

Microbial diagnostic microarray for food- and water-borne pathogens

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Summary

A microbial diagnostic microarray for the detection of the most relevant bacterial food- and water-borne pathogens and indicator organisms was developed and thoroughly validated. The microarray platform based on sequence-specific end labelling of oligonucleotides and the phylogenetically robust *gyrB* marker gene allowed a highly specific (resolution on genus/species level) and sensitive (0.1% relative and 10⁴ cfu absolute detection sensitivity) detection of the target pathogens. Validation was performed using a set of reference strains and a set of spiked environmental samples. Reliability of the obtained data was additionally verified by independent analysis of the samples via fluorescence *in situ* hybridization (FISH) and conventional microbiological reference methods. The applicability of this diagnostic system for food analysis was demonstrated through extensive validation using artificially and naturally contaminated spiked food samples. The microarray-based pathogen detection was compared with the corresponding microbiological reference methods (performed according to the ISO norm). Microarray results revealed high consistency with the reference microbiological data.

Introduction

Pathogens pose a significant threat to human, animal and agricultural health (Call *et al.*, 2003). The acute need for

reliable detection tools is further enhanced by the ever-increasing threat of biological weapons. Methods for pathogen detection need to allow rapid, reliable and sensitive analysis of a broad spectrum of targeted (micro)organisms from mostly complex environmental matrices (Hashsham *et al.*, 2004).

The feasibility of using microarrays for pathogen detection in food and environmental samples has been discussed repeatedly (Call *et al.*, 2003; Hashsham *et al.*, 2004; Lemarchand *et al.*, 2004; Sergeev *et al.*, 2004; Yoo *et al.*, 2004; Maynard *et al.*, 2005; Kostrzynska and Bachand, 2006). Microarray technology offers several advantages in comparison to conventional microbiological culture-based techniques and other molecular methods, including the possibility of parallel, specific and rapid detection of many different organisms in one single assay (Kostrzynska and Bachand, 2006). However, some major challenges, primarily those related to sensitivity and quantification potential during environmental scale application, need to be addressed (Zhou, 2003).

The sensitivity of microbial diagnostic microarrays (MDMs) can be defined as the lowest amount of nucleic acid (cells) needed for successful detection (absolute sensitivity) or as the ratio between targeted and non-targeted organisms (relative sensitivity). Relative sensitivity is the parameter most frequently limiting the applicability of MDMs. The reported detection threshold generally lies in the range of 1–5% (Bodrossy *et al.*, 2003; Deneff *et al.*, 2003; Tiquia *et al.*, 2004), which is not sufficient for reliable pathogen detection. In order to increase sensitivity and specificity of pathogen detection, microarrays that rely on species- or genus-specific PCR amplification have been developed (Sergeev *et al.*, 2004; Lee and Chao, 2005; Maynard *et al.*, 2005). However, these microarrays are limited to the detection of a narrow range of pathogens.

Previously, we reported on the development and optimization of an MDM approach that was characterized by both high specificity and high sensitivity (Kostić *et al.*, 2007). This approach was based on the combination of: (i) unique labelling method (SSELO, sequence-specific end labelling of the oligonucleotides), (ii) a novel concept of competitive oligonucleotides and (iii) the use of a house-keeping gene with a robust phylogenetic resolution at the species level (*gyrB*). Following these established and validated criteria we have developed a diagnostic microarray

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for the detection of the most relevant bacterial food- and water-borne pathogens as well as indicator organisms.

Results

Probe design and validation

The extended probe set (63 oligonucleotides targeting *gyrB* gene of 24 most common food- and water-borne pathogens and indicator organisms at the species and/or genus level) was validated using pure cultures of the reference strains. For microarray validation each strain was hybridized separately onto the microarray. All labelling reactions were performed using a mixture containing all reverse complement (RC) and competitive (CO) oligonucleotides (Appendices S1 and S2), therefore eliminating all predicted false positive results. This validation revealed only one false positive signal remaining on the array (Cam_2221 probe giving signal with *Enterococcus faecalis* target; weighted mismatch value 2.4). Design and experimental validation of the additional CO oligonucleotides targeting this probe–target pair are planned before further application of the microarray. The summary of validation data is presented in Fig. 1.

Interesting findings were made regarding previously developed *V. cholerae*-specific probes (Vch_1776, Vch_1795 and Vch_1839; Kostić *et al.*, 2007). Hybridization with DNA of the closely related *V. fluvialis* Sas27 and *V. mimicus* Sas23 isolates yielded a positive signal with three and two of these probes respectively. Subsequent comparative phylogenetic analysis of *gyrB* sequences of these species and Vch probes revealed that these probes were in fact also perfect-match probes for *V. fluvialis* and *V. mimicus* species. Detailed analysis of the *gyrB* alignment of these two strains revealed that there was not a single position left where a discriminating probe could be designed following previously described criteria (3' terminal cytosine residue).

Assay sensitivity and specificity

In order to ascertain absolute sensitivity (lowest number of detectable cells) of the newly developed microarray an overnight culture of *Salmonella* spp. was serially diluted and plated onto TSA-Y agar [from 4.7×10^8 colony-forming units (cfu) ml⁻¹ down to 0.47 cfu ml⁻¹]. Our results showed that the absolute sensitivity of the microarray is approximately 10⁴ cfu. In addition, relative sensitivity (lowest abundance of targeted pathogen in non-targeted background) of the microarray was tested by artificial contamination of water samples. The latter were prepared and analysed in parallel using microarray and fluorescence *in situ* hybridization (FISH) analysis. Detailed description of the spiked samples and an overview of the

results are summarized in Table 1. Samples A to C harbouring only *Salmonella* spp. were used as a positive control and for initial method optimization. Relative sensitivity of the microarray detection was demonstrated by the analysis of the spiked samples F to H. These samples harboured low amounts of targeted bacteria (*Escherichia coli*) spiked in a high amount of non-targeted bacteria (*Burkholderia phytofirmans* strain PsJN). As predicted, and confirmed by FISH, the relative abundance of *E. coli* in these samples was in the range of 0.04–0.4% (Table 1 – shaded values). Microarray analysis of these samples exhibited clearly detectable signals for all four *E. coli*-specific probes (Fig. 2A).

Reliability of parallel detection was shown with spiked samples D and E. All four pathogens that were spiked in the sample were successfully detected by the microarray (Fig. 2B). Quantification of relative abundance of each bacterium was done via FISH by performing four independent hybridizations for each sample always using a strain-specific probe labelled with Cy3 and EUB338 probe mix labelled with fluorescein (Table S4). In general, all strain-specific probes exhibited significantly weaker signals than the universal EUB338 probe mix. *Staphylococcus aureus* was not detectable using a specific probe (Sau), probably due to the too strong fixation (4% paraformaldehyde (PFA) solution, fixing agent for Gram-negative cells, was used on all filters; Gram-positive *S. aureus* would require ethanol as fixing agent). However, summarized relative abundances of the other three pathogens (established by using Daime software) corresponded to 90% of the total biovolume (represented by EUB338 stained cells).

Application of pathogen array for the analysis of food samples

In order to demonstrate the applicability of this microarray system for the detection and identification of food-borne pathogens, an extensive validation was performed using artificially and naturally contaminated food samples. Microarray results (summarized in Table 2) showed a high degree of reproducibility between replicate spike sets as well as good correlation to the results obtained by conventional microbiological analysis (data not shown). *Salmonella* spp. and *Listeria monocytogenes* were detectable at the level of 5 cfu per 25 g food after specific enrichment (RVS and Fraser respectively). The detection limit for *Yersinia enterocolitica* was found to be > 10 cfu per 25 g food (inoculum of 10 cfu per 25 g food could not be detected; inoculum of 30 cfu per 25 g food was clearly detectable). The presence of *Campylobacter* spp. in the spiked samples was confirmed by three probes (Cam_1556, Cam_2027 and Cam_2221). Ten cfu per 25 g food gave unambiguous positive signals on the microarray; lower amounts were not tested. Furthermore,

Table 1. Summary of the water spiking experiment.

Spiked sample	A	B	C	D	E	F	G	H
<i>S. enterica</i> DSM 17058	0.1	0.2	0.4	0.1	0.4			
	100	100	100	27,43	27,43			
<i>E. coli</i> DSM 5313	100	100	Not analysed	37,5	Not analysed			
				0.1	0.4	0.03	0.1	0.3
<i>S. aureus</i> ATCC 6538				32,44	32,44	0.04	0.13	0.38
				25,8	Not analysed	0.04	0.17	0.29
<i>P. aeruginosa</i> DSM 50071				0.1	0.4			
				7,54	7,54			
<i>B. phytofirmans</i> strain PsJN DSM 10436				Not detectable	Not analysed			
				0.1	0.4			
				32,58	32,58	1,0	1,0	1,0
				26,5	Not analysed	99,96	99,87	99,62
						99,97	99,83	99,71

a. Samples A to E were spiked with indicated volume of undiluted and samples F to H with indicated volume of 1:100 diluted overnight cultures (ONCs).

b. Values predicted by taking into consideration spiked volume and OD₆₀₀ of the ONCs.

c. Relative abundance was established by counting the probe- and DAPI-stained cells (samples A to C) or by using Daime software (samples D and F to H).

Samples A to H were spiked with one or more pathogens as indicated in the table. All samples were analysed using the microarrays and following the standard protocols as described in *Experimental procedures*. Several samples were re-analysed using FISH in order to confirm the results.

Campylobacter jejuni-specific probe Cje_2000 enabled unambiguous differentiation of *C. jejuni* and *C. coli* on the microarray.

Spiking of food matrix with multiple pathogens

In order to further test the application potential of the microarray for food analysis and to demonstrate the advantages of its multiplexing feature, a minced meat (pork and beef mix) matrix was spiked in parallel with *S. Typhimurium* DSM554, *L. monocytogenes* SLCC 2755 and *Y. enterocolitica* NCTC10460. Minced meat is a very complex and challenging matrix containing a native bacterial flora comprising, among others, *Proteus* sp., *Aeromonas* sp., *Citrobacter* sp. and *Y. enterocolitica* (as determined by microarray analysis of non-spiked samples after pre-enrichment in buffered peptone water; data not shown). Spiked meat samples were submitted to universal pre-enrichment (BPW for 18 h at 37°C) followed by selective enrichment (RVS, Fraser and ITC broths for *Salmonella enterica*, *L. monocytogenes* and *Y. enterocolitica* respectively; for 48 h at 37°C or 25°C). *Salmonella enterica* was the only spiked microorganism that could be detected already after buffered peptone water pre-enrichment. This was true for both microarray and classical microbiological (selective agar plates) analysis. Accordingly, *Salmonella* spp. specific signals could also be obtained from RVS enrichment. *Listeria monocytogenes* could only be detected after selective enrichment in Fraser broth. Correspondingly to the results obtained in the single spiking experiments, the detection limit for both *Salmonella* spp. and *L. monocytogenes* was 1–10 cfu per 25 g food. Even though *Y. enterocolitica* was present in the matrix as part of the native matrix flora it was suppressed below detectable levels during the course of biological enrichment. Both microbiological and microarray results for *Yersinia* spp. were negative.

Discussion

An ideal microbial diagnostic tool should allow sensitive, specific, reliable and parallel detection of the targeted microorganisms. All these criteria were taken into consideration during the development of the MDM for the detection of food- and water-borne pathogens and were thoroughly validated.

Detection sensitivity can be defined in two different ways. Relative sensitivity defines the lowest detectable abundance of targeted organism in a non-targeted background. Traditional MDMs, based on short-oligonucleotide probes, have a reported relative sensitivity in the range 1–5% of the total microbial community analysed (Bodrossy *et al.*, 2003). We were able to demonstrate that our microarray approach based on an

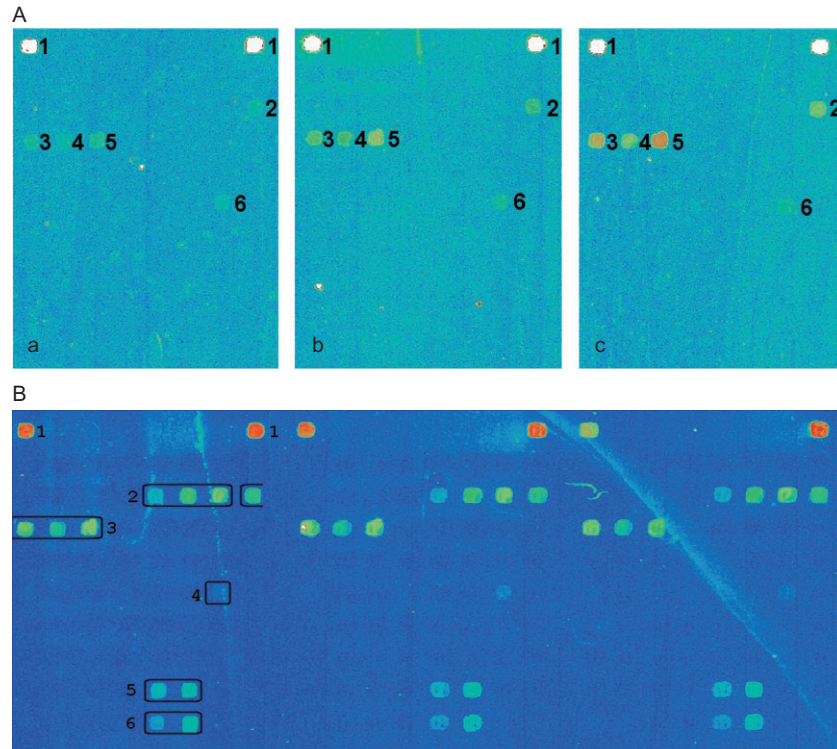


Fig. 2. A. Relative sensitivity of microarray detection. Microarray images showing hybridization results from spiked samples F to H (containing *Escherichia coli* DSM 5313 in *Burkholderia phytofirmans* strain PsJN DSM 10436; Table 2). Relative abundance of *E. coli* in the samples was 0.04% (a), 0.13% (b) and 0.38% (c). Images were scanned at 100% laser power, 1000 V PMT and are displayed in rainbow colour mode. Setting for both brightness and contrast is 50%. Only one of the three replicate subarrays per microarray is shown. 1, internal control Msi_294; 2, Eco_1402; 3, Eco_1404; 4, Eco_1472; 5, Eco_1521; 6, Vmimi_1684. Even though the normalized values of the *E. coli*-specific probes were below the cut-off value of 10% (of the control signal, Msi_294), hybridization profile undoubtedly indicated *E. coli*. Weak, but clear signal was obtained with all four *E. coli*-specific probes (2–5). Very weak cross-hybridization was also observed with one of the *Vibrio mimicus*-specific probes (6). However, this does not endanger high specificity of the detection since this signal is also far below cut-off value (normalized signal value approx 0.4 to 0.7) and all other (three) *V. mimicus*-specific probes as well as *Vibrio* spp. specific probe are clearly negative. These hybridization results also demonstrate how the multiple probe concept enables clear differentiation between weak signals representing low abundance pathogens and weak cross-hybridization biases.

B. Reliability of parallel detection. Microarray image showing hybridization results from spiked sample E (Table 2). Image was scanned at 100% laser power, 750 V PMT and is displayed in rainbow colour mode. Setting for both brightness and contrast is 50%. All three subarrays are shown. 1, internal control Msi_294; 2, *Salmonella* spp. specific probes (SaL_1451, SaL_1457, SaL_1950); 3, *Escherichia* spp. and *Shigella* spp. specific probes (Eco_1402, Eco_1404, Eco_1472, Eco_1521); 4, *Vibrio mimicus*-specific probe (Vmimi_1684); 5, *Pseudomonas aeruginosa*-specific probes (Paero_1207, Paero_1397); 6, *Staphylococcus aureus*-specific probes (Saur_2106, Saur_2320). These results clearly demonstrate the potential for reliable parallel detection of many different pathogens. A weak cross-hybridization observed with Vib_1684 probe (4) is not affecting detection specificity since the normalized value of this signal (1.0) is far below 10% cut-off limit, and furthermore, all other *V. mimicus*-specific probes (for reference see hybridization profile presented in Fig. 1) are clearly negative.

SSELO has a relative sensitivity in the range of 0.1%. These findings were initially obtained using mixed gDNA samples (Kostić *et al.*, 2007) and now confirmed by using spiked water samples. These samples confronted us with all the challenges to be expected in the intended downstream application (i.e. food and water quality monitoring). Thus, we were able to test the overall, true sensitivity of the analytical workflow (starting from sample preparation and ending with microarray analysis). Furthermore, we were able to confirm the obtained results by FISH.

The absolute sensitivity of the method (lowest number of detectable cells) was found to be approximately 10^4 cells. The main limiting factor was found to be the efficiency of the *gyrB* PCR amplification (decrease of three

log steps in comparison to standard 16S PCR amplification). This difference could explain the higher microarray sensitivity values, which have been reported for 16S based microbial diagnostic systems (Lee *et al.*, 2008). By improving PCR efficiency the overall sensitivity of the system can be further improved.

Despite the fact that the efficiency of the *gyrB* PCR needs to be improved to achieve higher sensitivity, the use of *gyrB* as a phylogenetic marker allowed the design of highly specific probes (e.g. in comparison to the 16S rRNA gene), resulting in unambiguous identification of closely related species belonging to the same genus. This high level of resolution was further enhanced through the use of short-oligonucleotide probes and competitive oli-

Table 2. Summary of the food spiking experiments.

Pathogen	Enrichment	Spike level	Microarray	Comments ^a
<i>L. monocytogenes</i>	Half Fraser bouillon (24 h at 30°C)	1–10	Negative	4 sets of samples
		10–100	Negative	3 different <i>L. monocytogenes</i> strains (NCTC 5105, SLCC 2755, NCTC 2945)
	Half Fraser bouillon (24 h at 30°C) + Fraser bouillon (24 h at 37°C)	1–10	Positive	2 food matrices – cheese & pâté
		10–100	Positive	
		100–1000	Positive	
<i>S. enterica</i>	buffered peptone water (18 h at 37°C) + RVS broth (24 h at 37°C)	1–10	Positive	3 sets of samples
		10–100	Positive	<i>S. Typhimurium</i> DSM 544 and <i>S. Enteritidis</i> DSM 9898
		100–1000	Positive	2 food matrices – egg & chicken
<i>C. coli</i> & <i>C. jejuni</i>	Bolton broth (48 h at 42°C; microaerophil)	1–10	Positive	2 sets of samples
		10–100	Positive	<i>C. coli</i> DSM 4689 and <i>C. jejuni</i> DSM 4688
		100–1000	Positive	2 food matrices – chicken & pork
<i>Y. enterocolitica</i>	ITC broth (48 h at 25°C)	1–10	Negative	2 sets of samples
		10–100	Positive	<i>Y. enterocolitica</i> NCTC 10460 1 food matrix – pork

a. DSMZ, German collection of Microorganisms and Cell Cultures; NCTC, National Collection of Type Cultures, London, UK; SLCC, Special Listeria Culture Collection, Würzburg, Germany [currently available at University College Cork (UCC), Environmental Research Institute, Cork, Ireland].

gonucleotides. However, specificity of probe design is directly influenced by the quality and extent of the sequence database. The comparison of available sequence data revealed a handicap that alternative markers have in comparison to the 16S rRNA gene (e.g. in July 2009 NCBI database (<http://www.ncbi.nlm.nih.gov>) contained more than 1.2 million 16S rRNA gene sequences versus approximately 14 000 *gyrB* sequences; at the time when probes were designed the number of available *gyrB* sequences was around 6000). The effect that the limited coverage of *gyrB* sequence database had on the specificity of the *in silico* probe design was revealed in the case of *V. cholera* probes, which were primarily assigned as *V. cholera* specific but finally proved to be *V. cholerae*, *V. mimicus* and *V. fluvialis* specific. Nevertheless, after update of the sequence database we were able to design specific probes for these species. In contrast, when examined in more detail (by performing BLAST search; <http://www.ncbi.nlm.nih.gov/blast>), some of the published *V. cholera*-specific probes targeting 16S rRNA proved to be less specific than claimed (e.g. by being perfect match with *V. mimicus* sequences). These findings demonstrate that a thorough 'wet-lab' validation with an extensive set of reference strains is still highly important and indispensable for the development of any new diagnostic tools.

There is an increasing demand for diagnostic methods with higher phylogenetical resolution (i.e. robust discrimination at the species level), which will necessitate a retreat from solely 16S rRNA gene-based diagnostic systems. In this case the utilization of alternative universal phylogenetic markers will be required, as also suggested by Santos and Ochman (2004). Universal markers are still

advantageous in comparison to species-specific markers, since the latter impose the need for the development and optimization of multiplex PCR systems, which frequently result in biased amplification efficiency (You *et al.*, 2008). The use of the *gyrB* based microarray allowed differentiation of *Vibrio* spp. similar to that published by Kong and colleagues (2009). However, our system was based on a single PCR amplification in comparison to the use of 12 primer pairs in a multiplex PCR system. Such complex systems are preferable in applications where improved differentiation potential is required (e.g. genotyping of pathogenic *E. coli* O157:H7 strains; Call *et al.*, 2001)

The most important advantage of microarray-based diagnostic systems is the parallelism of detection. This potential was demonstrated using artificially spiked water samples (Fig. 2B) and further confirmed by analysis of spiked and native food samples. Unfortunately, at present this potential is restricted in food and water analysis by the sensitivity limitation of microarray-based analysis, which necessitates the utilization of biological pre-enrichment of the targeted pathogen(s) from the sample. Considering the different growth requirements (such as substrate, oxygen concentration, temperature, etc.) and growth dynamics of different microorganisms, the dependence on biological pre-enrichment is the major factor limiting the potential for parallel DNA-based analysis in many cases. A potential solution is the development of molecular methods enabling the enrichment of specific target sequences within the template mixture.

The developed diagnostic microarray for the detection of the most relevant bacterial food- and water-borne pathogens was demonstrated to be an efficient and reliable alternative to the standard microbiological methods.

Despite the need of pre-enrichment, the microarray was able to fulfil legal requirements for the detection of pathogens in food in terms of both sensitivity and specificity. Moreover, even with the pre-enrichment step, the microarray-based pathogen detection was substantially more rapid than the microbiological reference ISO methods (3–4 days for microarray-based analysis versus 5–7 days required for ISO methods). Further developments in microarray technologies, such as the ArrayTube™ technology (Anjum *et al.*, 2007; Batchelor *et al.*, 2008; Felder *et al.*, 2009) and Luminex x-MAP system (Mahony *et al.*, 2007; Ginocchio and George, 2009), are expected to facilitate the transfer of the methodology described above into routine diagnostics.

Experimental procedures

Oligonucleotide probe design

The previously established *gyrB* sequence database (Kostić *et al.*, 2007) was extended by downloading new *gyrB* sequences from the NCBI database (<http://www.ncbi.nlm.nih.gov>) as well as by sequencing *gyrB* genes of strains used for microarray validation. Probes were designed using the ARB software package (Ludwig *et al.*, 2004) following already established and validated probe design criteria: (i) 3' terminal cytosine residue, (ii) placement of the diagnostic mismatch(es) as close to the 3' end of the probe as possible, (iii) similar melting temperature (targeted 60°C), and (iv) probe length between 17 and 28 nucleotides (Kostić *et al.*, 2007). CalcOligo 2.03 (Stralis-Pavese *et al.*, 2004) was used to create an Excel table indicating predicted melting temperatures (based on the nearest neighbour model and SantaLucia (SantaLucia *et al.*, 1996) parameters), length and GC content of the probes and the number of weighted mismatches between each probe–target pair. Nearest neighbour T_m values were calculated with concentration settings of 250 nmol for oligonucleotide and 50 mmol for Na⁺. Factors for weighing mismatches in CalcOligo were as follows: positions: 5' 1st 0.3; 5' 2nd 0.6; 5' 3rd 0.8; 3' 1st 4.0; 3' 2nd 2.0; 3' 3rd 1.2; all other positions 1.0. Base pairs: AC 1.2; TC 1.2; GU 0.7; TG 0.4; all other mismatched base pairs 1.0 (where first and second nucleotides refer to probe and target sequence respectively). Probe–target pairs with weighted mismatches values up to 0.5 were expected to yield positive hybridization under the conditions applied. Furthermore, based on the results obtained in the preceding study (Kostić *et al.*, 2007), additional CO oligonucleotides were designed in order to suppress potential false positive signals. Probe Msi_294 targeting *pmaA* gene of *Methylosinus trichosporium* OB3b was used as control oligonucleotide (Stralis-Pavese *et al.*, 2004). A complete list of the RC oligonucleotide probes and competitive oligonucleotides used in this study can be found in Tables S1 and S2.

Microarray fabrication

Oligonucleotides for immobilization were custom-synthesized (VBC Genomics, Vienna, Austria) with a 5' primary amino

group, followed by a C6 spacer and five thymidine residues preceding the probe sequence. A 384-well flat bottom plate (Ritter, Schwabmünchen, Germany) was prepared with 30 µl of 50 µM oligonucleotide solution in 50% DMSO spotting buffer. Microarrays were spotted with an OmniGrid spotter (1 TeleChem SMP3 pin) at 45% relative humidity and 21°C onto silylated slides containing aldehyde groups (CEL Associates, Pearland, TX, USA). Arrays were spotted in triplicates to allow a statistical correction of errors. Slide processing was carried out as described before (Stralis-Pavese *et al.*, 2004). Processed slides were stored desiccated at room temperature in the dark.

DNA templates for validation

Strains used for the validation of the new probe set are listed in Table S3. Strains were obtained from the University of Sassari and the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). DSMZ strains were cultivated according to the DSMZ (<http://www.dsmz.de>) users instructions. Total genomic DNA was isolated from the cultures that were homogenized using 0.1 mm glass beads (BioSpec Products, Bartlesville, OK, USA) and a bead mill homogenizer (Retsch, Haan, Germany) followed by standard organic extraction with phenol and chloroform (Sambrook and Russel, 2001). Nucleic acids were precipitated by the addition of 0.7 volumes of 2-propanol and 0.1 volumes of 3M sodium acetate (30 min at –20°C), pelleted by centrifugation, rinsed with 70% ethanol, air-dried and finally dissolved in 50 µl TE buffer containing RNaseA (1 µg µl⁻¹).

gyrB amplification

The *gyrB* gene was amplified using a mixture of universal primers UP1, UP1G, UP2r and UP2Ar (Yamamoto and Harayama, 1995). PCR reactions were performed in 100 µl aliquots using the FailSafe PCR PreMix E (Epicentre, Madison, WI, USA) and 4 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) with 50–100 ng DNA as template. Amplification conditions were 95°C for 5 min, followed by 35 cycles of: 1 min at 95°C, 1 min at 58°C, 2 min at 72°C, followed by a final elongation step of 10 min at 72°C (Kostić *et al.*, 2007). PCR products were subsequently purified using a commercial PCR purification kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions and eluted in 25 µl dH₂O. DNA concentration was measured using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to an end concentration of 50 ng µl⁻¹.

Cloning and sequencing

gyrB PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) and sequenced using M13 primers as described before (Kostić *et al.*, 2007). Sequences were analysed using Sequencher v 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA) and subjected to preliminary nucleotide – nucleotide BLAST analysis within the NCBI database (<http://www.ncbi.nlm.nih.gov/blast>). Accession numbers of obtained *gyrB* sequences are listed in Table S3. Subsequently, sequences were imported into the

gyrB database in ARB where phylogenetic analysis was performed.

Control *pmoA* PCR product

During each labelling and hybridization experiment a *pmoA* PCR product from *Methylosinus trichosporium* OB3b was included as an internal positive control and subsequently used for normalization of the results. Genomic DNA extraction and *pmoA* PCR amplification were performed as described before (Bodrossy *et al.*, 2003). Amplicons were purified using a commercial PCR purification kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. DNA concentration was measured using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the concentration was adjusted to 50 ng μl^{-1} .

DNA labelling

For the SSELO a modified protocol of Rudi and colleagues (2003) was applied. After purification, PCR products were treated with shrimp alkaline phosphatase (SAP) (Roche Diagnostics, Penzberg, Germany) in order to dephosphorylate remaining nucleotides. Samples consisting of 20 μl purified PCR product (50 ng μl^{-1}), 2 μl Thermo Sequenase reaction buffer (Amersham Biosciences, Piscataway, NJ, USA) and 4 μl SAP (1 U μl^{-1}) were incubated at 37°C for 30 min, followed by 10 min at 95°C to inactivate enzyme activity. The SAP-treated *gyrB* PCR products were then used as a template for the cyclic labelling reaction. The control *pmoA* PCR product was further diluted to an end concentration of 5 ng μl^{-1} . For the SSELO approach a set of RC oligonucleotides (see Table S1), lacking the 3' terminal cytosine residue, was custom synthesized (VBC Genomics, Vienna, Austria). Furthermore, a set of CO oligonucleotides (Table S2) was synthesized with a 3' phosphate modification. Lyophilized oligonucleotides were dissolved to an end concentration of 100 pmol μl^{-1} and stored at -20°C. For labelling reaction a mix of oligonucleotides, containing each RC and CO oligonucleotide at an end concentration of 1 pmol μl^{-1} , was prepared.

The cyclic labelling was performed in 10 μl aliquots consisting of 1 \times Thermo Sequenase reaction buffer, 10 ng SAP-treated control *pmoA* PCR product, 1 pmol of each reverse complement oligonucleotide, 10 pmol Tamra-ddCTP (PerkinElmer Life and Analytical Sciences, Boston, MA, USA), 10 pmol of each ddATP, ddTTP, ddGTP (Roche Diagnostics GmbH, Penzberg, Germany), 3 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 100 ng of SAP-treated *gyrB* PCR product. Reaction conditions were 25 cycles of 30 s at 95°C followed by 75 s at 60°C, carried out in a thermocycler. After cyclic labelling, samples were used directly for hybridization, without further purification.

Microarray hybridization

Hybridization was carried out as described before (Stralis-Pavese *et al.*, 2004). Labelled targets (10 μl) were mixed with hybridization buffer (pre-warmed to 65°C). Final concentration of the hybridization buffer was: 6 \times SSC, 1 \times Denhardt's

reagent (Sigma, St Louis, MO, USA), 0.1% SDS. Hybridization was performed for 2 h at 55°C. After hybridization, slides were washed in a 2 \times SSC, 0.1% SDS wash solution for 5 min, followed by two wash cycles for 5 min in 0.2 \times SSC, and a final wash for 5 min in 0.1 \times SSC, all at room temperature. Slides were dried with an oil-free air gun and scanned immediately.

Scanning and data analysis

Microarrays were scanned at 10 μm resolution using a GenePix 4000B laser scanner (Axon Instruments, Foster City, CA, USA). PhotoMultiplier Tube (PMT) gain was adjusted to scan the spots below the saturation level. Scanned images were saved as multilayer tiff images and analysed with the GenePix Pro 6.0 software (Axon Instruments, Foster City, CA, USA). Microsoft Excel was used for statistical analysis and presentation of the results. Microarray hybridization results were normalized to the signal obtained from the internal control oligonucleotide (Msi_294) and expressed as percentage, 100% equalling the signal of the control probe. Non-specific signals reached in some cases a value of up to 8% (of the control signal, Msi_294). Therefore, a cut-off value of 10% was chosen to allow for unambiguous differentiation of positive signals.

Artificially contaminated water samples

In order to validate the above-described experimental set-up and to confirm the detection threshold, spiking experiments were performed. Cultures of *E. coli* DSM 5313, *S. enterica* DSM 17058, *P. aeruginosa* DSM 50071, *S. aureus* ATCC 6538 and *B. phytofirmans* strain PsJN DSM 10436 were grown in 10% tryptic soy broth (Merck KGaA, Darmstadt, Germany) overnight at 37°C with agitation. Overnight cultures were characterized by measuring the optical density (OD) at 600 nm. Additionally, the cfu per ml were determined by plating serial dilutions on 10% Tryptic Soy Agar plates (Merck KGaA, Darmstadt, Germany). After incubation of the TSA-Y agar overnight at 37°C the colonies were counted. For each sample 2 l of sterile 0.9% NaCl solution was prepared and spiked with a defined volume of one or more cultures. Overview of the spiked samples is presented in Table 1.

For DNA extraction, microorganisms were harvested from 1 l aliquots by filtration through 0.22 μm pore size GS filters (Millipore Corporation, Billerica, MA, USA). Following filtration filters were thoroughly rinsed with 10 ml 0.9% NaCl solution and cells were pelleted by centrifugation. Pellets were resuspended in 800 μl supernatant and suspension was homogenized using 0.1 mm glass beads (BioSpec Products) and bead mill homogenizer (Retsch). Genomic DNA was extracted following standard protocol for organic extraction with phenol and chloroform (Sambrook and Russel, 2001).

Fluorescence in situ hybridization

For the FISH analysis 500 ml aliquots of the spiked water samples were filtered through 0.22 μm pore size polycarbonate filters (Millipore Corporation). After filtration, all fixation and washing steps were performed as described before (Loy

et al., 2005) and filters were stored at -20°C before *in situ* hybridization. Probes used for FISH analysis were selected using probeBase (Loy et al., 2003) and are listed in Table S4. Labelled probes were purchased from Thermo Electron GmbH (Ulm, Germany). Lyophilized oligonucleotides were dissolved to an end concentration of $300\text{ ng }\mu\text{l}^{-1}$ and stored at -20°C . For *in situ* hybridization working solutions of $30\text{ ng }\mu\text{l}^{-1}$ were prepared (with exception of EUB338 probe mix that was used at $50\text{ ng }\mu\text{l}^{-1}$ concentration). *In situ* hybridization on filters was performed following a previously published protocol (Glöckner et al., 1996). Polycarbonate filters (47 mm filter diameter, 35 mm diameter of effective filter area) containing paraformaldehyde-fixed bacteria were cut in eight sections. Each filter section was hybridized for 90 min with $30\text{ }\mu\text{l}$ of hybridization solution containing $3\text{ }\mu\text{l}$ of both Cy3-labelled specific probe and fluorescein-labelled EUB338 mix in an equilibrated chamber at 46°C . Following *in situ* hybridization filters were stringently washed for 15 min at 48°C , rinsed in ice-cold double-distilled water and dried on Whatman 3M paper. Subsequently, filters were stained with $100\text{ }\mu\text{g ml}^{-1}$ DAPI (4',6'-diamidino-2-phenylindole) solution for 5 min on ice, rinsed in ice-cold double-distilled water, followed by ice-cold 50% ethanol and dried on Whatman 3M paper. After drying filters were mounted on glass slides and embedded with Citiflour AFI (Citiflour Ltd, Canterbury, UK). Hybridized filters were analysed using an epifluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with a mercury lamp and appropriate fluorescence filter sets (Loy et al., 2005). For calculating total cell numbers probe- and DAPI-stained cells were counted at a magnification $1000\times$ in 10 randomly chosen fields. Assessment of relative abundance, related to biovolume, was performed by analysing 20 randomly taken images using the Daim software (Daims et al., 2005).

Artificially contaminated food samples

Generally, food samples, including meat, cheese and eggs, were purchased at the local retail level. Food samples were transported cooled at 4°C to the laboratory and immediately artificially contaminated. Twenty-five grams of each sample was inoculated with different spike levels: (i) 1–10 cfu (low level), (ii) 10–100 cfu (medium level), (iii) 100–1000 cfu (high level) and (iv) 0 cfu (negative control level) of selected food borne pathogen and enriched according to the ISO standard method (ISO 11290-1, 1996, ISO 6579, 2002, ISO 10273, 2003, ISO 10272-1, 2006). In detail, the spike strains included *S. enterica* ssp. *enterica* serovar Typhimurium DSM 544, *S. enterica* ssp. *enterica* serovar Enterica DSM 9898, *L. monocytogenes*: SLCC 2755 serovar 3a, NCTC 5105 serovar 1/2b and NCTC 7973 serovar 1/2a, *C. jejuni* DSM 4688, *Campylobacter coli* DSM 4689 and additionally *Y. enterocolitica* NCTC 10460. The spike strains stored at -80°C as cryobeads were cultivated overnight in brain heart infusion (BHI) broth (Merck KGa, Darmstadt, Germany) for $18 \pm 2\text{ h}$ at the optimal growth temperature (25°C for *Y. enterocolitica*, 37°C for *Salmonella* spp. and *Listeria monocytogenes*, 41.5°C for *Campylobacter* spp.). To obtain cells in a late-logarithmic growth phase $100\text{ }\mu\text{l}$ of the overnight cultures were re-inoculated into 10 ml BHI broth and grown for additional 6–8 h at the corresponding temperature. *Campy-*

lobacter sp. strains were incubated at 42°C for 44–48 h under microaerophilic conditions and then immediately used for spiking. All cultures were adequately diluted (in 10-fold steps; up to the level of 10^{-7} or 10^{-8}) in order to obtain the required amount of bacterial cells for spiking. The exact amount used was subsequently quantified by plate counting. All experiments were performed in triplicates resulting in nine sample sets. An overview of the spiked samples is presented in Table 2.

Precisely, 25 g food samples (chicken meat, egg) spiked with different spike levels of *Salmonella* spp. were pre-enriched in 225 ml buffered peptone water (BPW) (Merck KGaA, Darmstadt, Germany) for 18 h at 37°C according to ISO 6579. Subsequently, RVS broth (Oxoid Ltd, Cambridge, UK) was inoculated with $100\text{ }\mu\text{l}$ BWP and incubated at 42°C for 24 h. Quantification using plate count method was performed on XLD agar (Oxoid Ltd, Cambridge, UK). Twenty-five gram of each food sample (blue veined cheese, paté) spiked with *Listeria monocytogenes* was incubated in 225 ml of Half Fraser broth (Merck KGaA, Darmstadt, Germany) for 24 h at 30°C , followed by secondary enrichment in Fraser broth (Merck KGaA, Darmstadt, Germany) for 48 h at 37°C . Bacterial growth was determined using selective PALCAM (Solabia Biokar Diagnostics, Pantin Cedex, France) and OCLA agar (Oxoid Ltd, Cambridge, UK). Pork meat samples spiked with *Y. enterocolitica* were initially enriched in ITC broth (Merck KGaA, Darmstadt, Germany) for 48 h at 25°C . Quantification was carried out on CIN agar after alkali treatment (Oxoid Ltd, Cambridge, UK). Selective enrichment of chicken meat samples spiked with *Campylobacter* spp. was performed using Bolton broth (Oxoid Ltd, Cambridge, UK). Samples were incubated in an oxygen-reduced atmosphere (anaerobic jar with 5% oxygen and 10% carbon dioxide atmosphere) for 48 h at 42°C . Quantification on selective agar plates, DNA extraction and following microarray analysis were performed from each enrichment broth. Genomic DNA was isolated from each enrichment step following the Nucleospin tissue kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) user manual instructions and eluted in $100\text{ }\mu\text{l}$ buffer BE.

Spiking of food matrix with multiple pathogens

In order to further test the application potential of the microarray for food analysis and to demonstrate the advantages of its multiplexing feature, a minced meat (25 g of pork and beef mix) matrix was spiked in parallel with different spike levels: (i) 1–10 cfu (low level), (ii) 10–100 cfu (medium level), and (iii) 0 cfu (negative control level) of *S. Typhimurium* DSM554, *L. monocytogenes* SLCC 2755 and *Y. enterocolitica* NCTC10460.

gyrB amplification and target preparations from spiked samples

Considering the requirements of the routine food diagnostics in terms of easy handling, all tests with food samples were done without quantifying and adjusting the amount of template DNA. PCR amplification was performed in $100\text{ }\mu\text{l}$ aliquots with $5\text{ }\mu\text{l}$ DNA sample as a template. Subsequently, $20\text{ }\mu\text{l}$ of the purified PCR products was used for the SAP treatment. Other conditions were the same as described above.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Set of RC oligonucleotide probe set. All sequences are listed with the 3' terminal cytosine residue that was added only during the labelling reaction provided the template was present. Melting temperature and G+C % values were calculated using CalcOligo 2.03 and parameters indicated in *Experimental procedures*.

Appendix S2. Set of competitive oligonucleotides (COs). Positions of mismatches with the original probe (RC oligonucleotide, sequence in Table 1) are indicated by underlined capital characters.

Appendix S3. List of bacterial species used for microarray validation.

Appendix S4. Set of rRNA targeted probes used for fluorescence *in situ* hybridization (FISH). Details on oligonucleotide probes [sequence, specificity and formamide (FM) concentration] were acquired from probeBase (<http://www.microbial-ecology.net/probebase>).

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