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Development of a DNA Microarray for Enterococcal Species, Virulence, and Antibiotic Resistance Gene Determinations among Isolates from Poultry▽

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A DNA microarray (Enteroarray) was designed with probes targeting four species-specific taxonomic identifiers to discriminate among 18 different enterococcal species, while other probes were designed to identify 18 virulence factors and 174 antibiotic resistance genes. In total, 262 genes were utilized for rapid species identification of enterococcal isolates, while characterizing their virulence potential through the simultaneous identification of endogenous antibiotic resistance and virulence genes. Enterococcal isolates from broiler chicken farms were initially identified by using the API 20 Strep system, and the results were compared to those obtained with the taxonomic genes *atpA*, *recA*, *pheS*, and *ddl* represented on our microarray. Among the 171 isolates studied, five different enterococcal species were identified by using the API 20 Strep system: *Enterococcus faecium*, *E. faecalis*, *E. durans*, *E. gallinarum*, and *E. avium*. The Enteroarray detected the same species as API 20 Strep, as well as two more: *E. casseliflavus* and *E. hirae*. Species comparisons resulted in 15% (27 isolates) disagreement between the two methods among the five API 20 Strep identifiable species and 24% (42 isolates) disagreement when considering the seven Enteroarray identified species. The species specificity of key antibiotic and virulence genes identified by the Enteroarray were consistent with the literature adding further robustness to the redundant taxonomic probe data. Sequencing of the *cpn60* gene further confirmed the complete accuracy of the microarray results. The new Enteroarray should prove to be a useful tool to accurately genotype strains of enterococci and assess their virulence potential.

Enterococci are commensal bacteria found in the gastrointestinal tracts of humans, animals, and birds, as well as in soil and water. Sequenced *Enterococcus faecalis* and *E. faecium* genomes revealed the presence of virulence factors and mobile and/or exogenously acquired DNA giving some insight on how these bacteria made the transition from a commensal organism to a nosocomial pathogen (21, 46, 53). Enterococci can infect the endocardium, abdomen, urinary tract, burn or surgical wounds, and numerous other tissues, especially in immunocompromised patients (24, 59). *E. faecalis* causes the majority of infections, followed by *E. faecium* (53). Other enterococci such as *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. raffinosus*, and *E. solitarius* rarely cause infections but frequent misidentification by classical biochemical or microbiological methods suggests that their importance might be underestimated (14, 24, 35, 52, 55).

Resistance to antimicrobial agents can be intrinsic or acquired through several genetic mechanisms, including mutation, integration of foreign genetic material, or transfer of plasmids and transposons (21, 46, 52). While *E. gallinarum* and *E. casseliflavus* possess intrinsic low-level resistance to vancomycin, other enterococci have been shown to be resistant to aminoglycosides, ampicillin, and glycopeptides (30, 42, 55). Nosocomial infections involving vancomycin-resistant enterococci are difficult to treat with antimicrobial agents (2, 52). From an agricultural perspective, many antimicrobial agents have been approved for use in treating infections or to positively impact animal growth and feeding (15). Widespread use of antimicrobial agents in livestock and poultry has raised concerns that bacterial pathogens, having food animal reservoirs, could develop drug resistance and transfer these antimicrobial resistance (AMR) genes to humans through the consumption of retail meat (1, 49, 51). Consequently, there is an increasing interest in characterizing virulence factors and antibiotic resistance in enterococci causing nosocomial infections in hospitals or isolated from food products (17, 23, 25, 39, 43, 44, 47). Rapid and accurate identification of infection-related enterococcal species is crucial in choosing the appropriate treatment for patients (2).

Classical biochemical identification methods relying on enzymatic activity or sugar fermentation, such as the API 20 Strep, are time-consuming and only identify a limited number of species. More importantly, they have been shown to misidentify some species (5, 7, 22, 54, 57), underscoring the limi-

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TABLE 1. Prevalence of virulence factors represented on the Enteroarray

Virulence factor	Description[a]	Presence (% total) in:		
		E. faecalis	*E. faecium*	*E. hirae* plus *E. avium*
ace	Collagen binding cell wall protein	100	0	0
agg	Aggregative adherence protein	16	0	0
agrBfs	AgrBfs protein in *E. faecalis*	100	0	0
cad1	Pheromone cAD1 precursor lipoprotein	100	0	100
cAM373 gene	Sex pheromone cAM373 precursor	100	0	0
cCF10 gene	Pheromone cCF10 precursor lipoprotein	100	0	0
cob	Pheromone cOB1 precursor/lipoprotein	100	0	0
cpd1	Pheromone cPD1 lipoprotein	100	0	0
cylA	Hemolysin	6	0	0
cylB	Hemolysin	6	0	0
cylL	Hemolysin	3	0	0
cylM	Hemolysin	3	0	0
$efaA_{Efs}$	*E. faecalis* specific cell wall adhesin	100	0	0
$efmA_{Efm}$	*E. faecium* specific cell wall adhesin	0	90.1	0
esp (*E. faecalis*)	*E. faecalis* enterococcal surface protein	0	0	0
esp (*E. faecium*)	*E. faecium* enterococcal surface protein	0	0	0
gelE	Gelatinase	100	0	0

[a] No virulence factors were found in *E. casseliflavus*, *E. gallinarum*, and *E. durans* hybridized to the Enteroarray.

tations of classical microbiological/biochemical methods. To circumvent these limitations, molecular identification and typing methods such as PCR, multilocus sequence typing, and pulsed-field gel electrophoresis have been developed (11, 26, 27, 33, 38, 40, 41, 45, 54). However, these methods usually only target a few strains or species and generally lack information on the presence of specific virulence factors and AMR genes.

The parallel processing power of DNA microarrays allows the rapid identification and virulence potential assessment of microbial pathogens (4, 18–20, 31, 39). Microarrays targeting enterococci have appeared in the literature to identify several species or to detect virulence factors in *E. faecalis.* In addition, independent microarrays have been developed for AMR gene detection (4, 18–20, 31, 39). Due to the importance of enterococci in nosocomial infections, increasing resistance to antimicrobials, and the undefined role of different enterococcal species in food-borne illnesses, a single tool to comprehensively identify and characterize the virulence potential of isolated enterococcal species would be useful in understanding their role in enterococcus-related disease.

In the present study, a DNA microarray (Enteroarray) was designed with three distinct modules: taxonomic identifiers, virulence factors, and antibiotic resistance genes, making it a powerful instrument for the rapid and comprehensive species identification of enterococcal isolates, while providing valuable information on their virulence potential. Identification of enterococcal farm isolates by the classical API 20 Strep method and the Enteroarray confirmed that the array was extremely accurate in species identification by correctly identifying isolates misidentified by API 20 Strep while simultaneously characterizing the virulence potential and antibiotic resistance phenotype of each isolate.

MATERIALS AND METHODS

Enterococcal isolation. Enterococcal strains (n = 171) were isolated from various samples (fecal, cecal, and litter) from nine commercial and experimental broiler farms in the Fraser Valley of British Columbia. The commercial farms each raise, on average, 130,000 to 360,000 broilers annually, while approximately 3,600 broilers are produced annually on the experimental farms. The samples (5 to 6 g) in Carry-Blair medium (Quelab, Montreal, Quebec, Canada) were vigorously vortexed for 1 min, and then 10-fold serial dilutions were prepared in sterile saline. Presumptive *Enterococcus* populations were isolated by spreading 10-fold dilutions of individual samples from each farm onto KF streptococcal agar CM0701 (Oxoid, Nepean, Ontario, Canada) and incubating them at 37°C for 48 h as described elsewhere (9, 23). Six to eight typical presumptive colonies per sample were randomly selected, and a total of 171 isolates were confirmed as enterococci by Gram staining, catalase test, and API 20 Strep (bioMérieux, St-Laurent, Quebec, Canada). All 171 presumptive enterococcal isolates were subjected to the API 20 Strep (bioMérieux, St-Laurent, Quebec, Canada). Isolates from cecal samples were obtained at necropsy. Purified *Enterococcus* colonies were frozen in tryptic soy broth (TSB) containing 25% glycerol at −80°C for further analysis. Six *Enterococcus* species were obtained from the American Type Culture Collection (ATCC) as controls to validate the Enteroarray: *E. avium* ATCC 49463, *E. durans* ATCC 11576, *E. faecium* ATCC 35667, *E. gallinarum* ATCC 49608, *E. faecalis* ATCC 29212, and *E. hirae* ATCC 10541.

DNA extraction. Bacterial lysates were prepared for each isolate. A single colony was grown overnight in 1 ml of TSB in a 15-ml snap-cap tube. The cells were centrifuged at 12,000 × *g* for 10 min, and the supernatant was discarded before resuspending the pellet in 200 μl of high-pressure liquid chromatography water. Cells were lysed by boiling for 15 min and then placed on ice for 1 min. The lysed preparation was centrifuged at 12,000 × *g* for 5 min to pellet cell debris, and the supernatant containing the DNA was transferred in a sterile 1.5-ml Eppendorf tube.

Enterococcal microarray design. Our enterococcal microarray represents a composite of three separately designed modules: AMR genes, virulence genes, and taxonomic genes.

(i) Antibiotic resistance. We had previously designed and validated antibiotic resistance microarrays for use with both Gram-negative and Gram-positive bacteria (4, 20, 37) using a 70-mer probe length to maximize the overall sensitivity of the microarray (32). A total of 173 probes were used, including 166 antibiotic resistance genes and a class 1 integron. Sixty-five oligonucleotide probes printed on the microarray were selected directly from a previous study (18).

(ii) Virulence factors. OligoPicker software (56) was used to design 70-mer oligonucleotide probes targeting 15 virulence factors associated with *E. faecalis* and two associated with *E. faecium* (Table 1). The targeted virulence factors include hemolysins, gelatinases, surface adhesion proteins, aggregation substances, surface carbohydrates, collagen adhesins, and sex pheromones, which have been reported to be primarily found in isolates from foods of animal origin (13, 39).

(iii) Taxonomic identifiers. Taxonomic microarrays generally rely on 16S or 23S rRNA genes or the intervening sequence to identify bacterial species (31, 58). A previous enterococcus-specific array used 18-mer sequences to define species-specific probes. Because of the high similarities observed when the enterococcal 16S rRNA sequences were aligned, probes of >18 bases would lose

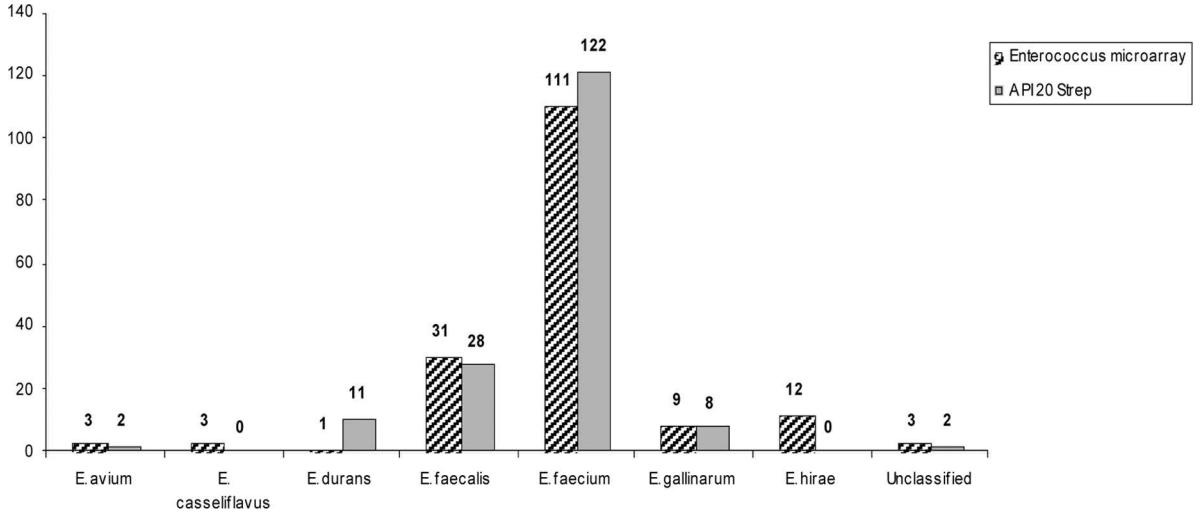


FIG. 1. Identification of enterococcal isolates by the Enteroarray and API 20 Strep tests. The results of the Enteroarray are presented in the hatched columns, while the gray columns represent the results of the API 20 Strep biochemical method. The biochemical method failed to identify all of the *E. casseliflavus* and *E. hirae* isolates.

their discriminatory ability if used on a microarray. Therefore, this approach could not be applied to the current virulence array when trying to match the length of the virulence factors and antibiotic resistance genes designed previously. Another more genetically diverse candidate gene, *cpn60* (heat shock protein), also frequently used in bacterial taxonomy (10, 34) was also eliminated for the same reasons as the 16S rRNA sequence since 28-mers were the longest species-specific probes that could be designed for enterococci.

To replace 16S and *cpn60*, we chose four genes—*ddl*, *pheS*, *atpA*, and *recA* (12, 28, 41, 50)—based on the availability of their sequences in GenBank, and their high level of interspecies sequence variation. However, specific 70-mer probes could not be designed for all enterococcal species, therefore we opted for 50-mers. The T_m of the 50-mer probes was close enough to that of the 70-mer virulence factor and antibiotic resistance gene probes to allow their printing on the same microarray since, at those lengths, any intensity differences between the different size probes are minor and have no effect on positive gene scoring.

Eighteen *Enterococcus* species most commonly used for the development of new identification methods (28, 31) were targeted for the Enteroarray. Confirmation of selected enterococcal isolates was performed by sequencing a segment of the *cpn60* gene amplified by the universal *cpn60* primers H729 (5′-CGC CAG GGT TTT CCC AGT CAC GAC GAI III GCI GGI GAY GGI ACI ACI AC-3′) and H730 (5′-AGC GGA TAA CAA TTT CAC ACA GGA YKI YKI TCI CCR AAI CCI GGI GCY TT-3′), including the M13F and M13R sequences, respectively (underlined) (34).

The universal eubacterial probe EUB338 was used as a positive control, and two types of negative controls were present, a shuffled version of the EUB338 probe and 50% dimethyl sulfoxide buffer with no oligonucleotide probe sequence. The microarrays were printed as previously described (3, 4).

***Enterococcus* DNA labeling.** Bacterial DNA was labeled using a Bioprime DNA labeling system (Invitrogen Life Technologies, Burlington, Ontario, Canada). A 10-μl portion of bacterial lysate was added to 10 μl of a random primer solution (750 μg of oligodeoxyribonucleotide primers [random octamers]/ml in 125 mM Tris-HCl [pH 6.8], 12.5 mM $MgCl_2$, and 25 mM 2-mercaptoethanol) and denatured at 95°C for 5 min. After a 5-min incubation on ice, the reaction mixture was completed by adding 5 μl of a deoxyribonucleotide triphosphate mix (1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dTTP, and 0.6 mM dCTP in 10 mM Tris [pH 8.0] and 1 mM disodium EDTA), 2 μl of 1 mM Cy5-dCTP and 0.5 μl of high-concentration DNA polymerase (Klenow fragment, 40 U/μl) for a total reaction volume of 27.5 μl. Labeling reactions were performed in the dark at 37°C for 3.5 h and stopped by the addition of 2.5 μl of 5 M disodium EDTA (pH 8.0). The labeled samples were then purified with a Pure-Link PCR purification kit (Invitrogen Life Technologies) according to the manufacturer's protocol. The amount of incorporated fluorescent Cy5 dye was then quantified by scanning the DNA sample with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) from 200 to 700 nm. The data were analyzed by using an online percent incorporation calculator (http://www.pangloss.com/seidel/Protocols/percent_inc.html).

Hybridization of labeled DNA. Microarrays were prehybridized at 50°C for 1 h under a Hybri Slip coverslip (22 by 60 mm; Sigma Chemical Co., St. Louis, MO) in a Slide Booster hybridization work station (model SB800; Advalytix, Germany) with 50 μl of DIG Easy Hyb buffer (Roche Diagnostics, Laval, Quebec, Canada) containing 5% (vol/vol) bovine serum albumin (1 mg/ml; New England BioLabs, Inc., Beverly, MA). The slide was then dipped in 0.1× SSC (15 mM NaCl plus 1.5 mM trisodium citrate [pH 7.0]) to remove the coverslip and air dried. Before hybridization, 1 μg of labeled DNA was dried by using a SpeedVac (DNA SpeedVac DNA110; Savant Scientific) and resuspended in 18 μl of DIG Easy Hyb buffer (Roche Diagnostics). The samples were denatured by heating 5 min at 95°C and pipetted under a Lifter cover slip (18 by 18 mm; Erie Scientific, Portsmouth, NH). Microarrays were then hybridized overnight at 50°C in the slide booster. The coverslips were removed by dipping the slide in 0.1× SSC–0.1% (wt/vol) sodium dodecyl sulfate (pH 7.2) and four stringency washes (three in 0.1× SSC–0.1% [wt/vol] sodium dodecyl sulfate and one in 0.1× SSC) were performed at 37°C for 5 min with agitation. The slide was then air dried and scanned at a resolution of 10 μm at 90% laser power with a ScanArray Lite fluorescent microarray analysis system (Perkin-Elmer, Mississauga, Ontario, Canada). Acquisition of fluorescent spots and quantification of fluorescent spot intensities were performed using the ScanArray Express software, version 2.1 (Perkin-Elmer, Foster City, CA). The data was normalized by subtracting local background intensity from the recorded spot intensities from one subarray. For each subarray, the median value for each set of triplicate-spotted oligonucleotides was compared to the median value for the whole subarray. Oligonucleotides with a signal-to-noise fluorescence ratio above 2 were considered positive. To identify an isolate down to the species level, a minimum of three of the four taxonomic probes designed for a particular species must show a positive signal before it was considered positively identified.

RESULTS

Taxonomy. A total of 171 presumptive enterococcal isolates were subjected to the API 20 Strep commercial identification system. Of the 171 isolates, all were confirmed to be enterococci (Fig. 1) and were distributed among five different enterococcal species as follows: *E. faecium* ($n = 122$), *E. faecalis* ($n = 28$), *E. durans* ($n = 11$), *E. gallinarum* ($n = 8$), and *E. avium* ($n = 2$).

The same 171 isolates were labeled and hybridized on the Enteroarray. Similar to the API 20 Strep results, all isolates were confirmed to be enterococci; however, they were distributed among seven different enterococcal species as follows: *E. faecium* ($n = 111$), *E. faecalis* ($n = 31$), *E. hirae* ($n = 12$), *E.*

TABLE 2. Differential isolate identification by API 20 Strep, Enteroarray, and *cpn60* sequencing

Isolate	Phenotypic identification API 20 Strep		Genotypic identification (Enteroarray)	*cpn60* sequencing BLAST result[a]		
	Species	% Identity		Species	% Identity	Accession no.
2954	*E. faecalis*	97.0	*E. avium*	ND		
2911	*E. faecium*	99.8	*E. avium*	*E. avium*	98	AF417583
2803	*E. faecium*	99.9	*E. casseliflavus*	*E. casseliflavus*	98	AF417584
2876	*E. faecium*	99.9	*E. casseliflavus*	*E. casseliflavus*	98	AF417584
2982	*E. faecium*	99.9	*E. casseliflavus*	*E. casseliflavus*	100	AF417584
2902	*E. durans*	91.9	*E. faecalis*	*E. faecalis*	98	DQ074968
1737	*E. faecium*	92.0	*E. faecalis*	*E. faecalis*	99	AF335185
2814	*E. faecium*	99.8	*E. faecalis*	*E. faecalis*	99	DQ074968
2934	*E. faecium*	69.3	*E. faecalis*	*E. faecalis*	99	DQ074968
2989	*E. faecium*	69.3	*E. faecalis*	*E. faecalis*	99	DQ074968
2990	*E. faecium*	69.3	*E. faecalis*	*E. faecalis*	99	DQ074968
2917	*E. durans*	90.1	*E. faecalis*	*E. faecium*	100	AF417582
1695	*E. faecalis*	99.9	*E. faecium*	*E. faecium*	98	AF417582
2804	*E. faecalis*	99.7	*E. faecium*	*E. faecium*	98	AF417582
1746	*E. gallinarum*	66.0	*E. faecium*	*E. faecium*	99	AF417582
1740	*E. gallinarum*	66.0	*E. faecium*	*E. faecium*	99	AF417582
2019	*E. gallinarum*	77.4	*E. faecium*	*E. faecium*	99	AF417582
2026	*E. gallinarum*	77.4	*E. faecium*	ND		
2033	*E. gallinarum*	80.6	*E. faecium*	*E. faecium*	98	AF417582
2020	*E. gallinarum*	93.8	*E. faecium*	*E. faecium*	99	AF417582
1734	*E. gallinarum*	99.6	*E. faecium*	*E. faecium*	99	AF417582
1745	*E. avium*	97.0	*E. gallinarum*	*E. gallinarum*	97	AF417587
2828	*E. faecium*	93.8	*E. gallinarum*	*E. gallinarum*	95	AF417587
2799	*E. faecium*	99.8	*E. gallinarum*	*E. gallinarum*	99	AF417587
2802	*E. faecium*	99.8	*E. gallinarum*	*E. gallinarum*	95	AF417587
2821	*E. faecium*	99.8	*E. gallinarum*	*E. gallinarum*	95	AF417587
2823	*E. faecium*	99.8	*E. gallinarum*	*E. gallinarum*	98	AF417587
2839	*E. faecium*	99.8	*E. gallinarum*	*E. gallinarum*	95	AF417587
2853	*E. faecium*	99.8	*E. gallinarum*	*E. gallinarum*	99	AF417587
2866	*E. durans*	91.7	*E. hirae*	*E. hirae*	97	AF417586
2906	*E. durans*	91.7	*E. hirae*	*E. hirae*	100	AF417586
1687	*E. durans*	95.0	*E. hirae*	*E. hirae*	98	AF417586
1696	*E. durans*	95.0	*E. hirae*	*E. hirae*	98	AF417586
1713	*E. durans*	95.0	*E. hirae*	*E. hirae*	99	AF417586
1722	*E. durans*	95.0	*E. hirae*	*E. hirae*	98	AF417586
1732	*E. durans*	95.0	*E. hirae*	*E. hirae*	99	AF417586
2029	*E. durans*	96.1	*E. hirae*	*E. hirae*	98	AF417586
1731	*E. faecium*	91.0	*E. hirae*	*E. hirae*	98	AF417586
1739	*E. faecium*	97.0	*E. hirae*	*E. hirae*	98	AF417586
1721	*E. faecium*	99.0	*E. hirae*	*E. hirae*	98	AF417586
2819	*E. faecium*	99.9	*E. hirae*	ND		
2998	*Aerococcus viridans*	99.3	No taxo probe	ND		
2941	*E. faecium*	99.8	*E. faecium* and *E. faecalis*	ND		
2985	Not valid	Not valid	One *E. faecalis* probe	ND		

[a] *cpn60* was amplified with universal primers (see Materials and Methods). ND, not done.

gallinarum ($n = 9$), *E. avium* ($n = 3$), *E. casseliflavus* ($n = 3$), and *E. durans* ($n = 1$). One isolate (isolate 2941) hybridized with probes representing both *E. faecium* and *E. faecalis*, suggesting that this sample was not obtained from a pure colony (Table 2). The same isolate was identified by API 20 Strep as being only *E. faecium*. All five ATCC strains were identified correctly on the Enteroarray. Therefore, between the two methodologies, an identification discrepancy was observed among 42 of the confirmed 171 enterococcal isolates. Unlike the 18 species that the Enteroarray was designed to identify, API 20 Strep only recognizes 5 and does not include *E. hirae* and *E. casseliflavus*. If the latter are removed from the pool of isolates, the discrepancies in species identification between the methods is ca. 15%. However, rather than indicating unknown species, API 20 Strep misclassified all three *E. casseliflavus* isolates as *E. faecium* at 99.9% certainty and all of the *E. hirae* as either *E. durans* or *E. faecium* at 91 to 99.9% certainty (Table 2). Therefore, a more conservative estimate of divergence between the methods is >24%.

Although all four taxonomic probes printed on the Enteroarray for each species always gave signals well above background, hybridization results from seven isolates gave signals for only three of the four positive probes. However, three isolates were identified as *E. casseliflavus* whose identification relies on only three taxonomic probes since the *recA* sequence was not available in GenBank. The built in redundancy of the multiple independent taxonomic probes allowed clear identification of the remaining four isolates based on the three positive signals. To further validate the accuracy of the Enteroarray, the identities of 38 isolates covering all combinations of species differences were confirmed by sequencing their *cpn60* gene, 34 of which were from misidentified isolates and 4 iso-

lates that were found to be the same species by the API 20 Strep and the Enteroarray. The top single species *cpn60* BLAST matches ranged between 95 and 100% identity with the second nearest species matching only at 89% or less. In all cases, sequencing of the *cpn60* gene agreed with the Enteroarray identification, including the four isolates that were in agreement by both methods (Table 2).

Virulence factors. All 31 isolates identified as *E. faecalis* by the Enteroarray also scored positively for a core of nine virulence factors: *ace*, $agrB_{Efs}$ (the *agrB* gene of *E. faecalis*) *cad1*, the cAM373 gene, the cCF10 gene, *cob*, *cpd1*, $efaA_{Efs}$ (the *efaA* gene of *E. faecalis*), and *gelE*. In addition to these nine virulence factors, five isolates were also positive for *agg*, one of which also possessed two hemolysins, *cylA* and *cylB*, and another with all four hemolysins (*cylA*, *cylB*, *cylL*, and *cylM*) for a total of 14 virulence factors in the same isolate (isolate 2968). Interestingly, the API 20 Strep system identified three fewer *E. faecalis* isolates than the Enteroarray. The three isolates were identified as an *E. avium* and two *E. faecium* strains. The possession of the same nine core virulence factors found in all of the Enteroarray identified isolates provides indirect support for their classification as *E. faecalis* strains as shown by the Enteroarray. None of the Enteroarray-identified isolates possessed the specific enterococcal surface protein (*esp*) from either *E. faecalis* or *E. faecium*. Esps are cell wall-associated proteins shown to be linked to urinary tract infections and are more frequently found in clinical isolates than commensal isolates (21).

Although all 31 *E. faecalis* isolates were positive for the cell wall adhesin ($efaA_{Efs}$), 100 of the 111 *E. faecium* isolates were positive for the *E. faecium*-specific cell wall adhesin $efmA_{Efm}$ (the *efmA* gene of *E. faecium*). Two other species scored positive signals for virulence factors. All *E. avium* and *E. hirae isolates* were positive for the *cad1* gene. This was the only virulence factor found in these two species, and no other virulence factors were found in species other than *E. faecalis* and *E. faecium.*

Antibiotic resistance. Many antibiotic resistance genes were identified among all of the enterococcal isolates. These genes are involved in resistance against aminoglycosides, kanamycin, neomycin, gentamicin, erythromycin, lincosamide, streptothricin, streptogramin, tetracycline, and bacitracin, and well as vancomycin. We also identified some genes associated with macrolide efflux pumps and transposons.

The *E. faecium* isolates possessed antibiotic resistance genes associated with 10 different antimicrobial agent classes, more than the six other species identified by the Enteroarray, where it ranged from two to eight classes (Table 3). Transposon-associated genes were found in all seven species and mostly in *E. casseliflavus*, *E. durans*, *E. faecium*, and *E. hirae*. Genes associated with tetracycline resistance were found in six species and were prevalent in 67 to 100% of the isolates for each species. Genes associated with aminoglycosides and bacitracin resistance were found in five species and in 100% of *E. durans* and *E. hirae* isolates for bacitracin and in 98% of the *E. faecium* isolates for aminoglycosides. Erythromycin-associated genes were found in four species, mostly in *E. faecalis* and *E. faecium*, and in ca. 50% of the *E. gallinarum* and *E. hirae* isolates. Vancomycin resistance genes were only present in *E. casseliflavus* and *E. gallinarum*, with all *E. gallinarum and E. casseliflavus* isolates carrying the *vanC1* and *vanC3* genes, respectively. The other antimicrobial agent classes were present in one to three species and in <45% of the isolates.

DISCUSSION

The custom-designed Enteroarray, possessing a taxonomic module in addition to virulence and antibiotic resistance gene modules, was able to accurately identify all isolates to the species level, unlike a standard methodology such as API 20 Strep, which had a 24% misidentification rate in our study.

Identification accuracy with the Enteroarray was high due to the built-in redundancy of using four ubiquitous enterococcal taxonomic probes per species. Validation of the Enteroarray with confirmed ATCC species, and later confirmation by *cpn60* sequence analysis of selected isolates showing disagreement between the two methods (Table 2), showed that the probes are highly specific since only one probe for *E. hermanniensis* showed cross-hybridization with all of the *E. hirae* isolates (data not shown). Although any one gene would have been sufficient to correctly identify most of the 18 enterococcal species targeted in the present study, the built-in redundancy helped bypass cases where genetic drift would be sufficient to reduce or eliminate hybridization of one of the genes. Indeed, there were 9 isolates among the 171 examined where one of the four genes did not result in a positive signal. Among those nine, only two taxonomic probes resulted in a false-negative result, *ddl* (three *E. gallinarum* isolates) and *recA* (five *E. faecalis* and one *E. faecium* isolate). As noted earlier, *E. casseliflavus* had only three probes designed for its identification.

In the present study, of the five species that API 20 Strep claims to identify, this methodology misidentified 15% of them. This underestimates the real rate (~24% in this study) since this method does not give a negative or "unknown" response to those enterococcal species not included here. For example, *E. casseliflavus* and *E. hirae* isolates were identified as *E. faecium* or *E. durans* to a high degree of certainty.

In addition to validating the taxonomic identification section of the Enteroarray using five ATCC *Enterococcus* species recognized by the API 20 Strep kit, we also picked other isolates from each of the seven species identified in the present study to independently confirm species identification by *cpn60* sequencing. Species identification of all sequences was confirmed by BLAST analysis and in all cases was in agreement with the Enteroarray results. It is especially important to note that of five API 20 Strep-identified *E. faecium* isolates at high levels of certainty, only one was correct, and the other four were misidentified as four separate species (*E. casseliflavus*, *E. faecalis*, *E. gallinarum*, and *E. avium*). Therefore, we can conclude that the *Enterococcus* microarray is a more powerful tool for the identification of enterococci to the species level than the API 20 Strep system that relies on biochemical tests. Although the cost of the microarray is higher than the API 20 Strep, the poor accuracy of the API 20 leaves the validity of any conclusions related to species identified by this methodology in doubt. One other publication has used a 16S/23S oligonucleotide DNA microarray to identify a range of enterococcal species similar to our study (31). Successful species discrimination was achieved by hybridizing specific PCR amplicons derived from either whole strains or *E. faecium*/*E. faecalis*-spiked milk. By

TABLE 3. Distribution of antimicrobial agents in enterococcal isolates

Species (no. of strains)	No. of strains (%)	Antibiotic resistance gene class(es)[a]	% Isolates with indicated ABR gene(s)
E. durans (1)	1 (100)	TE, B	TetO, 100; BcrR, 100
E. avium (3)	1 (33)	TE	Tetracycline, 67
	1 (33)	A, TE	*tetL*, 33
	1 (33)	No ABR gene	*tetM*, 67
			Aac(6), 33
E. casseliflavus (3)	3 (100)	V	VanC3, 100
	1 (10)		VanC1, 100
	5 (50)	V	Tetracycline, 90
E. gallinarum (10)	1 (10)	TE, V	*tetM*, 60
	1 (10)	E, TE, V	*tetS*, 30
	1 (10)	E, TE, V, B	*tetL*, 20
	1 (10)	A, E, TE, V	Erythromycin, 40
		A, E, TE, V, B	*ermAM*, 30
			ermB, 30
			ermA, 10
			BcrR, 20
			Aminoglycosides, 20
			aac(6′)-aph(2′), 10
			ant(9′)-Ia, 10
	1 (8)		BcrR, 100
	2 (17)	A, B	Tetracycline, 83
	1 (8)	TE, B	*tetM*, 58
	2 (17)	E, B	*tetL*, 50
E. hirae (12)	1 (8)	E, TE, B	*tetO*, 42
	1 (8)	A, L, TE, B	Aminoglycosides, 50
	1 (8)	A, E, TE, B	*aac(6)*, 25
	1 (8)	TE, STA, B	*aac(6′)-aph(2′)*, 17
	1 (8)	A, E, TE, STA, B	*ant(9)-Ia*, 17
	1 (8)	A, L, TE, B, STA	*aph(3′)-IIIa*, 8
		A, E, STC, TE, STA, B	Erythromycin, 50
			ermAM, 33
			ermB, 33
			ermA, 17
			SatG-VatE8, 33
			LinB, 17
			Sat4, 8
	3 (10)	E, B	Erythromycin, 90
	1 (3)	TE, B	*ermB*, 81
	1 (3)	E, TE	*ermAM*, 87
E. faecalis (31)	2 (6)	A, TE	Tetracycline, 87
	14 (45)	E, TE, B	*tetK*, 3
	1 (3)	A, E, STC, B	*tetL*, 71
	1 (3)	A, E, STC, TE	*tetM*, 84
	8 (26)	A, E, STC, TE, B	BcrR, 84
			Aminoglycosides, 39
			aac(6), 29
			aac(6′)-aph(2′), 6
			aph(3′)-IIIa, 29
			Sat4, 29
	3 (3)	A	Aminoglycosides, 99
	1 (1)	TE, B	*aac(6′)-Ii*, 99
	1 (1)	A, B	*aac(6)*, 55
	9 (8)	A, EES	*aac(6′)-aph(2′)*, 1
	2 (2)	A, L, TE	*ant(9)-Ia*, 5
	2 (2)	A, TE, B	*ant(2″)-Ia*, 1
	1 (1)	A, E, EES	*aph(3′)-IIIa*, 33
	2 (2)	A, STA, EES	Macrolide efflux system, 89
	6 (5)	A, B, EES	*mefA-mefE*, 16
	1 (1)	A, TE, EES	*msrC*, 85
	9 (8)	A, TE, B, EES	Tetracycline, 80
	8 (7)	A, E, TE, EES	*tetL*, 65
	1 (1)	A, TE, STA, EES	*tetM*, 78

Continued on following page

TABLE 3—*Continued*

Species (no. of strains)	No. of strains (%)	Antibiotic resistance gene class(es)[a]	% Isolates with indicated ABR gene(s)
	7 (6)	A, TE, STA, B, EES	*tetO*, 2
	5 (4)	A, E, TE, B, EES	*tetS*, 1
E. faecium (111)	1 (1)	A, L, TE, STC, B	BcrR, 74
	2 (2)	A, E, TE, STA, B	Erythromycin, 54
	3 (3)	A, L, TE, B, EES	*ermA*, 5
	2 (2)	A, L, TE, STA, B, EES	*ermB*, 42
	2 (2)	A, E, L, TE, B, EES	*ermAM*, 52
	2 (2)	A, E, TE, STA, B, EES	LinB, 43
	2 (2)	A, E, TE, STC, B, EES	SatG-VatE, 40
	1 (1)	A, E, L, TE, STA, EES	Sat4, 28
	1 (1)	A, E, TE, STC, STA, B, EES	
	5 (4)	A, E, L, TE, STA, B, EES	
	1 (1)	A, L, TE, STC, STA, B, EES	
	5 (4)	A, E, L, TE, STA, B, EES	
	1 (1)	A, E, L, TE, STC, STA, EES	
	11 (10)	A, E, L, TE, STC, B, EES	
	14 (13)	A, E, L, TE, STC, STA, B, EES	

[a] A, aminoglycosides; TE, tetracycline; B, bacitracin; L, lincosamide; STA, streptogramin A; V, vancomycin; E, erythromycin; STC, streptothricin; EES, erythromycin efflux system.

utilizing four different housekeeping genes rather than rRNA gene regions, we were able to increase the number of different specific probes per species to allow for a more robust assay. Moreover, with longer oligonucleotides (50 to 70 versus 18), the Enteroarray has increased target signal intensity. This also permits the addition of a wealth of different gene probes to extract a wide range of genomic information per hybridization with regard to virulence and antibiotic resistance gene content, thus representing a large improvement over PCR-based assays.

Infections caused by *E. faecium* are becoming more frequent, presumably due to increasing resistance to many antimicrobial agents commonly used in patient treatment regimes. *E. faecium* is equally responsible, along with *E. faecalis*, for enterococcus-related nosocomial infections in hospitals through two different mechanisms: antimicrobial agent resistance and virulence (53). According to the literature, no virulence factors have been characterized in enterococcal species other than *E. faecalis* and *E. faecium* (44). However, Fontana et al. (16) found *cad1* in *E. casseliflavus* but since this isolate did not display a *vanC* resistance phenotype, a hallmark of this species, its identity remains unclear. With the Enteroarray, the *cad1* gene probe was positive in all *E. faecalis*, *E. hirae*, and *E. avium* isolates, as well as in the ATCC strains used as positive controls for these species. It was not found in *E. casseliflavus*, *E. durans*, or *E. faecium*. Surprisingly, subsequent verification by PCR with primers directed against the *E. faecalis* gene sequence showed that only *E. faecalis* isolates produced an amplicon. To validate the genomic target sequence in either *E. hirae* and *E. avium*, we performed asymmetric genome walking to amplify the target for sequencing (48). Interestingly, sequence analysis from *cad1* primed amplicons from either *E. hirae* or *E. avium* produced a similar hypothetical protein. The lack of a positive *cad1* probe in *E. casseliflavus*, *E. durans*, or *E. faecium* indicates that this hypothetical protein is not universal, nor is it a variant of *cad1*, suggesting that our *cad1* probe needs to be redesigned.

There is a growing interest for the characterization of enterococci isolated from food since microbial AMR genes present in retail meat or dairies could possibly be transferred to the consumer (17, 23, 39, 43, 47, 49). Some AMR genes have been described in the literature as species characteristic and consequently have been used for their identification (5, 6). For example, Costa et al. (6) indicated that the presence of the *aac(6′)-Ii* gene, causing resistance to aminoglycosides was specific for *E. faecium*. With 110 of the 111 *E. faecium* isolates being positive for this gene, our data clearly support that observation. This gene was not found in any other isolate belonging to six other species. Therefore, the *aac(6′)-Ii* gene appears to be a good marker to identify *E. faecium* but only when present, as some *E. faecium* isolates can be found lacking this gene. This observation has also been observed by Kobayashi et al. (29), who detected *aac(6′)-Ii* exclusively in *E. faecium* but not in every isolate. Indirectly, the *aac(6′)-Ii* probe on our Enteroarray further confirms and supports the accuracy of the four taxonomic probes in properly identifying *E. faecium* isolates misidentified by the API 20 Strep method. The intrinsic low-level vancomycin resistance observed in *E. gallinarum* and *E. casseliflavus* conferred by the *vanC1* and *vanC3* genes, respectively, has become a species-specific characteristic and is commonly used as a molecular marker for their identification (5, 22, 54). All isolates in our study identified as *E. gallinarum* and *E. casseliflavus* by the Enteroarray, including the two ATCC strains, possessed either the *vanC1* or the *vanC3* gene. None of the isolates distributed among the other five species possessed either of these genes. This is consistent with the literature and also indirectly confirmed the identity of these isolates. The relationship between phenotypic antibiotic resistance and the genotype identified by the Enteroarray was presented elsewhere (8).

In addition to their use as therapeutic agents for animals, antimicrobial agents are also used to accelerate animal growth (15). The increase in the occurrence of antibiotic resistance genes in food is possibly a consequence of the use of antimicrobial agents in agriculture. In the present study, the treat-

ment with antimicrobial agents of the broiler chickens, from which the enterococci were isolated, may have influenced the occurrence and type of AMR genes associated with the different antimicrobial agent classes (8). Bacitracin was used as a growth promoter by all of the farms from which the chicken enterococcal samples were isolated. The *bcrR* gene, associated with bacitracin resistance, was present in most of the isolates but in none of the ATCC strains, which suggests that it could be related to the bacitracin used as in the study farm's feed supplement. This type of dissemination has also been observed in another study (36).

The presence of virulence factors and antibiotic resistance genes in the present study does not mean that these isolates can lead to difficulties in treating infections but rather that these genes are a good indicator of their potential as human pathogens. Consequently, their presence should not be underestimated. The Enteroarray is a rapid and robust tool for the identification and characterization of *Enterococcus* isolates to the species level. This microarray can be very useful in a clinical setting where enterococci causing nosocomial infections need to be characterized to direct subsequent patient treatment as well as in food safety and environmental assessment studies. In addition, it can be useful in agricultural settings to measure the impact of different feed or treatment methods on *Enterococcus* populations.

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