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Oligonucleotide microarray for identification of *Enterococcus* species

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Abstract

For detection of most members of the *Enterococcaceae*, the specificity of a novel oligonucleotide microarray (ECC-PhyloChip) consisting of 41 hierarchically nested 16S or 23S rRNA gene-targeted probes was evaluated with 23 pure cultures (including 19 *Enterococcus* species). Target nucleic acids were prepared by PCR amplification of a 4.5-kb DNA fragment containing large parts of the 16S and 23S rRNA genes and were subsequently labeled fluorescently by random priming. Each tested member of the *Enterococcaceae* was correctly identified on the basis of its unique microarray hybridization pattern. The evaluated ECC-PhyloChip was successfully applied for identification of *Enterococcus faecium* and *Enterococcus faecalis* in artificially contaminated milk samples demonstrating the utility of the ECC-PhyloChip for parallel identification and differentiation of *Enterococcus* species in food samples.

Keywords: *Enterococcus*; rRNA gene; Oligonucleotide; Microarray; Probe; Microbial diagnostics

1. Introduction

Bacteria of the genus *Enterococcus* are found in a wide variety of habitats such as soil, water, plants, fermented food, and in the gastrointestinal tracts of animals and humans [1]. In addition, members of this genus have recently attracted attention in clinical microbiology as emerging nosocomial, antibiotic-resistant pathogens causing bacteraemia, endocarditis, urethritis and other infections [2,3]. Their ability to survive adverse environmental conditions also makes some gastrointestinal enterococci suitable as indicators for hygienic quality in food and drinking water [4]. Rapid and accurate identification of enterococci at the species level is therefore an essential task in both clinical microbiology and food hygiene. Identification of enterococci isolates based on classical phenotypic and biochemical characterization is often difficult to accomplish due to considerable similarities among some of the species [5]. Therefore, commercial systems such as API (bioMérieux, Marcy l'Etoile, France) or MicroScan (Dade International, MicroScan Int., West Sacramento, CA, USA) often fail to correctly identify *Enterococcus* species [6–8].

Rapidly increasing data sets of rRNA sequences of prokaryotes [9,10] allow the design of specific hybridization probes (so-called "phylogenetic probes") for various taxa or phylogenetic entities at user-defined levels of resolution. Application of multiple probes targeting

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different sites on the rRNA (genes) significantly reduces the risk of misidentification and allows discrimination down to the species level [11]. This concept was applied previously to design a comprehensive rRNA-targeted oligonucleotide probe set of hierarchical and parallel specificity for most *Enterococcaceae* [12].

It was the aim of this study to extend and evaluate the previously developed nested phylogenetic probe set for enterococci [12] for reverse hybridization on microarrays. Although DNA microarrays are circulating for almost 10 years [13], they have only recently attracted attention as powerful diagnostic tools for the identification of microorganisms in complex environmental and clinical samples [14–24]. Here we show, by analyzing milk that was artificially contaminated with *Enterococcus* species, that the ECC-PhyloChip is a highly reliable tool to correctly identify and differentiate members of the *Enterococcaceae*.

2. Materials and methods

2.1. Reference strains

Reference organisms for evaluating the microarray are listed in Table 1 and were obtained either from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, (DSMZ, Braunschweig, Germany), the Laboratorium voor Mikrobiologie Gent, (LMG, Gent, Belgium) or the Institut für Lebensmitteltechnologie, Universität Hohenheim (LTH, Stuttgart, Germany). Strains were grown overnight in Brain–Heart-Infusion medium (Difco, Liverpool, UK) at 37 °C. One milliliter of culture was harvested by centrifugation (5 min at 7150*g*) and washed in phosphate-buffered saline (PBS: 130 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, pH 7.2) prior to DNA extraction.

Table 1
Reference strains

Species	Strain
Enterococcus asini	DSM 11492^T
Enterococcus avium	LMG 10744^T
Enterococcus casseliflavus	DSM 20680^T
Enterococcus cecorum	DSM 20682^T
Enterococcus columbae	DSM 7374^T
Enterococcus dispar	DSM 6630^T
Enterococcus durans	DSM 20633^T
Enterococcus faecalis	LMG 7937^T
Enterococcus faecium	DSM 20477^T
Enterococcus flavescens	DSM 7330^T
Enterococcus gallinarum	DSM 20628^T
Enterococcus hirae	DSM 20160^T
Enterococcus malodoratus	DSM 20681^T
Enterococcus mundtii	DSM 4838^T
Enterococcus pseudoavium	DSM 5632^T
Enterococcus raffinosus	DSM 5633^T
Enterococcus saccharolyticus	LMG 11427^T
Enterococcus solitarius	DSM 5634^T
Enterococcus sulfurous	DSM 6905^T
Lactococcus lactis	DSM 20481^T
Melissococcus plutonius	LTH 3442
Staphylococcus aureus	DSM 20232^T
Tetragenococcus halophilus	DSM 20339^T

2.2. Contaminated milk samples

Two different *Enterococcus* food isolates were obtained from the Bavarian State Institute for Food Surveillance (LUAS, Oberschleißheim, Germany) for artificial contamination of milk samples. One isolate was tentatively identified as *Enterococcus faecium* by selective plating and subsequent biochemical characterization of grown colonies by API 20 STREP (bioMérieux, Marcy l'Etoile, France) (LUAS, personal communication). The identity of the second isolate could not be determined to the species level by using this approach. For each *Enterococcus* isolate, one milliliter of ultra high temperature milk was inoculated with cells using a sterile loop. Five replicates each were prepared for *E. faecium* (S1–S5) and the unidentified *Enterococcus* species (S6–S10). An enrichment step was performed by incubating the milk aliquots for 16 h at 37 °C with 1 ml of Brain–Heart-Infusion medium. Subsequently, cells were harvested by centrifugation (5 min at 7150*g*), resuspended in 1 ml of digestion buffer (100 mM Na_2HPO_4, 150 mM NaCl, 10 mM EDTA, 40 mM NaOH), and incubated for 10 min at room temperature for protein denaturation [25]. After centrifugation at 7150*g* for 5 min at 4 °C, surface fat was removed by using a sterile swab and the supernatant was decanted. The protein denaturation step was repeated twice, and the retrieved cells resuspended in 200 μl PBS. Half of the cells were plated on oxolinic acid-esculin-azide enterococci selective agar [26], whereas the other half was used for extraction of nucleic acids.

2.3. Isolation of genomic DNA

Genomic DNA was isolated by enzymatic lysis of the cells and subsequent extraction of nucleic acids with chloroform/isoamyl alcohol as described previously [27]. Extracted DNA was resuspended in 50 μl double-distilled water and stored at −20 °C.

2.4. rRNA-targeted probes and microarray fabrication

Tables 2 and 3 list names, sequences, and intended specificities of the oligonucleotide probes used in this study. Further information (e.g., G+C content or molecular weight of each probe) can be accessed at the probeBase database (http://www.microbial-ecology.net/probebase/) [28]. The free hybridization energy, ΔG, of each probe to its perfectly matching target sequence was calculated with the 2-state hybridization server

Table 2
23S rRNA gene-targeted oligonucleotide probes for *Enterococcaceae*

Name[a]	*Escherichia coli* position	Sequence [5′–3′]	GC content (%)	ΔG (kcal mol^{-1})	Specificity[b]	Reference
Enc38i	847	AGA ATG ATG GAG GTA GAG	44.4	−17.5	Most *Enterococcaceae*	[12]
Eamprs09	142	CAC TGA AAA GTA ACA TCC	38.9	−16.9	*E. avium* *E. malodoratus* *E. pseudoavium* *E. raffinosus* *E. sulfureus*	[12]
Eacdfg57	1447	AGA CAT ATC CAT CAG TCT	38.9	−17.1	*E. asini* *E. casseliflavus* *E. dispar* *E. flavescens* *E. gallinarum*	[12]
Eampr18	343	GGT GCC AGT CAA ATT TTG	44.4	−18.8	*E. avium* *E. malodoratus* *E. pseudoavium* *E. raffinosus*	[12]
Edfm57	1456	CTG CTT GGA CAG ACA TTT	44.4	−18.8	*E. durans* *E. faecium* *E. mundtii*	[12]
Eduhi09	142	CAC GCA AAC GTA ACA TCC	50.0	−20.0	*E. durans* *E. hirae*	[12]
Esa38	835	ATT CTC AAC TTC GAC GCT	44.4	−19.5	*E. asini* *E saccharolyticus*	[12]
Ecafl09i	142	GGA TGT TAC GTC TGC GTG	55.5	−20.6	*E. casseliflavus* *E. flavescens*	[12]
Ecoce58	1490	AGT GAC AAG CAT TTG ACT	38.9	−18.4	*E. cecorum* *E. columbae*	[12]
Esasu58i	1487	GAG AGT CAA ATG CTT TCA	38.9	−17.7	*E. saccharolyticus* *E. sulfureus*	[12]
Esoha57	1452	TGG ACA GAC CTT TCC ATT	44.4	−18.9	*E. solitarius* *T. halophilus*	[12]
Eas09	136	CGT AAC ATC CTA TCA AAG	38.9	−16.6	*E. asini*	[12]
Eav58i	1494	AAA TGC TTA CAT CTC TAA	27.8	−15.7	*E. avium*	[12]
Ece09	142	CAC TTA AAG GTA ACA TCC	38.9	−16.6	*E. cecorum*	[12]
Eco09i	142	GGA TAT TAC CCT TAA GTG	33.3	−16.0	*E. columbae*	[12]
Eca58	1502	AGC TTG TCC GTA CAG GTA	50.0	−20.4	*E. casseliflavus*	This study
Edr58	1500	CTT ACT CGT GTA GAC AGA	44.4	−18.0	*E. durans*	This study
Efa54	1399	CAA AAA CAA CTG GTA CAG	38.9	−17.3	*E. faecalis*	[12]
Efm09	142	CAC ACA ATC GTA ACA TCC	44.4	−18.3	*E. faecium*	[31]
Efl58i	1500	TTC TAC CTA TAC GGA CAA	38.9	−17.1	*E. flavescens*	[12]
Ega09	142	CAC AAC TGT GTA ACA TCC	44.4	−18.1	*E. gallinarum*	[12]
Ehr58	1500	CTT GCT CGT ACA GAC AGA	50.0	−19.6	*E. hirae*	This study
Ema58i	1497	TGC TTG CAT CTC TAA GGA	44.4	−19.1	*E. malodoratus*	[12]
Emu58	1498	GTC CTT AAA GTT AGA AGC	38.9	−16.6	*E. mundtii*	[12]
Eps58	1497	TCC TTA TAG ACG TAA GCA	38.9	−17.6	*E. pseudoavium*	[12]
Era58	1499	TGT CCT TAA AGA CGT AAG	38.9	−17.1	*E. raffinosus*	[12]
Esa09	142	CAC TAA TAA GTA ACA TCC	33.3	−15.2	*E. saccharolyticus*	[12]
Enc01aV[d]	1	AGG TTA AGT GAA TAA GGG	38.9	−16.8	*Enterococcus* spp., *Vagococcus* spp., not *E. solitarius*	[12]
Enc01bV[d]	1	AGG TTA AGT AAG AAA GGG	38.9	−16.8	*E. solitarius, T. halophilus*	[12]
Enc01cV[d]	1	AGG TTA AGT GAA CAA GGG	44.4	−18.2	*M. plutonius*	[12]
Esasu58[d]	1487	TGA AAG CAT TTG ACT CTC	38.9	−17.7	*E. saccharolyticus* *E. sulfureus*	[12]
Eso18i[d]	276	ACA CGA TCT TTT AGA CGA	38.9	−18.3	*E. solitarius*	[12]

(*continued on next page*)

Table 2 (*continued*)

Name[a]	*Escherichia coli* position	Sequence [5′–3′]	GC content (%)	ΔG (kcal mol^{-1})	Specificity[b]	Reference
Eso58[d]	1496	GTG AAC AAG AAA AAG CCT	38.9	−18.1	*E. solitarius*	[12]
Eso58i[d]	1496	AGG CTT TTT CTT GTT CAC	38.9	−18.1	*E. solitarius*	[12]
Edr58i[d]	1500	TCT GTC TAC ACG GAT AAG	44.4	−18.0	*E. durans*	This study
Eav58[d]	1494	TTA GAG ATG TAA GCA TTT	27.8	−15.7	*E. avium*	This study
Edi38[d]	835	ATT CTT CAC TTC CAA ATT	44.4	−16.2	*E. dispar*	[12]
Efs18i[d]	343	CGA AAT GCT AAC AAC ACC	44.4	−18.7	*E. faecalis*	Modified from [31][c]
Efi58[d]	1476	TGA CTC CTC TCC AGA CTT	44.4	−19.1	*E. faecium*	[12]
Efm09i[d]	142	GGA TGT TAC GAT TGT GTG	44.4	−18.3	*E. faecium*	Modified from [31][c]
Esu18[d]	346	CTA GGT GCA TAC CAA ATT	38.9	−17.4	*E. sulfureus*	[12]
Mpl15i[d]	268	AAA CCA ACG AGC ATG CTT	44.4	−20.2	*M. plutonius*	This study
Mpl58i[d]	1502	ACT CTG TAA GGA TGA GTT	38.9	−17.3	*M. plutonius*	This study
Tha09[d]	126	GAT GAA AAA TGC GAG GTT	38.9	−18.3	*T. halophilus*	[12]

[a] Suffix "i" in the probe name indicates that this probe cannot be used to detect 23S rRNA. The reverse complementary version of this probe targets 23S rRNA.
[b] Target organisms having a perfectly matching probe target site.
[c] The inverse complementary version of the published probe was used.
[d] Probe was excluded from the final ECC-PhyloChip because it gave either false-positive signals with many non-target reference strains or a false-negative signal with the target strain.

Table 3
16S rRNA gene-targeted oligonucleotide probes for *Enterococcaceae*

Name	*Escherichia coli* position	Sequence [5′–3′]	GC content (%)	ΔG (kcal mol^{-1})	Specificity[a]	Reference
EUB338	338	GCT GCC TCC CGT AGG AGT	66.7	−22.4	Most *Bacteria*	[32]
Enc131	131	CCC CTT CTG ATG GGC AGG	66.7	−21.8	Most *Enterococcus* spp., *M. plutonius*	[12]
Ecf459	461	GGG ATG AAC ATT TTA CTC	38.9	−16.8	*E. pseudoavium* *E. casseliflavus* *E. flavescens* *E. dispar*	[12]
Ecg191	193	GCG CCT TTC AAC TTT CTT	44.4	−19.5	*E. gallinarum* *E. casseliflavus* *E. flavescens*	[12]
Ecc461	462	AGG GAT GAA CTT TCC ACT	44.4	−18.7	*E. cecorum* *E. columbae*	[12]
Enc93	93	GCC ACT CCT CTT TTT CCG	55.6	−20.3	*E. hirae* *E. faecium*	[12]
Edi131	131	CCC CCG CTT GAG GGC AGG	77.8	−24.4	*E. asini*	[12]
Ece92	92	CCA CTC ATT TTC TTC CGG	50.0	−19.2	*E. cecorum*	[12]
Edi137	138	ATG TTA TCC CCC GCT TGA	50.0	−20.3	*E. dispar*	[12]
Efs129	129	CCC TCT GAT GGG TAG GTT	55.6	−19.7	*E. faecalis*	[12]
Esa452	453	CAT TCT CTT CTC ATC CTT	38.9	−16.9	*E. saccharolyticus*	[12]
Eso193	194	ACG CAC AAA GCG CCT TTC	55.6	−22.2	*E. solitarius*	[12]
Esu90	90	CAC TCC TCT TAC TTG GTG	50.0	−18.4	*E. sulfureus*	[12]
Mplu464	465	GTC ACG AGG AAA ACA GTT	44.4	−18.9	*M. pluton*	[12]
Enc145[b]	146	GGG ATA ACA CTT GCA AAC	44.4	−18.4	*Enterococcus* spp., not *E. dispar*, *E. asini*, *E. solitarius*, *E. columbae*, *E. caecorum*, and *E. faecalis*	[12]
Enc1259[b]	1260	GAA GTC GCG AGG CTA AGC	61.1	−21.7	*Enterococcus* spp., not *E. solitarius*, *E. columbae*, *E. caecorum*, and *E. faecalis*	[12]

[a] Target organisms having a perfectly matching probe target site.
[b] Probe was excluded from the final ECC-PhyloChip because it gave either false-positive signals with many non-target reference strains or a false-negative signal with the target strain.

(concentration of Na^+ and temperature were set to 0.829 M and 42 °C, respectively) at the mfold website (http://www.bioinfo.rpi.edu/applications/mfold/) [29]. Each oligonucleotide was tailed at the 5′ end with a 15 dTTP spacer element and synthesized with a 5′-terminal amino-modification (MWG-Biotech, Ebersberg, Germany). Spotting of the modified oligonucleotide probes (50 pmol/μl in 50% dimethylsulfoxide) onto aldehyde-group-coated CSS-100 glass slides (CEL Associates, Houston, USA) was performed using a GMS 417 (Affymetrix, Santa Clara, USA) contact printing device. All probes were immobilized on the microarray in duplicate. Microarrays were dried over night at room temperature for effective cross-linking. Reduction of free reactive aldehyde groups with sodium borohydride and washing of slides was performed as described previously [19].

2.5. PCR amplification and fluorescent labeling

For subsequent microarray hybridization, ≈4.5-kb DNA fragments containing large parts of the 16S and the 23S ribosomal RNA genes were PCR-amplified from DNA of reference organisms or contaminated milk samples by using the primer pair 616V–985R [12]. PCR mixtures were prepared in 100 μl volume containing 50 pmol of each primer, 200 μM of dNTPs, 10 μl of 10× Ex Taq™ reaction buffer, and 2.5 U of Ex *Taq* polymerase (Takara, Biomedicals, Japan). Thermal cycling was performed by using an initial denaturation step at 94 °C for 2 min, followed by 32 cycles of denaturation at 94 °C for 1 min, 52 °C annealing for 1 min 30 s, and elongation at 72 °C for 2 min 30 s. Cycling was completed by a final elongation step at 72 °C for 5 min. For the milk samples, PCR were run in duplicates. One reaction contained 10 μl of the sample DNA while the second reaction additionally contained 1 ng of *E. faecium* pure culture DNA, serving as a control for successful amplification (absence of PCR inhibitors). Negative controls with no template DNA were also included in all PCR amplification experiments. Presence and size of amplification products were analyzed by 1% agarose gel electrophoresis. Purified PCR amplicons were fluorescently labeled with Cy5 by random priming according to an established protocol [19].

2.6. Microarray hybridization

Vacuum-dried Cy5-labeled PCR products (400 ng) and 0.5 pmol of the Cy5-labeled control oligonucleotide CONT-COMP [19] were resuspended in 20 μl of hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% blocking reagent [Roche, Mannheim, Germany], 0.1% *n*-lauryl sarcosine, 0.02% SDS, 5% formamide), denatured for 10 min at 95 °C, and immediately placed on ice. Then the solution was pipetted onto the microarray, covered with a cover slip, and inserted into a watertight custom-made hybridization chamber containing 100 μl of hybridization buffer for subsequent equilibration. Hybridization was performed overnight at 42 °C in a water bath. After hybridization, the slides were washed immediately for 5 min in 50 ml washing buffer [containing 3 M tetramethylammoniumchloride (TMAC), 50 mM Tris–HCl, 2 mM EDTA, and 0.1% SDS]. For optimization of the washing conditions, separate microarrays were washed at 46, 48, 49, 50, and 52 °C, respectively. Subsequent microarray evaluation experiments were performed at the optimal washing temperature of 49 °C. After the stringent washing, slides were washed twice with ice-cold double-distilled water, air dried, and stored in the dark at room temperature. Fluorescent images were recorded with a GMS 418 fluorescent scanner (Affymetrix, Santa Clara, USA) and quantitatively analyzed by using the ImaGene 4.0 software (BioDiscovery, Inc., Los Angeles, CA). Signal-to-noise ratios (SNRs) were determined for each probe as outlined previously [19]. Probe spots with SNRs equal to or greater than 2.0 were considered as positive.

2.7. 16S rRNA sequence retrieval from contaminated milk

For confirmation of microarray results, almost-complete 16S rRNA gene fragments were amplified from contaminated milk DNA (sample S6) by using the primer pair 616V–630R and cloned with the TOPO TA cloning kit (Invitrogen Corp., San Diego, USA) as described previously [19]. Insert sequences were partially sequenced and phylogenetically analyzed by using the ARB program package [9] as outlined previously [30].

3. Results

3.1. Evaluation of the ECC-PhyloChip

A total of 52 previously published [12,31] and 7 newly designed rRNA-targeted oligonucleotide probes for members of the family *Enterococcaceae* (Tables 2 and 3) was spotted together with probe EUB338 that targets most bacteria including *Enterococcaceae* [32,33]. The microarray additionally included probes NONSENSE and CONT which served as controls for unspecific binding and hybridization efficiency, respectively [19].

Initially, the optimal washing temperature was determined experimentally as the best compromise between signal intensity and stringency for some of the probes by hybridizing the ECC-PhyloChip with fluorescently labeled target DNA of *E. faecium* and *E. faecalis* under increasing stringencies (data not shown). All following experiments were performed at the optimized washing temperature of 49 °C. Subsequently, specificities of all

probes were evaluated by hybridizing fluorescently labeled target DNA from each of the 21 *Enterococcaceae* reference organisms and 2 control organisms (*Lactococcus lactis*, *Staphylococcus aureus*) to a separate ECC-PhyloChip. Based on the hybridization results, 19 probes had to be excluded from the final microarray (listed separately in Tables 2 and 3) because they did not show a positive signal with target organisms or exhibited non-specific binding to many non-target organisms. The remaining 41 probes showed a positive signal with the respective perfectly matching DNA and had calculated free hybridization energies in the range of -15.2 to $-24.4\ \mathrm{kcal\,mol^{-1}}$ (Tables 2 and 3). Thirty-five of the fourty-one probes (85%) of the final version of the ECC-PhyloChip hybridized exclusively to their perfectly matching target organisms while six probes also cross-hybridized with a few non-target organisms having up to 3 mismatches in the respective probe target sites (Fig. 1). Nevertheless, for each of the 21 *Enterococcaceae* reference organisms, including *Melissococcus plutonius* and *Tetragenococcus halophilus*, a characteristic hybridization pattern was obtained with the final version of the ECC-PhyloChip (Fig. 1).

3.2. *Identification of* Enterococcus *species in contaminated milk by ECC-PhyloChip analysis*

The developed microarray was tested with two artificially contaminated milk samples (each sample consisted of five replicates, S1–S5 and S6–S10, respectively). Milk samples were either inoculated with *E. faecium* or an *Enterococcus* species which could not be identified with the API 20 STREP test. ECC-PhyloChip analysis was performed after a pre-cultivation step of the milk samples in enrichment media. Microarray hybridization patterns were identical for replicates S1 to S5. Signal intensities for probes EUB338, Enc38i, Enc131, Edfm57, Enc93 targeting *Enterococcus* species at higher hierarchical levels and the species-specific probe Efm09 were above the threshold value. This hybridization pattern is indicative for *E. faecium* (data not shown). Hybridization patterns of replicates S6 to S10 were also identical to each other but differed from samples S1 to S5. Positive signals for the hierarchically nested probes EUB338, Enc38i, Enc131, Edfm57, Enc93, and for the species-specific probes Efm09 (targeting *E. faecium*), Efs129 (targeting *E. faecalis*), and Efa54 (targeting

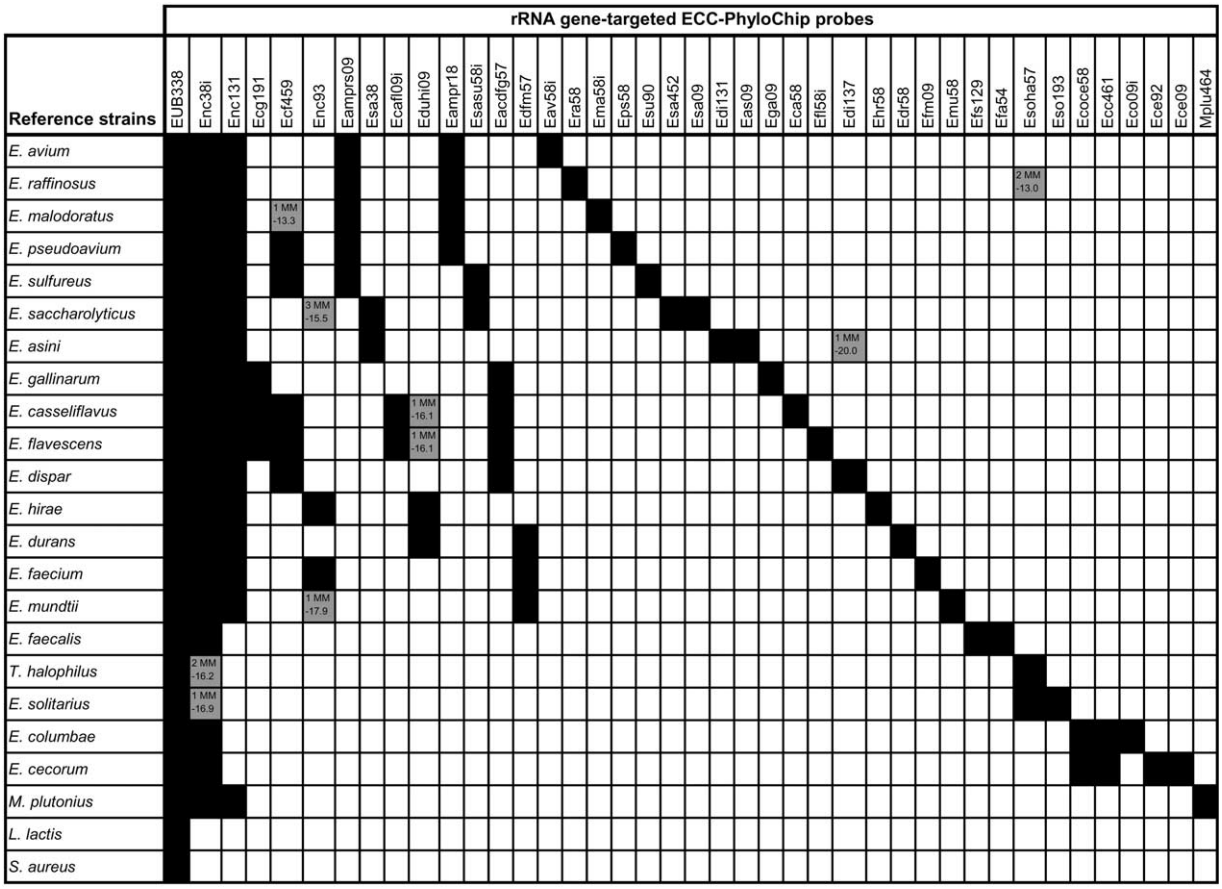

Fig. 1. Evaluation of the ECC-PhyloChip by hybridization with fluorescently labeled target DNA of reference organisms. Positive signals of perfectly matching and mismatching probe-target hybrids are shown by black and grey boxes, respectively. For each cross-hybridization event the number of mismatching base pairs (MM) and the free energy, ΔG ($\mathrm{kcal\,mol^{-1}}$), are indicated.

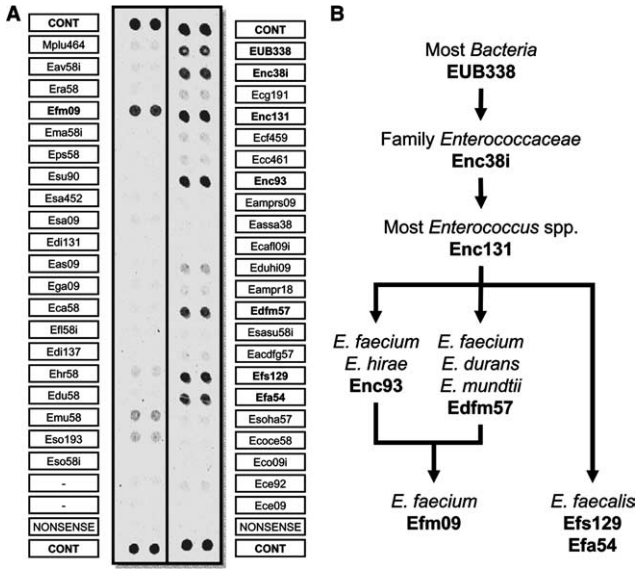

Fig. 2. (A) ECC-PhyloChip hybridization pattern of contaminated milk sample S6. Each probe was spotted in duplicate. Probe names are located next to each probe pair and indicate the position of the probe spots on the microarray. Perfectly matching target organisms of each probe are listed in Tables 2 and 3. Positive probes with an SNR above two are indicated in boldface type. (B) Translation of the microarray hybridization pattern indicating the presence of *E. faecium* and *E. faecalis* in milk samples S6–S10.

E. faecalis) unexpectedly indicated co-contamination of the milk with *E. faecium* and *E. faecalis* (Fig. 2). These microarray data were confirmed by comparatively analyzing cloned 16S rRNA gene sequences from replicate S6. A total of 10 clones was analyzed; eight of them were affiliated with *E. faecium* and two with *E. faecalis* (data not shown).

4. Discussion

4.1. Specificity of the ECC-PhyloChip

A previously developed set of oligonucleotide probes for detection and identification of members of the *Enterococcaceae* [12] was extended and spotted as microarray. One advantage of microarrays compared to more conventional hybridization formats is that miniaturized microarrays require lower amounts of labeled target nucleic acids for successful hybridization. Thus, the final version of the ECC-PhyloChip could be hybridized with 400 ng of labeled PCR product in total, whereas 4920 ng (120 ng per probe and cavity) would be needed for hybridization of the same probe set in microwell plates [12].

Because of its specific nucleotide composition and the number, position, and types of mismatches to non-target organisms, theoretically each individual probe on the microarray would require specific hybridization conditions to ensure its optimal specificity [12,18]. However, such flexibility can neither be achieved with the microarray format used in this study nor with most commercially available microarray systems which only allow performing the hybridization and/or washing step under constant (monostringent) conditions. Therefore, the design of the microarray probes and the experimental conditions were adapted for this setup using the approach of Loy et al. [19]. All probes had the same length and TMAC was added to the wash buffer to minimize the influence of GC-content differences between probes on their melting behavior. Furthermore, the optimal wash temperature was determined experimentally. Applying these optimized conditions, some of the 60 probes still showed cross-hybridizations with many non-target species and were thus removed from the microarray. Of the remaining 41 probes (14 16S and 27 23S rRNA gene-targeted), 85% hybridized exclusively to their perfectly matching target species. Only six probes of the final version of the ECC-PhyloChip hybridized with mismatching DNA from a few non-target organisms (Fig. 1). Unspecific hybridizations of some microarray probes to not fully complementary target DNA are not unexpected under monostringent conditions and can at least partly be predicted by analyzing the thermodynamic properties of a given probe-target duplex [24]. One such property, the free hybridization energy ΔG can be calculated according to the nearest neighbor model, which takes into account base pairing and base stacking interactions of probe and target molecules [29,34]. We observed that for most of the positive probe-non-target combinations on the final version of the ECC-PhyloChip, the calculated theoretical ΔG values (-20.0 to $-15.5\,\mathrm{kcal\,mol^{-1}}$) (Fig. 1) were in the range of ΔG values of all perfectly matched probe-target hybrids (-24.4 to $-15.2\,\mathrm{kcal\,mol^{-1}}$) (Tables 2 and 3). The only exceptions were probe Ecf459 with *Enterococcus malodoratus* and probe Esoha57 with *Enterococcus raffinosus* having ΔG values of -13.3 and $-13.0\,\mathrm{kcal\,mol^{-1}}$, respectively (Fig. 1). Thus, although not all unspecific hybridization events can be explained by high theoretical hybridization energies, our results confirm that theoretical ΔG values are useful indicators of the actual association/dissociation behavior of a given probe-(non)target combination [24,35]. It should be noted that the nearest neighbor algorithms for calculating thermodynamic properties of probe-target duplexes were established based on hybridizations in solution. It is thus likely that the prediction of microarray hybridization events will improve further when optimized algorithms for probes immobilized on solid supports become available.

Despite the few cross-hybridizations of some probes under monostringent experimental conditions, the ECC-PhyloChip allowed unambiguous identification of all target strains (if analyzed as pure cultures) because

each tested member of the *Enterococcaceae* is targeted by at least three ECC-PhyloChip probes having nested or parallel specificities (Fig. 1). For example, the hierarchical probe set allows differentiation of *T. halophilus* from *Enterococcus* species, although the species-specific probe Tha09 needed to be removed due to lacking specificity from the final version of the microarray (Table 2). *T. halophilus* is unambiguously identified by positive signals of probes EUB338 (targeting most bacteria), Enc38i (targeting most *Enterococcaceae*), and Esoha57 (targeting *E. solitarius* and *T. halophilus*) if presence of *E. solitarius* can concurrently be excluded by a negative signal of probe Eso193 (targeting *E. solitarius*). Furthermore, the hybridization patterns of the reference strains (Fig. 1) also demonstrated that all 19 *Enterococcus* species tested on the ECC-PhyloChip can be differentiated and identified even if they occur in any mixtures in the analyzed samples. The only exception is if a sample is co-contaminated with *Enterococcus asini* and *Enterococcus dispar* in the presence of some other enterococci (e.g., *Enterococcus casseliflavus*). In this situation *E. dispar* cannot be unambiguously identified. Furthermore, specific identification of *T. halophilus* in a complex sample is not possible with this array if the sample also contains *E. raffinosus*.

4.2. ECC-PhyloChip analyses of food samples

The ability to correctly identify *Enterococcus* species in selected food samples by ECC-PhyloChip hybridization was proven using artificially contaminated milk samples. An enrichment step was included prior to DNA isolation in order to increase the number of target organisms and thus the detection sensitivity of the assay. As expected, the microarray fingerprints of the milk replicates S1 to S5 were identical to the pure culture fingerprint of the inoculum *E. faecium* (Fig. 1). As the milk samples were artificially contaminated with single enterococcal isolate, the identification of two distinct *Enterococcus* species, *E. faecium* and *E. faecalis*, in replicates S6 to S10 by ECC-PhyloChip analysis came as a surprise (Fig. 2). However, this result was confirmed by 16S rRNA gene sequencing and demonstrated that (i) the developed ECC-PhyloChip is well suited to analyze samples contaminated with more than one *Enterococcus* species and that (ii) the culture used for contamination of milk replicates S6 to S10 consisted of two *Enterococcus* species.

Similar to a recently developed multiplex PCR method for the genus- and species-specific amplification of superoxide dismutase genes (*sodA*) of enterococci [36], the unambiguous identification of novel *Enterococcus* species by ECC-PhyloChip hybridization of isolates or environmental samples is not possible. Nevertheless, positive signals for probes targeting enterococci at broader specificity (e.g., EUB338, Enc38i, and Enc131), combined with negative signals for species-specific probes targeting already recognized enterococci, are strongly indicative for the presence of yet unknown (or not targeted) *Enterococcus* species. If such a result is obtained, comparative 23S or 16S rRNA gene sequence analysis is recommended for phylogenetic assignment of the novel *Enterococcus* species.

4.3. Conclusions and outlook

Routine identification of enterococci is a laborious and time-consuming process involving cultivation and subsequent phenotypic characterization of isolates. The ECC-PhyloChip described here is suitable for rapid monitoring of most recognized *Enterococcus* species ($n = 19$) at high resolution, allows large numbers of samples to be analyzed in a short time period, and has the potential for full automation. We have not systematically tested sensitivity (i.e., the lowest absolute and/or relative abundance of target organisms that are detectable) of the ECC-PhyloChip, due to the inclusion of a pre-enrichment step in the protocol. If one would attempt rendering the ECC-PhyloChip assay completely independent from cultivation one could expect a detection limit for the relative abundance of the target organisms of about 5% of the total bacterial cells in the sample [17,37]. If required, several strategies are available to further increase the sensitivity of a diagnostic microarray approach [38–40]. For example, the use of target group-selective primers (instead of general bacterial primers) allows the detection of organisms representing less than 1% of all bacteria in a complex sample [24]. In this context, it is important to note that *Enterococcaceae*-specific primer pairs suitable for the amplification of large 16S and 23S rRNA gene fragments are already available [12] and could be used for cultivation-independent ECC-PhyloChip-based detection of low-abundant *Enterococcus* species in complex food and clinical samples.

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