB* sequences. Other bacteria, such as *V. cholerae* and *V. parahaemolyticus*, produced longer and shorter bands, respectively, than the amplified partial *carB* band (lanes 14 and 15). These nonspecific fragments may have been generated from sequences in their genomic DNA being similar to the forward primer Sal_220f; such sequences were discovered in the NCBI BLAST search. The results of the *carB* PCRs indicated that the designed universal primer set was able to successfully and specifically amplify a partial *carB* gene from *S. enterica* subsp. *enterica* serotypes.

Serotype-level detection of *carB*-based oligonucleotide microarray. To examine the ability of the constructed *carB*-based oligonucleotide microarray to perform specific serotype-level analysis, PCR amplicons labeled fluorescently with Alexa Fluor 647 were used in hybridization reactions. The specific spots of each target *S. enterica* subsp. *enterica* serotype are indicated by a yellow-dotted box in the raw hybridization images shown in Fig. 3A. It is known that discrimination between *S.* Choleraesuis and *S.* Enteritidis is difficult using a 16S rRNA-based oligonucleotide microarray (13). Importantly, three serotypes of *S. enterica* subsp. *enterica* were specifically analyzed and clearly discriminated without any nonspecific spots by our *carB*-based oligonucleotide microarray (Fig. 3A). We also analyzed the 16S rRNA gene (see Tables S4 and S5 in the supplemental material) to explain the reason for the superior serotype discrimination ability of the *carB*-based

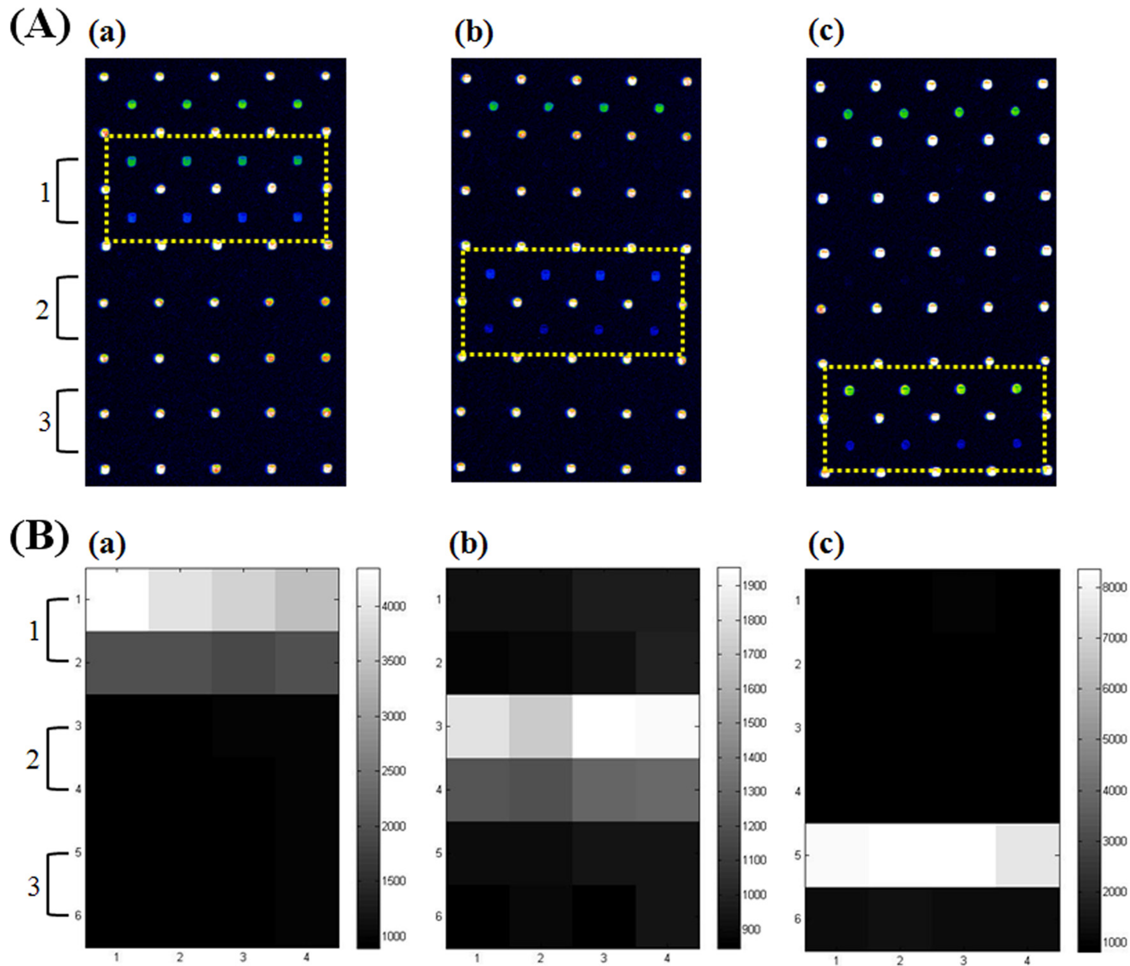

FIG 3 Detection of three *S. enterica* subsp. *enterica* serotypes using the *carB*-based oligonucleotide microarray. (A) Raw hybridization data with each amplified target at 50°C in hybridization buffer for 1 h and (B) 2D visualization plots of serotype-specific spots. The intensities are represented by gray gradation: the maximum intensity is white, and the minimum intensity is black. (a) *S.* Choleraesuis; (b) *S.* Enteritidis; (c) *S.* Typhimurium. A set of two serotype-specific capture probes was used for *S.* Choleraesuis (1), *S.* Enteritidis (2), and *S.* Typhimurium (3).

microarray system to that of the 16S rRNA-based microarray. Through comparison of mutations and numbers of the capture probe candidate regions for each gene, we concluded that the sequence differences of *carB* were sufficient to design the specific capture probes for *S. enterica* subsp. *enterica* serotypes, while those of the 16S rRNA gene were insufficient.

Because it is difficult to realize a quantitative analysis using raw hybridization images, we employed a 2D visualization tool, which can represent a gray gradient system using Matlab m-files, to facilitate our quantitative analysis and simplify the discernment of the spots using intensity-scale enlargement (Fig. 3B). The gray gradient system employed white as the maximum intensity and black as the minimum intensity. The 2D visualization images of both *S.* Choleraesuis and *S.* Enteritidis clearly show the sections of their specific spots, which had the highest and second highest fluorescence intensities, respectively (Fig. 3Ba and Bb). The intensity difference between the first and the second specific spots was relatively small, within the range of 2- to 3-fold. However, one section of specific spots was only observed in the 2D visualization image of *S.* Typhimurium (Fig. 3Bc) because the fluorescence intensity of the first specific spots was much higher (approximately 15-fold) than that of the second specific spots. Thus, the large intensity difference resulted in only the first specific spots being detected in the 2D visualization image, even though the signals of the second specific spots were also shown in the raw data (Fig. 3Ac). The reason for the large difference in intensity can be explained by the complementary sequence of the ST-1 capture probe, which has a similar T_m and GC% to the other capture probes, being located at an easily accessible site in the secondary structure of the target single-strand DNA during hybridization (34, 36, 37).

Selectivity and sensitivity of *carB*-based oligonucleotide microarray. We performed detection analyses to examine the cross-reactivity of the *carB*-based serotype-specific capture probes on the oligonucleotide microarray with nontarget pathogenic bacteria: *S.* Newport, *E. coli*, *S. boydii*, *S. dysenteriae*, *S. sonnei*, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *Y. enterocolitica*. In the case that the signal-to-noise (S/N) ratio of a spot was larger than 2, the spot was regarded as having a positive signal (38). Detectable positive signals from any of the serotype-specific capture spots were not observed for the 9 nontarget pathogenic bacteria (Table 2). Accordingly, we determined that the *carB*-based serotype-specific capture probes do not have cross-reactivity with other patho-

TABLE 2 Cross-reactivity of *carB*-based oligonucleotide microarray with each common food-borne pathogenic bacterium

Pathogenic species	Detection result from[a]:	
	Serotype-specific spots	*Salmonella* positive-control spots
S. enterica subsp. *enterica* serotype Newport	−	+
Escherichia coli	−	−
Shigella boydii	−	−
Shigella dysenteriae	−	−
Shigella sonnei	−	+
Vibrio cholerae	−	−
Vibrio parahaemolyticus	−	+
Vibrio vulnificus	−	+
Yersinia enterocolitica	−	+

[a] +, positive signal (S/N ratio of >2); −, negative signal (S/N ratio of <2).

genic bacteria, including another serotype, *S*. Newport, and genetically closely related species, such as *E. coli* and *Shigella* spp., which have approximately 90% identical *carB* sequences. Some bacteria, such as *S*. Newport, *S. sonnei*, *V. parahaemolyticus*, *V. vulnificus*, and *Y. enterocolitica*, exhibited positive signals on the *Salmonella* positive-control spots (Table 2). It was reasonable to analyze out the positive-control spots with *S*. Newport due to the common sequences of the *Salmonella* positive-control probe in *S. enterica* subsp. *enterica*. The *carB* gene sequence of *Y. enterocolitica* has two base pair differences from the *Salmonella* positive-control probe, equivalent to 9.09% dissimilarity. Therefore, this low sequence difference was able to generate nonspecific *Salmonella* positive-control spots. However, another three bacteria (*S. sonnei*, *V. parahaemolyticus*, and *V. vulnificus*) also generated nonspecific positive-control spots; based on their high *carB* gene sequence differences; we suspect that nonspecifically amplified DNA contaminants might have similar sequences.

Next, the dynamic detection range according to the target DNA concentration was assayed to determine the sensitivity of our *carB*-based oligonucleotide microarray. We prepared six concentrations (1.6 to 50 nM) of target DNA from *S*. Typhimurium as the representative serotype and performed hybridization and quantification analyses. A dose-response curve was plotted based on the normalized fluorescence intensities, which were corrected using artificial standard spot intensities (Fig. 4). The normalized florescence intensities of the ST-1-specific spots increased linearly in the range of 1.6 to 12.5 nM target DNA and showed a tendency to saturate when the DNA concentration was above 25 nM. Thus, the detection limit of the ST-1 serotype-specific capture probe was evaluated as approximately 1.6 to 3.1 nM. Because the intensities of the ST-2 spots were much lower than those of the positive-control spots and the ST-1 spots in particular, the ST-2 spot intensities appeared to be unchanged in the dose-response curve. The detection limit of the ST-2-specific capture probe was estimated in the range of 6.2 to 12.5 nM, which was relatively higher than that of the ST-1 probe. Consequently, at least 1.6 nM target DNA will be necessary to detect the *S*. Typhimurium serotype using this *carB*-based oligonucleotide microarray. This value is less sensitive than other oligonucleotide microarrays based on 16S rRNA information for bacterial identification, with detection limits approximating 2.5 to 5.0 fM (39). For more sensitive detection, it might be necessary to employ signal improvement strategies, such as nanoparticle labeling (40, 41).

In real contaminated foods, the target pathogenic bacterium is often present with naturally occurring microbes or different types of pathogens, which might cause interference in the selective identification of a specific pathogen (42–46). Thus, we examined the selective detection of the *S. enterica* subsp. *enterica* serotypes from a mock sample comprising a mixture of diverse bacterial DNAs. For the mock sample, the DNA template for PCR was prepared by directly mixing the chromosomes of *S*. Typhimurium and eight pathogenic bacteria. As shown in the raw hybridization images (Fig. 5A), the serotype-specific spots for *S*. Typhimurium were clearly analyzed from the mock sample without any nonspecific spots. To compare the signal intensities of the serotype-specific spots for pure *S*. Typhimurium and the mock samples on two microarrays, we corrected the fluorescence intensities by employing the intensities of artificial standard spots to minimize the chip-to-chip variation. The normalized fluorescence spot intensities of the sample containing the bacterial DNA mixture were decreased by as much as 65 to 70% compared to those of the pure *S*. Typhimurium sample (Fig. 5B). These reductions might be caused by the reduced amount of target DNA from the mock sample, which contained nonspecific DNA fragments, and/or the interference from nonspecific DNA contaminants during the hybridization between the target DNA and the serotype-specific capture probes. However, the reduction in the signal was not significant. These results demonstrated that efficient selective detection and analysis of *S. enterica* subsp. *enterica* serotypes, even in the presence of other bacteria, are possible using our *carB*-based oligonucleotide microarray system.

Furthermore, we expect that our *carB*-based microarray approach can be expanded in terms of its potential utilization for the detection of subspecies or serotypes of other bacteria, because the *carB* genes of other bacteria, including *E. coli*, *Shigella*, and *Vibrio*, also have species-specific conserved regions for universal primers and serotype- or subspecies-specific sequences for capture probes, which can be supported by the multiple alignment of *carB* genes (see Fig. S1 in the supplemental material). Even though we suc-

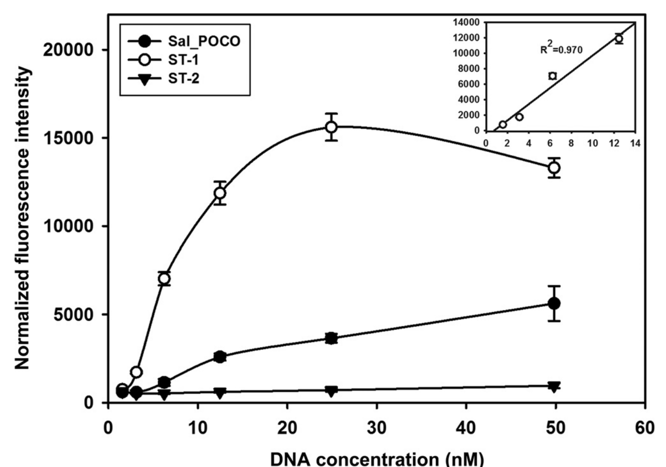

FIG 4 Dynamic detection range of the *carB*-based oligonucleotide microarray. Shown are normalized fluorescence intensity plots for the positive-control (closed circle), specific ST-1 (open circle), and specific ST-2 (closed triangle) spots according to various *S*. Typhimurium DNA concentrations. The inset is the plot for the specific ST-1 spot at a lower range of DNA concentrations. Each value is the mean of 4 repeated spots, and the error bars represent standard deviations.

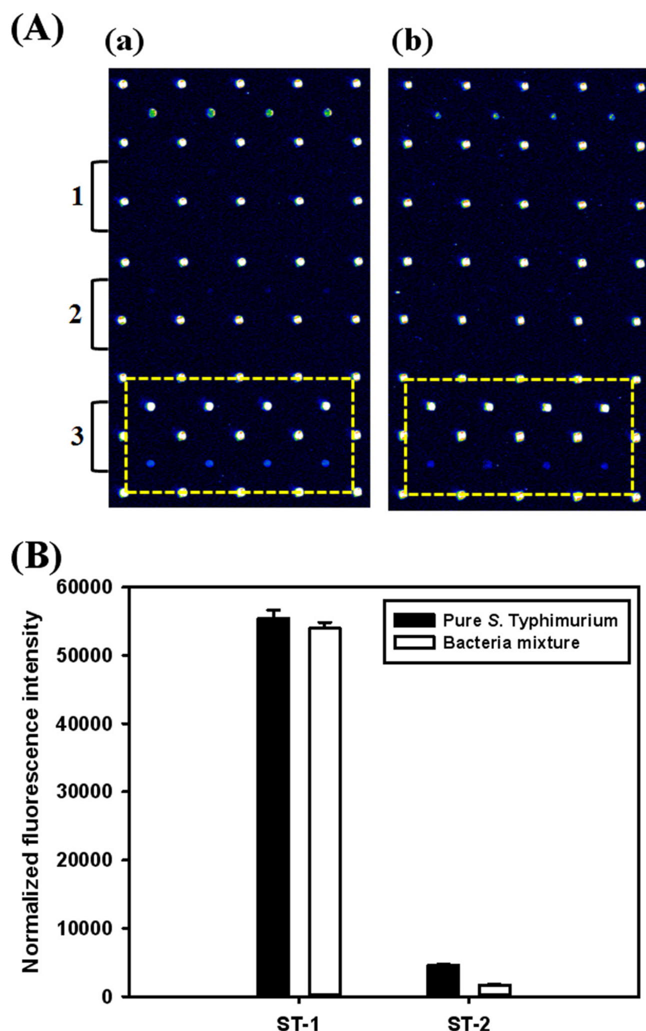

FIG 5 Detection of *S.* Typhimurium from samples of pure DNA (a) and a diverse bacterial-DNA mixture (b). Shown are raw hybridization images (A) and the bar graph (B) of the normalized spot intensities from the serotype-specific spots (ST-1 and ST-2). A set of two serotype-specific capture probes was used for (1) *S.* Choleraesuis, (2) *S.* Enteritidis, and (3) *S.* Typhimurium. Each value is the mean of 4 repeated spots, and the error bars represent standard deviations.

cessfully discriminated three target serotypes using the *carB*-based serotype-specific capture probes without nonspecificity in the present work, it might be possible to show false-positive signals among phylogenetically close serotypes if many serotypes are coexisting in a sample, because the same sequences might be shared for several thousand serotypes of *S. enterica* subsp. *enterica*. This potential limitation could be overcome by adding more serotype-specific capture probes that can be further selected from sufficient candidates of *carB* biomarker. In addition, our suggested method is the genotype-based identification of serotypes. To detect pathogenicity or antibiotic resistance, it is necessary to add extra biomarker information related to virulence factors or antibiotic resistances.

In summary, we designed six serotype-specific capture probes, consisting of two types of probes for each target serotype and one *Salmonella* positive-control probe, based on the *carB* sequence, which was selected as a competent biomarker through genetic analysis, and constructed an oligonucleotide microarray to detect and discriminate three pathogenic *S. enterica* subsp. *enterica* serotypes: Choleraesuis, Enteritidis, and Typhimurium. Using the developed oligonucleotide microarray and the designed universal primer set to prepare a labeled fragment of the target *carB* gene, three serotypes of *S. enterica* subsp. *enterica* were specifically and clearly analyzed and discriminated. The serotype-specific capture probes did not display cross-reactivity with common pathogenic bacteria, including other *S. enterica* subsp. *enterica* serotypes and genetically closely related bacteria. The developed *carB*-based oligonucleotide microarray could detect as little as 1.6 to 3.1 nM target DNA. In addition, the technique was able to selectively analyze *S. enterica* subsp. *enterica* serotypes, even in a diverse bacterial DNA mixture, although the specific spot signals were slightly lower than those from the pure serotype sample. Thus, we expect that the developed *carB*-based oligonucleotide microarray can be used as a potential biosensor system to detect and distinguish the major *S. enterica* subsp. *enterica* serotypes efficiently, rapidly, selectively, and reliably.

ACKNOWLEDGMENTS

This work was supported by the Technology Development Program for Agriculture and Forestry from the Ministry for Agriculture, Food and Rural Affairs, South Korea, and the Marine Biotechnology Program funded by the Ministry of Oceans and Fisheries, South Korea.

REFERENCES

1. **Swaminathan B, Gerner-Smidt P, Barrett T.** 2006. Focus on *Salmonella*. Foodborne Pathog. Dis. **3**:154–156. http://dx.doi.org/10.1089/fpd.2006.3.154.
2. **Voetsch AC, Van Gilder TJ, Angulo FJ, Farley MM, Shallow S, Marcus R, Cieslak PR, Deneen VC, Tauxe RV, Emerging Infections Program FoodNet Working Group.** 2004. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. Clin. Infect. Dis. **38**:S127–S134. http://dx.doi.org/10.1086/381578.
3. **Fricke WF, Mammel MK, McDermott PF, Tartera C, White DG, LeClerc JE, Ravel J, Cebula TA.** 2011. Comparative genomics of 28 *Salmonella enterica* isolates: evidence for CRISPR-mediated adaptive sublineage evolution. J. Bacteriol. **193**:3556–3568. http://dx.doi.org/10.1128/JB.00297-11.
4. **Chan K, Baker S, Kim CC, Detweiler CS, Dougan G, Falkow S.** 2003. Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar Typhimurium DNA microarray. J. Bacteriol. **185**:553–563. http://dx.doi.org/10.1128/JB.185.2.553-563.2003.
5. **Grassl GA, Finlay BB.** 2008. Pathogenesis of enteric *Salmonella* infections. Curr. Opin. Gastroenterol. **24**:22–26. http://dx.doi.org/10.1097/MOG.0b013e3282f21388.
6. **Layton AN, Galyov EE.** 2007. *Salmonella*-induced enteritis: molecular pathogenesis and therapeutic implications. Expert Rev. Mol. Med. **9**:1–17.
7. **Liebana E, Guns D, Garcia-Migura L, Woodward MJ, Clifton-Hadley FA, Davies RH.** 2001. Molecular typing of *Salmonella* serotypes prevalent in animals in England: assessment of methodology. J. Clin. Microbiol. **39**:3609–3616. http://dx.doi.org/10.1128/JCM.39.10.3609-3616.2001.
8. **Mertes F, Biens K, Lehrach H, Wagner M, Dahl A.** 2010. High-throughput universal probe *Salmonella* serotyping (UPSS) by nanoPCR. J. Microbiol. Methods **83**:217–223. http://dx.doi.org/10.1016/j.mimet.2010.09.005.
9. **Harvey RW, Price TH.** 1979. Principles of *Salmonella* isolation. J. Appl. Bacteriol. **46**:27–56. http://dx.doi.org/10.1111/j.1365-2672.1979.tb02580.x.
10. **Perales I, Audicana A.** 1989. Semisolid media for isolation of *Salmonella* spp. from coastal waters. Appl. Environ. Microbiol. **55**:3032–3033.
11. **Croci L, Delibato E, Volpe G, De Medici D, Palleschi G.** 2004. Comparison of PCR, electrochemical enzyme-linked immunosorbent assays, and the standard culture method for detecting *Salmonella* in meat products. Appl. Environ. Microbiol. **70**:1393–1396. http://dx.doi.org/10.1128/AEM.70.3.1393-1396.2004.
12. **Eom HS, Hwang BH, Kim DH, Lee IB, Kim YH, Cha HJ.** 2007. Multiple

detection of food-borne pathogenic bacteria using a novel 16S rDNA-based oligonucleotide signature chip. Biosens. Bioelectron. **22:**845–853. http://dx.doi.org/10.1016/j.bios.2006.03.005.

13. **Hwang BH, Cha HJ.** 2010. Pattern-mapped multiple detection of 11 pathogenic bacteria using a 16S rDNA-based oligonucleotide microarray. Biotechnol. Bioeng. **106:**183–192. http://dx.doi.org/10.1002/bit.22674.
14. **Hwang BH, Shin HH, Seo JH, Cha HJ.** 2012. Specific multiplex analysis of pathogens using a direct 16S rRNA hybridization in microarray system. Anal. Chem. **84:**4873–4879. http://dx.doi.org/10.1021/ac300476k.
15. **Pathirana ST, Barbaree J, Chin BA, Hartell MG, Neely WC, Vodyanoy V.** 2000. Rapid and sensitive biosensor for *Salmonella*. Biosens. Bioelectron. **15:**135–141. http://dx.doi.org/10.1016/S0956-5663(00)00067-1.
16. **Call DR, Borucki MK, Loge FJ.** 2003. Detection of bacterial pathogens in environmental samples using DNA microarrays. J. Microbiol. Methods **53:**235–243. http://dx.doi.org/10.1016/S0167-7012(03)00027-7.
17. **Guschin DY, Mobarry BK, Proudnikov D, Stahl DA, Rittmann BE, Mirzabekov AD.** 1997. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl. Environ. Microbiol. **63:**2397–2402.
18. **Schena M, Shalon D, Davis RW, Brown PO.** 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270:**467–470. http://dx.doi.org/10.1126/science.270.5235.467.
19. **Severgnini M, Cremonesi P, Consolandi C, De Bellis G, Castiglioni B.** 2011. Advances in DNA microarray technology for the detection of food-borne pathogens. Food Bioprocess Technol. **4:**936–953. http://dx.doi.org/10.1007/s11947-010-0430-5.
20. **Call DR.** 2005. Challenges and opportunities for pathogen detection using DNA microarrays. Crit. Rev. Microbiol. **31:**91–99. http://dx.doi.org/10.1080/10408410590921736.
21. **Chandler DP, Newton GJ, Small JA, Daly DS.** 2003. Sequence versus structure for the direct detection of 16S rRNA on planar oligonucleotide microarrays. Appl. Environ. Microbiol. **69:**2950–2958. http://dx.doi.org/10.1128/AEM.69.5.2950-2958.2003.
22. **Liu WT, Mirzabekov AD, Stahl DA.** 2001. Optimization of an oligonucleotide microchip for microbial identification studies: a non-equilibrium dissociation approach. Environ. Microbiol. **3:**619–629. http://dx.doi.org/10.1046/j.1462-2920.2001.00233.x.
23. **Peplies J, Lachmund C, Glockner FO, Manz W.** 2006. A DNA microarray platform based on direct detection of rRNA for characterization of freshwater sediment-related prokaryotic communities. Appl. Environ. Microbiol. **72:**4829–4838. http://dx.doi.org/10.1128/AEM.02949-05.
24. **Rudi K, Skulberg OM, Skulberg R, Jakobsen KS.** 2000. Application of sequence-specific labeled 16S rRNA gene oligonucleotide probes for genetic profiling of cyanobacterial abundance and diversity by array hybridization. Appl. Environ. Microbiol. **66:**4004–4011. http://dx.doi.org/10.1128/AEM.66.9.4004-4011.2000.
25. **Choi SH, Woo JH, Lee JE, Park SJ, Choo EJ, Kwak YG, Kim MN, Choi MS, Lee NY, Lee BK, Kim NJ, Jeong JY, Ryu J, Kim YS.** 2005. Increasing incidence of quinolone resistance in human non-typhoid *Salmonella enterica* isolates in Korea and mechanisms involved in quinolone resistance. J. Antimicrob. Chemother. **56:**1111–1114. http://dx.doi.org/10.1093/jac/dki369.
26. **Kim S, Chun SG, Lim OY, Park MS, Kang YH, Park YH, Lee BK.** 2004. Genomic relationship of *Salmonella enterica* serovar Typhimurium DT104 isolates from Korea and the United States. J. Microbiol. **42:**14–19.
27. **Hwang BH, Cha HJ.** 2008. Quantitative oligonucleotide microarray data analysis with an artificial standard probe strategy. Biosens. Bioelectron. **23:**1738–1744. http://dx.doi.org/10.1016/j.bios.2008.01.024.
28. **Holden HM, Thoden JB, Raushel FM.** 1999. Carbamoyl phosphate synthetase: an amazing biochemical odyssey from substrate to product. Cell. Mol. Life Sci. **56:**507–522. http://dx.doi.org/10.1007/s000180050448.
29. **Javid-Majd F, Mullins LS, Raushel FM, Stapleton MA.** 2000. The differentially conserved residues of carbamoyl-phosphate synthetase. J. Biol. Chem. **275:**5073–5080. http://dx.doi.org/10.1074/jbc.275.7.5073.
30. **Nyunoya H, Lusty CJ.** 1983. The *carB* gene of *Escherichia coli*: a duplicated gene coding for the large subunit of carbamoyl-phosphate synthetase. Proc. Natl. Acad. Sci. U. S. A. **80:**4629–4633. http://dx.doi.org/10.1073/pnas.80.15.4629.
31. **Hong J, Salo WL, Lusty CJ, Anderson PM.** 1994. Carbamyl phosphate synthetase III, an evolutionary intermediate in the transition between glutamine-dependent and ammonia-dependent carbamyl phosphate synthetases. J. Mol. Biol. **243:**131–140. http://dx.doi.org/10.1006/jmbi.1994.1638.
32. **Lawson FS, Charlebois RL, Dillon JA.** 1996. Phylogenetic analysis of carbamoyl phosphate synthetase genes: complex evolutionary history includes an internal duplication within a gene which can root the tree of life. Mol. Biol. Evol. **13:**970–977. http://dx.doi.org/10.1093/oxfordjournals.molbev.a025665.
33. **Seong WJ, Kwon HJ, Kim TE, Lee DY, Park MS, Kim JH.** 2012. Molecular serotyping of *Salmonella enterica* by complete *rpoB* gene sequencing. J. Microbiol. **50:**962–969. http://dx.doi.org/10.1007/s12275-012-2547-x.
34. **Koehler RT, Peyret N.** 2005. Effects of DNA secondary structure on oligonucleotide probe binding efficiency. Comput. Biol. Chem. **29:**393–397. http://dx.doi.org/10.1016/j.compbiolchem.2005.09.002.
35. **Lane S, Evermann J, Loge F, Call DR.** 2004. Amplicon secondary structure prevents target hybridization to oligonucleotide microarrays. Biosens. Bioelectron. **20:**728–735. http://dx.doi.org/10.1016/j.bios.2004.04.014.
36. **Hagan MF, Chakraborty AK.** 2004. Hybridization dynamics of surface immobilized DNA. J. Chem. Phys. **120:**4958–4968. http://dx.doi.org/10.1063/1.1645786.
37. **Lima WF, Monia BP, Ecker DJ, Freier SM.** 1992. Implication of RNA structure on antisense oligonucleotide hybridization kinetics. Biochemistry **31:**12055–12061. http://dx.doi.org/10.1021/bi00163a013.
38. **Bowtell D, Sambrook J.** 2003. DNA microarrays. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
39. **Francois P, Charbonnier Y, Jacquet J, Utinger D, Bento M, Lew D, Kresbach GM, Ehrat M, Schlegel W, Schrenzel J.** 2006. Rapid bacterial identification using evanescent-waveguide oligonucleotide microarray classification. J. Microbiol. Methods **65:**390–403. http://dx.doi.org/10.1016/j.mimet.2005.08.012.
40. **Oaew S, Karoonuthaisiri N, Surareungchai W.** 2009. Sensitivity enhancement in DNA hybridization assay using gold nanoparticle-labeled two reporting probes. Biosens. Bioelectron. **25:**435–441. http://dx.doi.org/10.1016/j.bios.2009.07.031.
41. **Lee G, Cho YS, Park S, Yi GR.** 2011. Synthesis and assembly of anisotropic nanoparticles. Korean J. Chem. Eng. **28:**1641–1650. http://dx.doi.org/10.1007/s11814-011-0183-5.
42. **Lampel KA, Orlandi PA, Kornegay L.** 2000. Improved template preparation for PCR-based assays for detection of food-borne bacterial pathogens. Appl. Environ. Microbiol. **66:**4539–4542. http://dx.doi.org/10.1128/AEM.66.10.4539-4542.2000.
43. **Layton MC, Calliste SG, Gomez TM, Patton C, Brooks S.** 1997. A mixed foodborne outbreak with *Salmonella heidelberg* and *Campylobacter jejuni* in a nursing home. Infect. Control Hosp. Epidemiol. **18:**115–121. http://dx.doi.org/10.1086/647565.
44. **Mandal P, Biswas A, Choi K, Pal U.** 2011. Methods for rapid detection of foodborne pathogens: an overview. Am. J. Food Technol. **6:**87–102. http://dx.doi.org/10.3923/ajft.2011.87.102.
45. **Meehan PJ, Atkeson T, Kepner DE, Melton M.** 1992. A foodborne outbreak of gastroenteritis involving two different pathogens. Am. J. Epidemiol. **136:**611–616.
46. **Zhao CW, Ge BL, De Villena J, Studler R, Yeh E, Zhao SH, White DG, Wagner D, Meng JH.** 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, DC, area. Appl. Environ. Microbiol. **67:**5431–5436. http://dx.doi.org/10.1128/AEM.67.12.5431-5436.2001.
47. **Nyunoya H, Broglie KE, Lusty CJ.** 1985. The gene coding for carbamoyl-phosphate synthetase I was formed by fusion of an ancestral glutaminase gene and a synthetase gene. Proc. Natl. Acad. Sci. U. S. A. **82:**2244–2246. http://dx.doi.org/10.1073/pnas.82.8.2244.