

Waterborne Pathogen Detection by Use of Oligonucleotide-Based Microarrays

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A small-oligonucleotide microarray prototype was designed with probes specific for the universal 16S rRNA and *cpn60* genes of several pathogens that are usually encountered in wastewaters. In addition to these two targets, *wecE*-specific oligonucleotide probes were included in the microarray to enhance its discriminating power within the *Enterobacteriaceae* family. Universal PCR primers were used to amplify variable regions of 16S rRNA, *cpn60*, and *wecE* genes directly in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium genomic DNA mixtures (binary); *E. coli*, *S. enterica* serovar Typhimurium, and *Yersinia enterocolitica* genomic DNA mixtures (ternary); or wastewater total DNA. Amplified products were fluorescently labeled and hybridized on the prototype chip. The detection sensitivity for *S. enterica* serovar Typhimurium was estimated to be on the order of 0.1% (10^4 *S. enterica* genomes) of the total DNA for the combination of PCR followed by microarray hybridization. The sensitivity of the prototype could be increased by hybridizing amplicons generated by PCR targeting genes specific for a bacterial subgroup, such as *wecE* genes, instead of universal taxonomic amplicons. However, there was evidence of PCR bias affecting the detection limits of a given pathogen as increasing amounts of a different pathogen were spiked into the test samples. These results demonstrate the feasibility of using DNA microarrays in the detection of waterborne pathogens within mixed populations but also raise the problem of PCR bias in such experiments.

Microbial water quality assessment and the rapid detection of pathogenic microorganisms remain great challenges in ecotoxicology, public health maintenance, and, more recently, bioterrorism prevention (25). The main origin of human waterborne pathogens is the guts of humans and animals. Water contamination events often result from discharges from wastewater treatment facilities, overflowing sanitary sewer systems, waste materials that find their way into domestic and industrial sewage, and runoff of animal fecal matters during storm events (21). Bacteria within contaminated water represent a highly diversified group. Numerous *Salmonella* spp., *Escherichia* spp., *Shigella* spp., *Yersinia* spp., and *Klebsiella* spp. within the *Enterobacteriaceae* are human waterborne pathogens found in domestic wastewater. In particular, *Escherichia coli* strains constitute an important set of waterborne pathogens composed of numerous Shiga toxin-producing strains and enterohemorrhagic (e.g., O157: H7) strains associated with potable (14, 32) and recreational (7, 22) water.

Several nucleic acid-based methods have been developed for the rapid detection of pathogens in food, soil, and water with high degrees of sensitivity and specificity and without the need for complex cultivation (3, 4, 9, 24, 40). In general, these

methods allow detection within hours, rather than days as is normally required by culture techniques. Due to its high sensitivity and specificity, PCR is the most commonly employed molecular tool (16). A major limitation to this approach is the utilization of one specific primer pair per gene detection reaction. Although multiple primer sets may be successfully combined in one reaction, they rarely exceed more than six primer sets due to the generation of nonspecific products or false negatives. Another difficulty with multiplex PCR is that it requires additional postamplification analysis to discriminate the products. Size separation by electrophoresis is frequently used to discriminate multiplex PCR products, but this requires additional labor and that the amplicons of each reaction be significantly different in size, which can limit the primer pairs that can potentially be multiplexed. Consequently, general pathogen detection by PCR can be both labor-intensive and costly.

Microarrays represent an important advance in molecular detection technology, allowing the simultaneous detection of specifically labeled DNAs from many different pathogenic organisms on a small glass slide containing thousands of surface-immobilized DNA probes. Both basic types of microarrays, i.e., immobilized oligonucleotide probes and PCR amplicons, have been used successfully to detect (40) and/or characterize (5) pathogens. As the sensitivity of microarrays hybridized with total genomic DNA from complex mixtures is usually inadequate to provide detection of low pathogen concentrations (30), the hybridized DNA (target) usually consists of PCR

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TABLE 1. Binary and ternary mixture compositions

Mixture	DNA (ng)				%	
	<i>Y. enterocolitica</i>	<i>S. enterica</i> serovar Typhimurium	<i>E. coli</i>	Extracted wastewater	<i>Salmonella</i>	<i>Yersinia</i>
Binary	0	0.50	49.5	0	1	0
	0	5×10^{-2}	50.0	0	0.1	0
	0	5×10^{-3}	50.0	0	0.01	0
	0	5×10^{-4}	50.0	0	0.001	0
Ternary	0.50	5×10^{-2}	49.5	0	0.1	1
	5×10^{-2}	5×10^{-2}	50.0	0	0.1	0.1
Complex environmental DNA	0	2.50	0	47.5	5.0	0
	0	0.50	0	49.5	1.0	0
	0	5×10^{-2}	0	50.0	0.1	0

amplicons (10, 40). This mode of pathogen detection necessitates the combination of many PCRs prior to their hybridization on microarrays. Wilson et al. (40) used 140 amplicons to characterize 18 pathogenic species, thus constraining the use of microarrays for routine detection of pathogens in wastewater. Target DNA amplification with universal primers to ubiquitous genes prior to microarray hybridization can circumvent this limitation (12, 23, 28, 36). The *cpn60* gene codes for GroEL, an essential, highly conserved chaperonin protein which displays moderate DNA sequence diversity, making this gene useful in bacterial taxonomy applications (8, 17, 20). However, within the *Enterobacteriaceae*, 16S rRNA and *cpn60* sequences may share sufficient similarity to generate cross-hybridization reactions, even when short oligonucleotides are used as probes. As the majority of water pathogens belong to this family, discrimination on the basis of 16S rRNA and *cpn60* sequences is challenging. However, sequence diversity within the *wecE* gene, an *Enterobacteriaceae*-specific gene which forms part of the *wec* gene cluster involved in enterobacterial common antigen biosynthesis, has been shown to discriminate among the *Enterobacteriaceae* most frequently found in water (2).

The aim of this study was to assess the detection efficacy of an oligonucleotide-based microarray designed with probes specific for the universal targets of 16S rRNA and *cpn60* genes in addition to the *Enterobacteriaceae*-specific *wecE* genes of several pathogens usually encountered in wastewater.

MATERIALS AND METHODS

Environmental wastewater sampling and concentration. Raw wastewater samples (20 liters) were collected from the Fabreville wastewater treatment plant influent (Laval, Quebec, Canada) and stored at 4°C in sterile plastic containers until centrifugation (approximately 1 to 2 h). To concentrate raw wastewater solids, 900-ml volumes of raw wastewater were centrifuged in a swinging-bucket rotor (Beckman JS-4.2) at $3,000 \times g$ for 15 min at room temperature. Each wastewater pellet was resuspended in 10 ml of conserved supernatant. After this concentration step, the pellet was stored at -20°C until DNA extraction. Bacterial cell concentrations in raw water were determined by culturing on plate count agar for heterotrophic counts and on mTEC agar for *E. coli* counts (34, 35). All cell counts were calculated by averaging results from three separate plating experiments.

Genomic DNA preparation. Genomic DNA was extracted using a boiling method (13) from pure cultures of *E. coli* K-12, *Salmonella enterica* serovar Typhimurium (ATCC 14028), and *Yersinia enterocolitica* (ATCC 23715). A bead beating technique was used to extract genomic DNA from wastewater samples, followed by ammonium acetate purification (41). A final polyvinylpyrrolidone column purification step, modified from the procedure of Berthelet et al.

(6), was performed on the precipitated wastewater DNA. Briefly, 1 ml of acid-washed polyvinylpyrrolidone in 20 mM potassium phosphate (pH 7.0) was added to a microspin column (Amersham Biosciences Inc., Québec, Canada), placed inside a 2-ml collection tube, and centrifuged for 3 min at $700 \times g$ at room temperature, and the column was placed into a new sterile collection tube. The wastewater DNA extract was warmed for 10 min at 37°C and loaded onto the column. The column was centrifuged for 3 min at $700 \times g$ at room temperature, and the purity of the collected DNA was verified by spectrophotometry using the A_{260}/A_{280} ratio.

PCR amplification conditions of DNA mixtures. The specific compositions of the different complex genomic DNA mixtures used as PCR templates are presented in Table 1. To generate 16S rRNA gene, *cpn60*, or *wecE* amplicons from these mixtures, different amounts *S. enterica* serovar Typhimurium, *E. coli*, and *Y. enterocolitica* genomic DNA, ranging from 50 ng to 50 fg, were added in the master mix as templates for the PCRs. Published universal primers (15) were used for amplification of 16S rRNA (F1, 5'-GAGTTTGATCCTGGCTCAG-3'; R2, 5'-GWATTACCGCGGCKGCTG-3'). For *cpn60* amplicons, the primers wdF (5'-GAIHIGCIGGIGAYGGNCANACANAC-3') and wdR (5'-KIYKTCIC CRAANCCNGGNGCYTT-3') were used. These are based on published primers H279 and H280 (20), with the modification that inosines near the 3' ends of the primers were replaced by mixed base positions to decrease nonspecific priming. The *wecE* gene primers (*wecE1*, 5'-AGGGCGTGATGTCCACTAC-3'; *wecE2*, 5'-GAAGAAGTGGCTGCGGTTAG-3') were newly designed.

The PCR mixture included 5 µl of $10 \times$ PCR buffer (100 mM Tris-HCl, pH 9.0, 15 mM MgCl₂, and 500 mM KCl), 0.5 µl of 20 mM deoxynucleoside triphosphates, 1 µl of each of the forward and reverse primers (stock concentration, 25 µM), 0.5 µl (2.5 units) of *Taq* DNA polymerase (Amersham Biosciences), 50 ng of genomic DNA extracted from pure culture or environmental wastewater samples, and sterile distilled water added to give a 50-µl final volume. For *cpn60* amplification, 1 µl of 100 mM MgCl₂ solution was also added to the master mixture.

The annealing temperatures used during amplification were 52°C for 16S rRNA gene, 60°C for *cpn60*, and 55°C for *wecE* primers. Amplifications were performed in a GeneAmp PCR system 9700 (Perkin-Elmer) according to the following scheme: a hot start for 3 min at 94°C; 40 amplification cycles of 1 min at 94°C, 1 min at the annealing temperature described above, and 30 s (*wecE* amplification) or 1 min (16S rRNA and *cpn60* amplifications) at 72°C; and a final extension for 7 min at 72°C. The lengths of the amplicons generated were approximately 528 bp for the 16S rRNA gene, 555 bp for *cpn60*, and 188 bp for *wecE*. An aliquot (5 µl) of each amplification reaction product was electrophoresed in a 1.5% (wt/vol) agarose gel containing ethidium bromide and $1 \times$ Tris-acetate buffer (pH 8). DNA bands were visualized under UV light. Amplicons were purified with the QIAquick PCR purification kit (QIAGEN Inc., Ontario, Canada) according to the manufacturer's instructions before being labeled.

Oligonucleotide probe design. 16S rRNA and *cpn60* sequences of different pathogenic strains were compiled from GenBank, the ARB database (27), the Ribosomal Database Project (11), and the *cpn60* database (19). Specific 16S rRNA and *cpn60* oligonucleotides were designed using OligoPicker software (39). Initial design criteria were as follows: length between 18 and 26 bases, a maximum of no more than 11 continuous matches between a probe and nontarget species, no more than six repetitive bases, and a minimum melting temper-

TABLE 2. Probes

Probe name according to reference 1	Sequence	Target species	Accession no. of reference file	Position of probe in reference file
S-G-Salm-0455-a-A-24	TTG CTG CGG TTA TTA ACC ACA ACA	<i>Salmonella</i> spp.	Z47544	471–448
S-S-E.coli-0462-a-A-23	CGT CAA TGA GCA AAG GTA TTA AC	<i>E. coli</i> , <i>Shigella</i> spp.	J01859	483–461
S-S-E.coli-0453-a-A-25	GAG CAA AGG TAT TAA CTT TAC TCC C	<i>E. coli</i> , <i>Shigella</i> spp.	J01859	476–452
S-S-Y.ente-0452-a-A-25	CAC AAA GGT TAT TAA CCT TTA TGC C	<i>Y. enterocolitica</i>	Z75316	450–426
S-G-Yers-0079-a-A-22	CGC CGG CAA AGT AGT AAA CTA C	<i>Yersinia</i> spp.	Z75316	73–52
S-G-Vibr-0154-a-A-22	GTA TTA GCC ATC GTT TCC AAT G	<i>Vibrio</i> spp.	X76337	168–147
S-F-Ente-0383-a-S-22	AGC CTG ATG CAG CCG TAT GCA G	<i>Enterobacteriaceae</i>	U92194	382–403
S-G-Salm-0467-a-S-22	TAC CGC AGC TAA TTG ACG TTA C	<i>Salmonella</i> spp.	U92195	466–487
S-Ss-S.parA-0222-a-S-22	ATC ACA TGT GCC CAG ATG CCA T	<i>Salmonella</i> serovar Paratyphi A	U88546	222–243
S-Ss-S.parC-1006-a-S-22	CTT TCC AGA GAT GAG TTT GTG C	<i>Salmonella</i> serovar Paratyphi C	U88548	1005–1026
S-Ss-S.entH-0994-a-S-22	ACA TCC ACG GGA AGT TTT CAG A	<i>Salmonella</i> serovar Heidelberg	AF276989	966–987
S-Ss-S.entA-0076-a-S-22	GAA GCA GCT TGC ACG TAG CTG A	<i>Salmonella</i> serovar Agona	U92197	76–97
S-Ss-S.entE-1015-a-S-22	GAT CCA TTT GTG CCT TAG GGA A	<i>Salmonella</i> serovar Enteritidis	U90318	1014–1035
S-Ss-S.entT-1088-a-S-22	GGT TAA GTC CCG CTA CGA TCG C	<i>Salmonella</i> serovar Thompson C1	AF057363	1092–1113
S-D-Bact-0338-a-A-18	GCT GCC TCC CGT AGG AGT	Domain <i>Bacteria</i>		
C-S-A.thal-0896-a-A-25 ^a	AAC AAC AAC ATC TGG CGT AAG AGT G	<i>Arabidopsis thaliana</i>	AY042821	920–896
C-S-A.thal-0871-a-A-25 ^a	CCA AAC AAT GCT TGG CCA GCC CAC C	<i>Arabidopsis thaliana</i>	AY042821	895–871
C-S-E.coli-0369-a-A-22	TTC TTC AAC TGC AGC GGT AAC A	<i>E. coli</i>	X07850	860–840
C-S-Y.ente-0470-a-A-21	GTG CAA TCA GCT CAC CCA CAG	<i>Y. enterocolitica</i>	X68526	490–470
C-S-Y.ente-0623-a-A-23	AAG TTC AAT AGA ACC GGT TTC TG	<i>Y. enterocolitica</i>	X82212	645–623
C-S-V.chol-0447-a-A-21	GAC GAG AGG CTT TCG CTA CGC	<i>Vibrio cholerae</i>	AF230940	467–447
C-S-V.chol-0464-a-A-24	GCC CAC GCT AGA GTC TGA GTT AGC	<i>V. cholerae</i> , <i>V. mimicus</i>	AF230940	205–182
C-Ss-S.Typh-0366-a-A-22	TTC AAC CGC CGC AGC AAC CGC T	<i>Salmonella</i> serovar Typhimurium	AB033231	886–865
W-Ss-Salm-0588-a-S-22	TCG TAC GCT AAT TGA ACG CGC G	<i>Salmonella</i> serovar Typhimurium	AE008883	8741–8762
W-Ss-Salm-0497-a-S-22	TAG GGA CGA TCG GTC ACA TCG G	<i>Salmonella</i> serovar Typhimurium	AE008883	8650–8671
W-S-E.coli-0497-a-S-22	TGG GAA CCA TTG GTC ATA TTG G	<i>E. coli</i> , <i>Shigella</i> spp.	M87049	28762–28783
W-G-Kleb-unk.-a-S-20	ATC GCA CGC TGG TGG AAC GC	<i>Klebsiella</i> spp.	Unpublished	
W-G-Serr-unk.-a-S-20	ATC CGG CCC TGA TCG ATC GG	<i>Serratia</i> spp.	Unpublished	
W-G-Yers-unk.-a-S-25	CAC TAT CGG CCA TAT TGG TTG CTT T	<i>Yersinia</i> spp.	Unpublished	
W-G-Yers-unk.-a-S-23	GCT TAT CAA TGA CCC GTC ACT GA	<i>Yersinia</i> spp.	Unpublished	
W-S-Y.pest-0517-a-S-23	TCT GGG TAC CAT TGG CCA TAT TG	<i>Yersinia pestis</i>	AJ414159.1	81913–81891
W-G-Yers-unk.-b-S-23	CAC TAT CGG CCA TAT TGG TTG CT	<i>Yersinia</i> spp.	Unpublished	
W-G-Entb-unk.-a-S-21	TCG TGC GCT GGT TGA GCG TGC	<i>Enterobacter</i> spp.	Unpublished	
W-F-Ente-0523-a-S-21	TTT AGC TTC CAT GAA ACC AAA	<i>Enterobacteriaceae</i>	AL627279	22787–22767
W-F-Ente-0526-a-S-20	AGC TTC CAT GAA ACC AAA AA	<i>Enterobacteriaceae</i>	AL627279	22784–22765
W-G-Prot-unk.-a-S-26	CAT CTA TTT TTA CCC TTA TGA AGG GC	<i>Proteus</i> spp.	Unpublished	
W-G-Prov-unk.-a-S-24	CGC TTT ATA GGA CGC GAA TTG TAC	<i>Providencia</i> spp.	Unpublished	
W-G-Citr-unk.-a-S-24	AAA ATT AGT CGA ACG TGC AGA GAT	<i>Citrobacter</i> spp.	Unpublished	

^a Negative controls.

ature of 55°C. The *wecE* probes were chosen from an earlier publication (2). Specificity of candidate probes was verified by BLAST searches against GenBank. Multiple DNA alignments used to design generic probes were performed by using the CLUSTALW program (33). Oligonucleotide probes were purchased and synthesized by Integrated DNA Technologies (Coralville, IA). The probe sequences, their sizes, the references used, and the corresponding bacterial indicators are listed in Table 2. For a positive control, the general 16S rRNA probe S-D-Bact-0338-a-A-18 was used to detect the presence of bacteria. Negative controls were composed of two plant-specific *Arabidopsis* oligonucleotide sequences in addition to three printing buffer spots.

Microarray prototype and printing. Our water pathogen microarray prototype contains a combination of short, 18- to 26-mer oligonucleotide probes specific for 16S rRNA (15 probes) and *cpn60* (six probes). Fifteen short *wecE* oligonucleotide probes (~20 bases) were also printed on the prototype to enhance its

discriminating power. The sequences of all printed 18- to 26-mer oligonucleotides are listed in Table 2.

In preparation for printing, lyophilized oligonucleotides were suspended in water to obtain a stock concentration of 100 pmol/μl. These solutions were diluted in dimethyl sulfoxide (50%, vol/vol) to a final concentration of 50 pmol/μl. Ten microliters of each probe was transferred into a 384-well microplate and stored at –20°C until printing onto Corning GAPS II slides (Corning Co., Corning, N.Y.) with a Virtek ChipWriter, using Telechem SMP3 microspotting pins. Each probe was printed in triplicate. Slides were processed through UV cross-linking (600 mJ) followed by heat treatment (80°C overnight) and stored in the dark at room temperature until use. Quality control of the printing was assessed by terminal transferase labeling of the printed material (38). Printed slides showed uniform spot intensity and morphology, with an average spot fluorescence of 54,000 pixels.

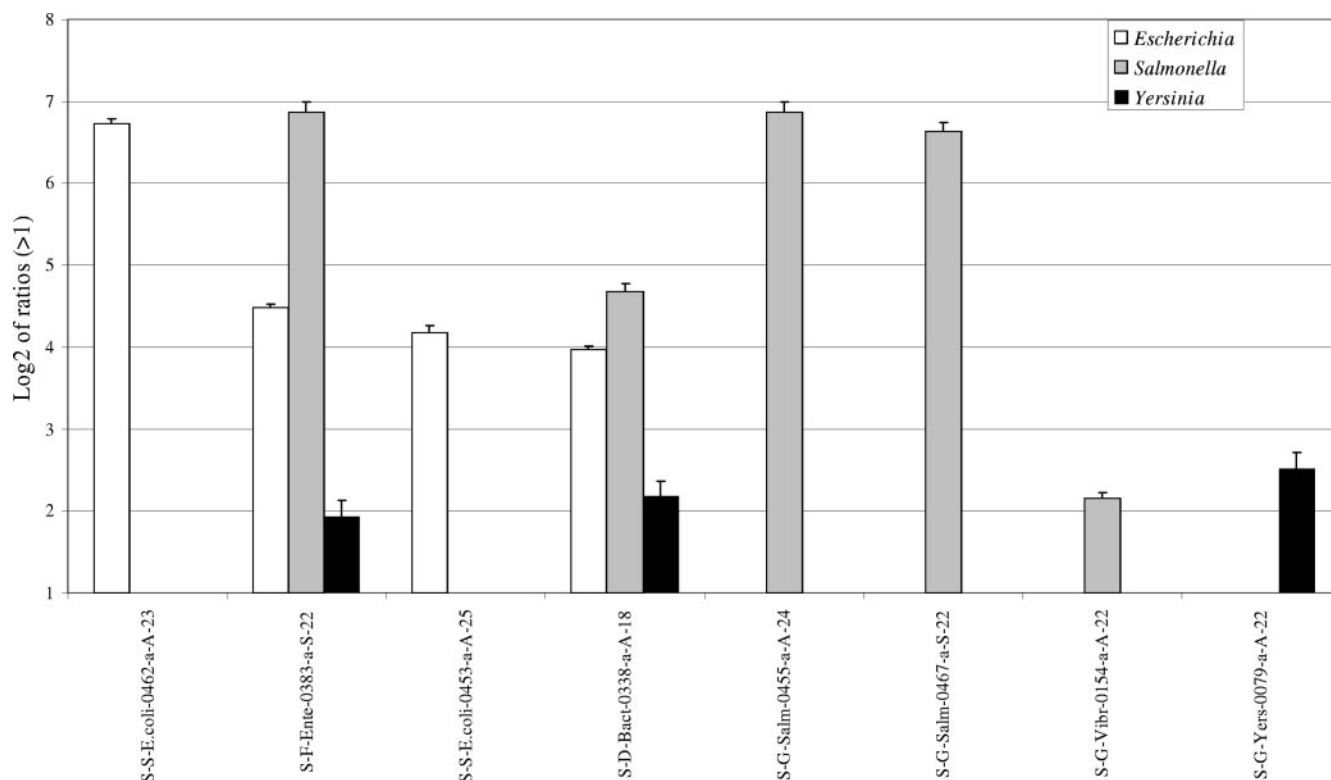


FIG. 1. Hybridization of 16S rRNA fragments amplified from purified genomic *S. enterica* serovar Typhimurium, *E. coli*, and *Y. enterocolitica* DNAs on the prototype microarray. Hybridization of 25 ng of 16S rRNA amplicons from genomic DNAs of *E. coli*, *S. enterica* serovar Typhimurium, and *Y. enterocolitica* is shown. Results are shown as the logarithm (base 2) ratio of the probe's fluorescence intensity relative to control (buffer) spots, after normalization. The error bars represent standard errors, and each result represents the average of six spot intensities derived from two different microarray hybridizations.

Amplicon labeling and hybridization. Two micrograms of purified amplicons was chemically labeled with a Mirus Cy5 Label IT nucleic acid labeling kit (Mirus, Madison, Wis.) according to the manufacturer's instructions. As the labeling reaction was performed in a small volume (30 μ l), a quick spin was performed after 30 min of incubation to minimize evaporation loss. The tubes were incubated for 4 h at 37°C in the dark, after which unreacted reagents were removed using a QIAquick PCR purification kit (QIAGEN Inc., Ontario, Canada).

Microarrays were prehybridized at 37°C for 1 hour with 14 μ l of prewarmed (37°C) DIG Easy Hyb buffer (Hoffmann-La Roche Limited, Ontario, Canada) containing 10 μ g of denatured salmon sperm DNA (Invitrogen Life Technologies, Ontario, Canada) under 22- by 22-mm coverslips (Sigma-Aldrich Canada Ltd., Ontario, Canada). Afterwards, the coverslips were removed by dipping the slides into 0.1 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and the slides dried by centrifugation at room temperature in a 50-ml conical tube for 5 min at 150 \times g.

For detection threshold determination, 1 μ g of labeled amplicons obtained after the amplification of binary or ternary DNA mixtures or total environmental genomic DNA was dried in a Speedvac (Savant model no. SVC200H). For specificity experiments, 25 ng of amplicons obtained from pure genomic DNA was dried in a Speedvac. Dried DNA was resuspended into 10.5 μ l of prewarmed (37°C) DIG Easy Hyb buffer and 1 μ l of salmon sperm DNA. DNA was then chemically denatured and neutralized for 5 min at room temperature as described by the manufacturer (Mirus Cy5 Label IT nucleic acid labeling kit). Microarrays were hybridized with the labeled DNA mix under a coverslip for 4 hours at 37°C. After hybridization, coverslips were removed in 0.1 \times SSC, the microarrays were washed three times in prewarmed (37°C) 0.1 \times SSC-0.1% (vol/vol) sodium dodecyl sulfate for 5 min and one time in 1 \times SSC for 10 min, and the slide was dried by centrifugation (150 \times g, 5 min, room temperature). All hybridizations were done in triplicate.

Data acquisition and analysis. Hybridized arrays were imaged using a fluorescence scanner (ScanArray; Canberra-Packard, Mississauga, Ontario) and ScanArray software version 2.1. Three complete arrays were printed on each slide and could be hybridized simultaneously yet independently using separate

coverslips. This approach minimizes array variation resulting from minor fluctuations in different external parameters such as temperature. Fluorescent spot intensities were quantified using QuantArray software version 3.0 (Canberra-Packard) after normalizing the data by subtracting local background from the recorded spot intensities from arrays on the same slide. The median value for each set of triplicate spotted probes was compared to the median for the buffer spots, and probes that had a signal-to-noise fluorescence ratio of greater than 2.0 (i.e., \log_2 of ratios >1) on replicate arrays were considered positive (26).

RESULTS

PCR amplification sensitivity on pure cultures. The PCR amplification sensitivities for three different taxonomic genes (16S rRNA gene, *cpn60*, and *wecE*) were estimated using different *S. enterica* serovar Typhimurium genomic DNA amounts ranging from 50 ng to 50 fg as PCR templates. The detection thresholds, assessed by visualization of the appropriately sized fragment after electrophoresis in agarose gels, were similar to those reported by other groups (40). Under our standard PCR conditions, the 16S rRNA and *wecE* amplifications gave similar thresholds, where 500 fg was the minimum amount of *S. enterica* serovar Typhimurium DNA template required for detection (data not shown). As 5 fg of genomic DNA represents approximately one *S. enterica* serovar Typhimurium genome, the PCR detection limit for these two targets corresponds to approximately 10^2 bacterial genomes. For the *cpn60* amplification reactions, the detection limit was higher

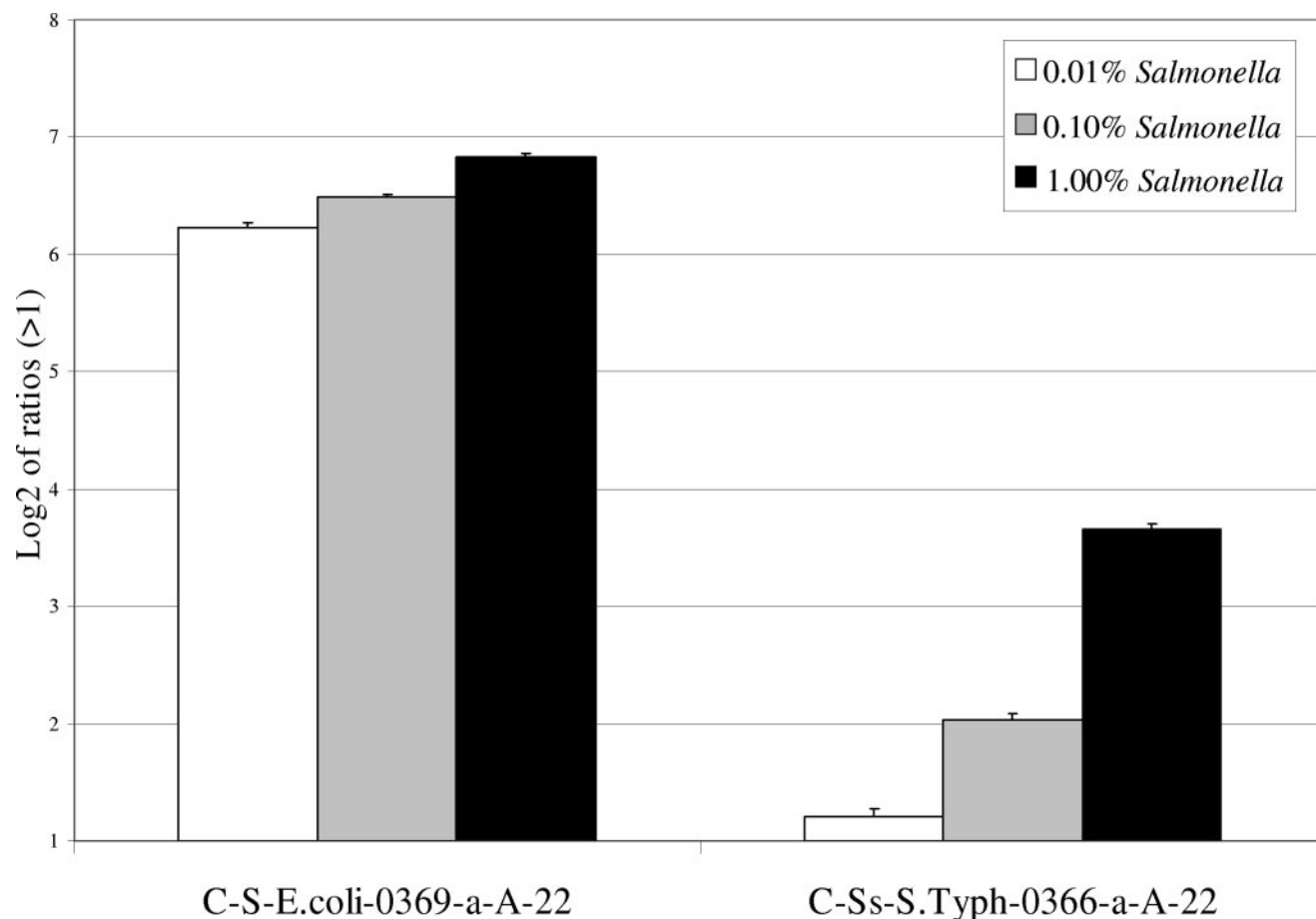


FIG. 2. Hybridization of *cpn60* fragments amplified from different binary (*E. coli*-*Salmonella*) mixtures on the prototype microarray. Hybridization of 1 μ g of *cpn60* fragments amplified from mixtures containing 0.01%, 0.10%, and 1.00% *S. enterica* serovar Typhimurium is shown. Results are shown as the logarithm (base 2) ratio of the probe's fluorescence intensity relative to control (buffer) spots, after normalization. Only two array probes specific for the *cpn60* gene of either *E. coli* or *Salmonella* showed a positive hybridization signal. The error bars represent standard errors, and each result represents the average of six spot intensities derived from two different microarray hybridizations.

than the previous estimate and corresponded to 5 pg or 10^3 genomes (data not shown).

Microarray validation. To evaluate the specificities of the printed oligonucleotide probes, 25 ng of Cy5-labeled 16S rRNA gene, *cpn60*, or *wecE* amplicons, produced from either *E. coli*, *S. enterica* serovar Typhimurium, or *Y. enterocolitica* genomic DNA, was individually hybridized to the water pathogen microarray. Hybridization with 16S rRNA *E. coli* or *Y. enterocolitica* amplicons resulted in positive signals detected with their specific probes as well as the general bacterial S-D-Bact-0338-a-A-18 probe (Fig. 1). Hybridization of the 16S rRNA amplicons from *S. enterica* serovar Typhimurium resulted in positive signals for the S-G-Salm-0455-a-A-24 and S-G-Salm-0467-a-S-22 oligonucleotides, which are homologous to *S. enterica* serovar Typhimurium as well as to a number of other *Salmonella* species (Fig. 1). As expected, a positive signal was again detected with the S-D-Bact-0338-a-A-18 probe. All three 16S rRNA amplicons (*E. coli*, *S. enterica* serovar Typhimurium, and *Y. enterocolitica*) hybridized with the general S-F-Ente-0383-a-S-22 probe. One cross-hybridization was observed with the 16S rRNA probe for *Vibrio* spp. when *Salmonella* amplicons were hybridized (Fig. 1). By con-

trast, testing the microarrays with *cpn60* amplicons gave only the expected signals at a ratio of intensities of >2 , while results for *wecE* also showed the expected signals, with weak cross-hybridization only to heterologous probes (C-Ss-S.Typh-0366-a-A-22 and S-G-Salm-0467-a-S-22) (data not shown).

Detection thresholds in mixed genomic DNA backgrounds. To evaluate the microarray prototype detection limits in a more complex environment, different amounts of pure culture genomic DNA were added to create either binary (*E. coli* and *S. enterica* serovar Typhimurium) or ternary (*E. coli*, *S. enterica* serovar Typhimurium, and *Y. enterocolitica*) genomic DNA mixtures or were added to total DNA extracted from wastewater (Table 1). In wastewater environments, *E. coli* strains are generally predominant in comparison to *Salmonella* spp. or *Yersinia* spp., and heterotrophic plate counts of our samples showed that $1.2 (\pm 0.1) \times 10^6$ CFU/ml were present in the raw wastewater. In the same samples, the *E. coli* concentration was determined to be $1.0 (\pm 0.1) \times 10^4$ CFU/ml when plated on mTEC medium. In order to mimic these conditions, the binary and ternary DNA samples had a large excess of purified *E. coli* genomic DNA. To obtain well-defined hybridization signals,

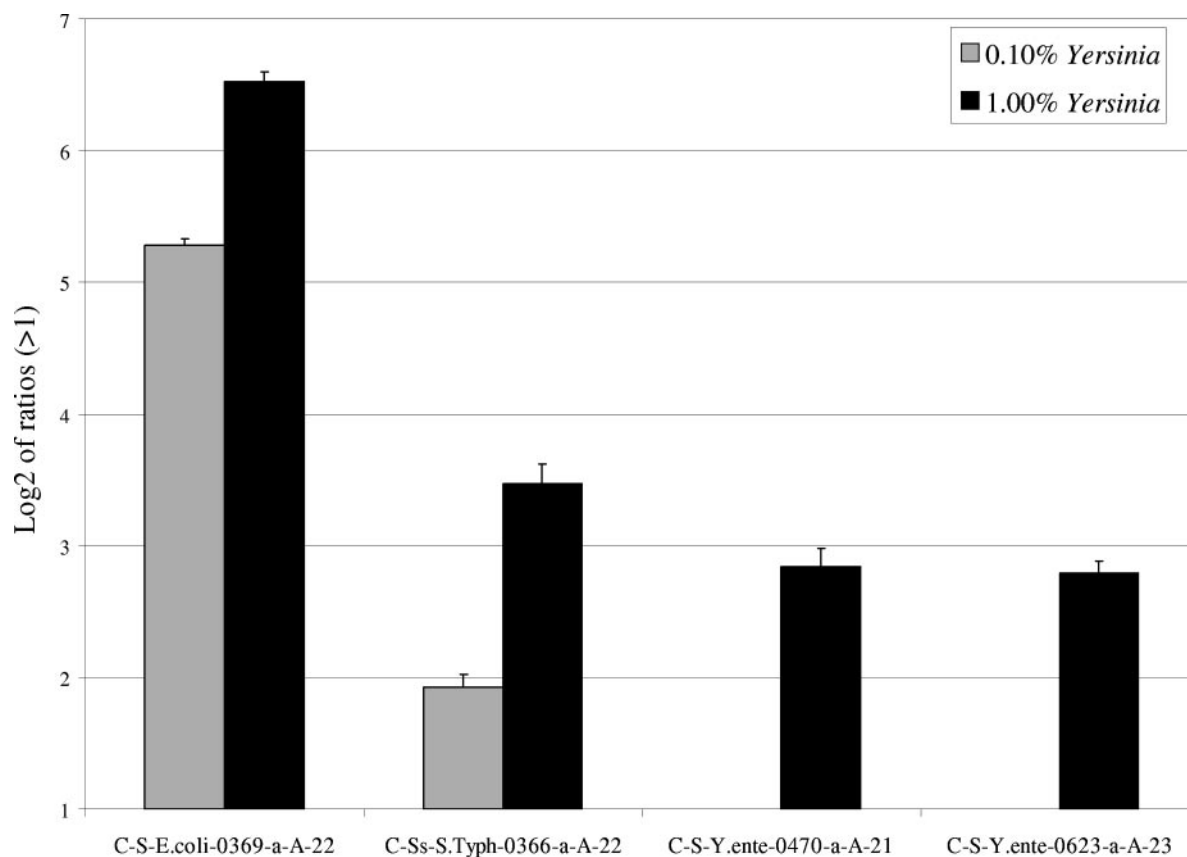


FIG. 3. Hybridizations of *cpn60* fragments amplified from different ternary (*E. coli*-*Salmonella*-*Yersinia*) mixtures on the prototype microarray. Hybridization of 1 μ g of *cpn60* fragments amplified from mixtures of 0.1% *S. enterica* serovar Typhimurium and either 0.1% or 1% *Yersinia enterocolitica* is shown. Results are shown as the logarithm (base 2) ratio of the probe's fluorescence intensity relative to control (buffer) spots, after normalization. The error bars represent standard errors, and each result represents the average of six spot intensities derived from two different microarray hybridizations.

1 μ g of total labeled taxonomic amplicons, generated from each type of DNA mixture, was hybridized on the microarray.

When decreasing amounts of *S. enterica* serovar Typhimurium genomic DNA were added to *E. coli* DNA, amplification and hybridization of total *cpn60* amplicons showed that *S. enterica* serovar Typhimurium was clearly detectable in the 0.1% (50-pg) or 1% (500-pg) *Salmonella* binary mixtures (Fig. 2). A weak signal was obtained with the 0.01% (5-pg) mixture but was <2 in intensity ratio. On the basis that 50 ng of genomic DNA is equivalent to 1×10^7 *S. enterica* serovar Typhimurium cells, the threshold of the method corresponds to the presence of 10^3 to 10^4 specific genomes. The presence of *S. enterica* serovar Typhimurium DNA at levels lower than 0.01% gave negative results. Similar detection limits were obtained for both the 16S rRNA and *wecE* sets of amplicons from the binary mixtures (data not shown).

In the ternary DNA mixtures where *Yersinia* genomic DNA was added at 1.0% to fixed concentrations of *S. enterica* serovar Typhimurium and *E. coli* DNA (Table 1), universal amplification and hybridization of *cpn60* amplicons showed that this gene was specifically detected for all three organisms (Fig. 3). The strongest signals were observed for *E. coli* and *Salmonella* probes that are specific for 99% and 0.1% of the total DNA, respectively. *Yersinia*, present at an equivalent concentration as

S. enterica serovar Typhimurium (0.1%), failed to produce a signal (Fig. 3). If *Yersinia* DNA was added at a 10-fold-higher concentration than *Salmonella*, the signal increased to a level approximating that of *Salmonella* (Fig. 3). In parallel experiments, a similar pattern was observed for *Yersinia* 16S rRNA amplicons but not *wecE*, which had similar intensities as *Salmonella* at similar concentrations (data not shown).

When variable amounts of *S. enterica* serovar Typhimurium DNA were added to a highly complex DNA sample (wastewater-extracted total DNA), hybridizations with either 16S rRNA (Fig. 4A) or *cpn60* (Fig. 4B) amplicons generated from the wastewater mixture produced specific fluorescent signals for the *Salmonella*-specific probes at as low as 50 pg of DNA (0.1% of total added *S. enterica* serovar Typhimurium DNA). No signals were observed for the *Salmonella*-specific probes when hybridizing Cy5-labeled 16S rRNA amplicons generated from the wastewater DNA in non-*Salmonella*-supplemented wastewater DNA. In contrast to the absence of signals observed with *S. enterica* serovar Typhimurium- or *E. coli*-specific probes for the 16S rRNA gene, positive signals for the *Salmonella*-specific *cpn60* and *wecE* probes were observed after hybridization with either total *cpn60* (Fig. 4B) or *wecE* (Fig. 4C) amplicons produced from non-*Salmonella*-supplemented wastewater DNA. In addition to the *Salmonella* results, it was also found that the

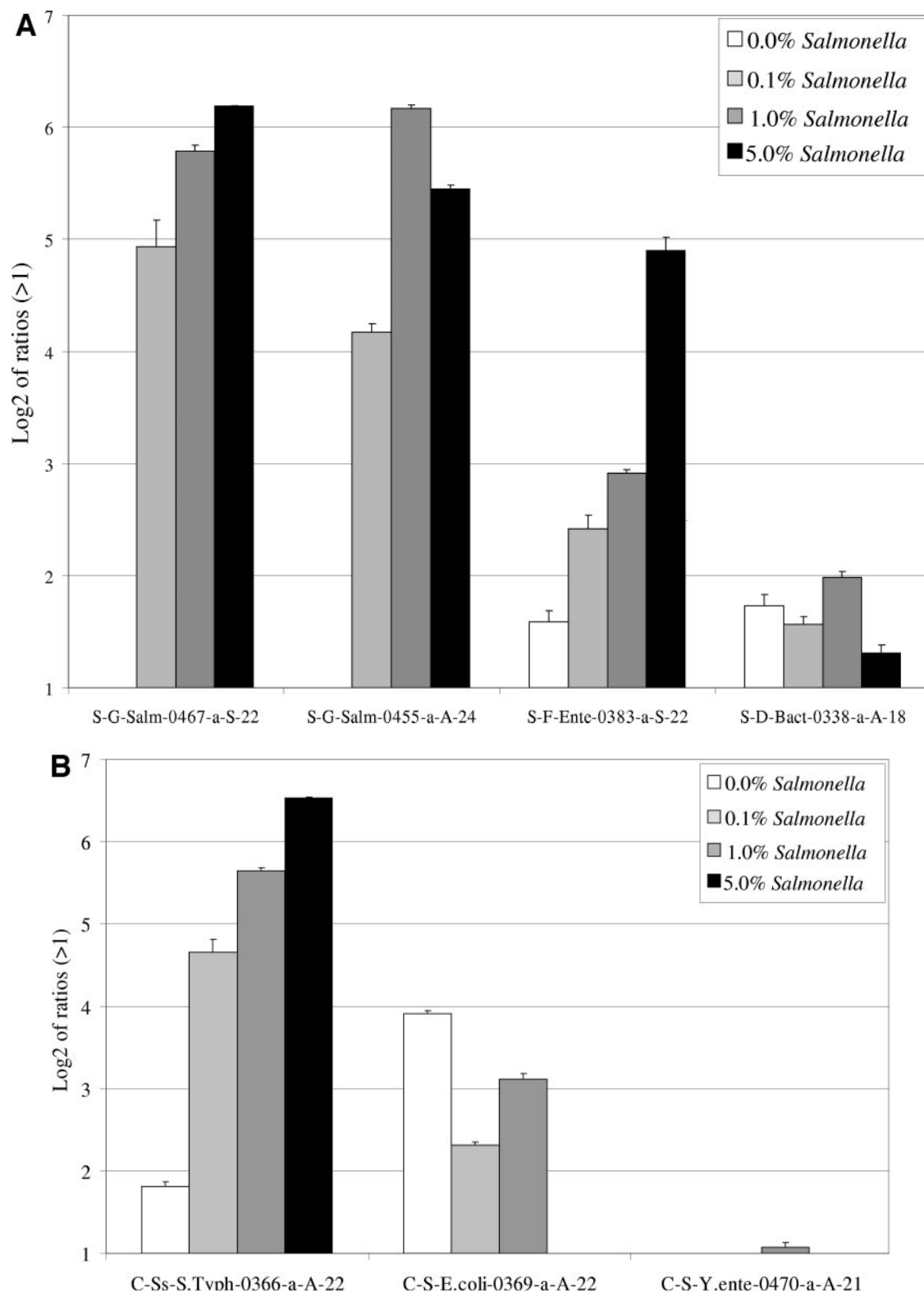


FIG. 4. Hybridization of 16S rRNA, *cpn60*, or *wecE* amplicons generated from different ratios of *S. enterica* serovar Typhimurium DNA in wastewater DNA. Variable amounts of *S. enterica* serovar Typhimurium DNA were added to wastewater DNA (0%, 0.1%, 1.0%, and 5.0% final concentrations). The mixtures were subjected to PCR using universal 16S rRNA (A), *cpn60* (B), or *wecE* (C) primers. One microgram of each amplicon was labeled and separately hybridized to the prototype microarray. Results are shown as the logarithm (base 2) ratio of the probe's fluorescence intensity relative to control (buffer) spots, after normalization. The error bars represent standard errors, and each result represents the average of six spot intensities derived from two different microarray hybridizations.

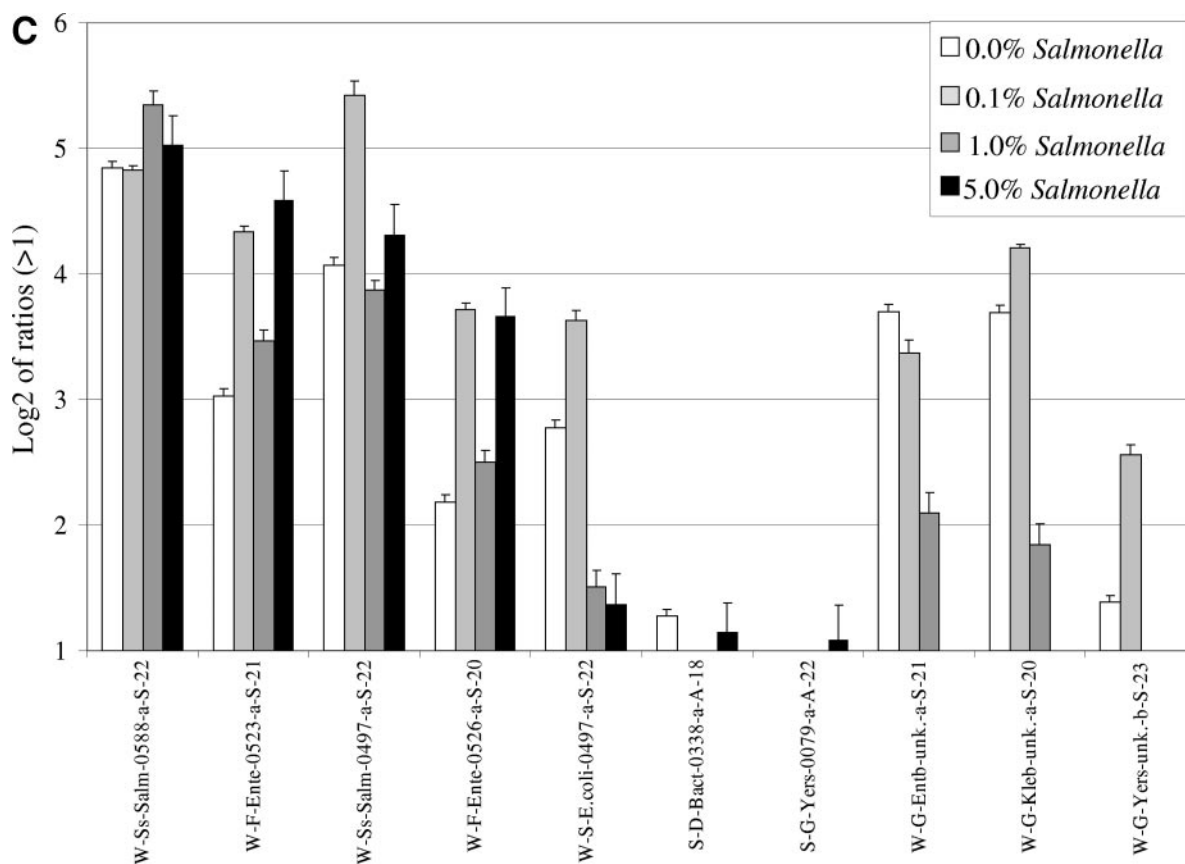


FIG. 4—Continued.

presence of *E. coli* in wastewater samples was detected by the *cpn60* and *wecE* probes in non-*Salmonella*-supplemented DNA. The signal for *Salmonella*-specific *cpn60* was relatively weak compared to the *E. coli* signal (Fig. 4B), whereas the *Salmonella* *wecE* signal was stronger than the *E. coli* *wecE* signal (Fig. 4C).

Hybridization of total *wecE* amplicons derived from non-*Salmonella*-supplemented wastewater DNA produced signals with general *Salmonella* species-specific probes (W-Ss-Salm-0588-a-S-22 and W-Ss-Salm-0497-a-S-22) as well as with the *E. coli*, *Enterobacter*, *Yersinia*, and *Klebsiella* *wecE* probes (Fig. 4C). For the most part, we observed that the signals for *Salmonella* and *Enterobacteriaceae* probes increased with the addition of *S. enterica* serovar Typhimurium DNA. While the signals for *Enterobacter*, *Yersinia*, and *Klebsiella* decreased and disappeared completely at the highest concentration of *S. enterica* serovar Typhimurium DNA, the signal for *E. coli* decreased but never disappeared. A small amount of cross-hybridization, just above the ratio of 2, was also observed with two 16S probes, S-D-Bact-0338-a-A-18 and S-G-Yers-0079-a-A-22.

DISCUSSION

Discrimination between closely related bacterial species or genera is relatively difficult when using a single specific taxonomic gene in PCR-based assays, especially within complex

environmental samples (29). Although incorporating more taxonomic identifiers would circumvent this problem, the increase in cost and labor rapidly becomes unrealistic. In this feasibility study aimed at detecting pathogens in wastewater samples, both points were addressed, first by employing three different taxonomic genes and second by harnessing the parallel processing power of DNA microarrays. With regard to the taxonomic genes, two correspond to universal target sequences (16S rRNA and *cpn60* genes), and the third (*wecE*) belongs to the *wec* cluster specific to the *Enterobacteriaceae* family (2). Inclusion of a nonuniversal target, such as the *wecE* gene, is advantageous when higher sensitivity is desired. Another important factor behind the choice of genes capable of discriminating among different bacterial species is that they can be amplified from a complex genomic DNA mixture by using a single set of universal primers. Hybridization of the individually amplified 16S rRNA gene, *cpn60*, and *wecE* fragments from *E. coli*, *S. enterica* serovar Typhimurium, or *Y. enterocolitica* strains showed that all the *cpn60* and *wecE* oligonucleotide probes printed on the microarray can specifically discriminate between the three bacterial species. The *S. enterica* serovar Typhimurium 16S cross-hybridization that occurred with the S-G-Vibr-0154-a-A-22 probe can be explained by a high percent similarity (87%) between them.

Although PCR amplification remains an important tool for bacterial detection, precise identification of PCR products requires additional measures such as the use of specific beacons.

However, in addition to their cost, the design of beacons for real-time PCR can face difficult design issues with complex samples such as feces (31). Microarrays coupled with PCR can serve as a set of parallel dot blots to enhance microbial detection and identification. Under our PCR conditions, the detection limits of the 16S rRNA and *wecE* PCRs using genomic DNA from a single strain are in the same range (approximately 100 bacterial genomes). Therefore, the sensitivity of the these two reactions is sufficiently high to be applicable for the detection of low levels of bacteria in complex mixtures. For the *cpn60* PCR, the detection limit was unexpectedly higher (10-fold) than those of the two other reactions but remains at acceptable levels.

When complex samples are used as templates for PCR amplification, PCR bias may occur and manifest itself by a non-proportional amplification of the less abundant species (37). To address this concern, hybridization thresholds for a specific species were assessed using variable amounts of a specific genomic DNA in binary, ternary, and complex DNA mixtures. The binary and ternary mixtures were composed mainly of genomic DNA from *E. coli* to mimic conditions encountered in wastewater, where *E. coli* is predominant. By using amplicons produced from binary or ternary genomic DNA mixtures (*S. enterica* serovar Typhimurium and *E. coli* genomic DNAs), we demonstrated that our 16S rRNA gene, *cpn60*, and *wecE* microarray oligonucleotide probes can detect between 10^3 and 10^4 *S. enterica* serovar Typhimurium genomes in a 50-ng DNA sample. The addition of genomic DNA from *Y. enterocolitica* to the mixture to a ratio of 1% does not affect the detection limit of the microarray for *S. enterica* serovar Typhimurium; therefore, detection sensitivity was not affected by the addition of a third DNA component in the mixture. Since the observed thresholds were identical when binary and ternary mixtures were tested, it is reasonable to conclude that little or no PCR bias had occurred under our conditions. However, the sensitivity of the microarray appears to vary slightly depending on the microorganisms tested. The intensities of the 16S rRNA and *cpn60* signals for *Yersinia* DNA were lower than those obtained with *Salmonella* DNA when both bacterial DNAs were added at a ratio of 0.1% in the ternary mixture. The reason for this variation is unknown, since the 16S rRNA and *cpn60* genetic sequences of *Salmonella* and *Yersinia* share similar G/C ratios. Moreover, hybridization conditions favoring *Salmonella* over *Yersinia* amplicons can be eliminated, since the amplicon sizes and the annealing temperatures of the printed probes specific for both bacteria are identical. Using amplicons generated from *Salmonella*-wastewater DNA mixtures, a detection threshold of $>10^4$ *S. enterica* serovar Typhimurium genomes was obtained. This environmental detection limit constitutes only an estimate, since the initial quantity of *S. enterica* serovar Typhimurium present in the original wastewater sample was unknown. Previous culture studies on domestic wastewater treatment have shown that the amount of *S. enterica* serovar Typhimurium cells is generally two orders of magnitude lower than that of *E. coli* (18).

An unexpected result occurred in our *wecE* amplicon hybridizations in that the intensities of some non-*Salmonella* hybridization signals (W-G-Kleb-unk.-a-S-20 and W-G-Entb-unk.-a-S-21 probes) decreased when the ratio of *S. enterica* serovar Typhimurium DNA in the mixture was increased (Fig. 4C).

This observation might be explained by PCR amplification bias, where the PCR seems to favor amplification of the most abundant bacterial species to the detriment of the less abundant ones. This observation can be problematic if a bacterial species is predominant in an environmental mixture in comparison to others. A weaker or absent PCR amplification might be obtained for the less abundant bacteria, and therefore, the detection threshold of the microarray for these bacteria might be affected.

Of the three taxonomic genes used in this study, only *wecE* hybridization signals were observed for *Salmonella* when the genes were amplified from wastewater DNA not spiked with *S. enterica* serovar Typhimurium DNA. Thus, the detection sensitivity for the *wecE* amplicons is higher than that for either the 16S rRNA or *cpn60* gene. This increased sensitivity could be due to at least three factors. First, since the size of the *wecE* amplicons (188 bp) is 2.8-fold lower than that of either the 16S rRNA (528 bp) or *cpn60* (555 bp) amplicons, using 1 μ g of labeled *wecE* amplicons would represent a higher number of molecules hybridized on a molar basis. Second, the lower detection limit of the *wecE* amplicons can also be explained by the fact that *wecE* amplification is specific for one eubacterial family (*Enterobacteriaceae*), in contrast to the ubiquitous 16S rRNA or *cpn60* gene. Therefore in community DNA, the diversity of *wecE* would be lower than that of either 16S rRNA or *cpn60*, resulting in a higher relative abundance. Finally, the *wecE* probe (Table 2) was designed to detect several species of *Salmonella* other than *S. enterica* serovar Typhimurium. Since the *wecE* primers amplified the *wecE* genes for only a limited diversity of microorganisms, they might favor the detection of more specific bacteria than with the 16S rRNA or *cpn60* primers, which would amplify the appropriate gene from all bacteria present in the wastewater sample. Thus, to increase the sensitivity of the prototype for wastewater pathogens, it may be advantageous to target amplicons generated from PCRs targeting a limited group of bacteria instead of universal PCRs specific for all bacteria.

In summary, we have designed a specific and sensitive microarray that can be utilized for the detection of several bacterial species in wastewater samples. Amplification and fluorescent labeling of the 16S rRNA, *cpn60*, and *wecE* genes from extracted community DNA show specific detection of each of the microorganisms studied when hybridized to oligonucleotide probes printed on the wastewater prototype microarray. Although sensitivity may vary depending on the microorganisms tested, detection sensitivity can be increased by targeting amplicons specific for a limited group of bacteria instead of universal taxonomic amplicons from a broad spectrum of bacteria.

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