Front. Biol. 2012, 7(2): 167–177
DOI 10.1007/s11515-012-1183-5

RESEARCH ARTICLE

Enterococcus faecalis can be distinguished from *Enterococcus faecium* via differential susceptibility to antibiotics and growth and fermentation characteristics on mannitol salt agar

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Abstract ***Enterococcus faecalis*** **and** ***Enterococcus faecium*** **are both human intestinal colonizers frequently used in medical bacteriology teaching laboratories in order to train students in bacterial identification. In addition, hospitals within the United States and around the world commonly isolate these bacteria because they are a cause of bacteremia, urinary tract infections, endocarditis, wound infections, and nosocomial infections. Given that enterococci are becoming more of a world health hazard, it is important for laboratories to be able to distinguish these bacteria within hospitalized patients from other bacterial genera. In addition, laboratories must differentiate different species within the** ***Enterococcus*** **genus as well as different strains within each species. Though enterococci are differentiated from other bacterial genera via classical culture and biochemical methods, nucleic acid sequencing is required to differentiate species within the genus—a costly, time consuming, and technically challenging procedure for laboratory technicians that, in itself, does not necessarily lead to speedy identification of bactericidal antibiotics. In this study, we perform antibiogram analysis to show (1) that penicillin can be rapidly employed to distinguish strains and clinical isolates of** ***E. faecalis*** **from** ***E. faecium*****, (2)** ***E. faecalis*** **is susceptible to ampicillin, and (3) that vancomycin resistance in enterococci shows no sign of abating. Additionally, we show that** ***E. faecalis*** **can grow on mannitol salt agar and ferment mannitol, while** ***E. faecium*** **lacks these phenotypes. These data reveal that we now have rapid, cost effective ways to identify enterococci to the species, and not just genus, level and have significance for patient treatment in hospitals.**

Keywords enterococci, *Staphylococcus*, antibiotic resistance, bacteriology, microbiology

Introduction

The enterococci are a genus of catalase-negative, Gram-positive bacteria that comprise 38 different species, two of the most important of which are *Enterococcus faecalis* and *Enterococcus faecium* (Devriese et al., 2006; Murray et al., 2009; Teixeira et al., 2011; Vu and Carvalho, 2011). Both are human intestinal colonizers frequently used in medical bacteriology teaching laboratories in order to train clinical microbiology students in bacterial identification. In addition, hospitals around the world commonly isolate these bacteria from patients because they are a cause of bacteremia, urinary tract infections, endocarditis, wound infections, meningitis, intraabdominal and pelvic infections, and nosocomial and iatrogenic infections (Megran, 1992; Moellering, 1992; Emori and Gaynes, 1993; Murray et al., 2009; Teixeira et al., 2011; Vu and Carvalho, 2011). Aside from hospital patients, *Enterococcus faecalis* has also been discovered in the waters and sands of the world's beaches, such as the coastline of Southern California, where sewage contamination and river runoff have increased its numbers (Yamahara et al., 2007).

As with diseases caused by other bacteria, those induced by

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the enterococci have grown in importance as a direct result of the recent decline in the clinical microbiology workforce (Carvalho, 2011). The lack of clinical microbiologists prevents hospitals from rapidly and effectively diagnosing pathogens. Consequently, hospital physicians end up treating patients without laboratory identification or the antibiotic susceptibility profile of the disease-causing microbe (Carvalho, 2011). As a result, most physicians prescribe "broad spectrum" antibiotics to resolve patient symptoms. Though this approach quickly offers some patients desired relief, antibiotic-resistant bacteria can emerge, such as with respect to vancomycin-resistant *Enterococcus faecalis* (Noble et al., 1992; Clark et al., 1993; Woodford et al., 1995; Courvalin, 2006; Rice, 2006; Carvalho, 2011). Indeed, treatment of hospitalized patients with broad-spectrum antibiotics alone or in combination with improper patient usage can often result in the spread of normal enterococcal flora from the intestinal tract to other mucosal and skin surfaces where they can cause disease (Murray et al., 2009).

Given the ubiquity of enterococci within the human population, it is important for laboratories to be able to distinguish these agents within hospitalized patients from other bacterial genera and also *differentiate* different species within the *Enterococcus* genus as well as different strains within each species. Similarly, it is important to perform surveillance of the antibiotic susceptibility profile of *Enterococcus* strains or clinical isolates in order to better identify the most efficacious bactericidal medicines to be administered to infected patients. With regards to the first point, enterococci grow on enriched sheep blood agar (Lehman et al., 2007; Murray et al., 2009; Teixeira et al., 2011) and can be discriminated from other bacterial genera on the basis of catalase-negativity (to differentiate from *Staphylococcus*, *Micrococcus*, and *Stomatococcus*), ability to grow in high NaCl (to differentiate from *Streptococcus*), optochin resistance (to differentiate from *Streptococcus pneumoniae*), resistance to bile dissolvability (to differentiate from *Streptococcus pneumoniae*), and production of L-pyrrolidonyl arylamidase (PYR) (to differentiate from *Streptococcus*) (Devriese et al., 2006; Lehman et al., 2007; Fisher and Phillips, 2009; Murray et al., 2009; Teixeira et al., 2011). Though enterococci are differentiated from other bacterial genera via these culture and biochemical methods, molecular technology like nucleic acid sequencing is presently required to differentiate species within the genus—a costly, time consuming, and technically challenging procedure for laboratory technicians (Domig et al., 2003; Lehman et al., 2007; Murray et al., 2009). Indeed, our medical bacteriology laboratories at CSUDH presently do not employ nucleic acid sequencing on a daily basis to identify laboratory unknowns of enterococci to the species level given the expense of this procedure and the time constraints within the laboratory agenda. More rapid methods are required for student teaching laboratories where many unknowns are being diagnostically worked on simultaneously (though students are, of course, informed of sequencing technology). A parallel scenario exists in developing world hospitals because sequencing technology is too costly and these hospitals lack the trained personnel to do the sequencing.

With regards to the second point, identification of *Enterococcus* species does not automatically yield information about what antibiotics are needed to relieve patient symptoms or to cure patients of infection. Full antibiogram diagnostic testing is normally performed to acquire such data. Given the personnel shortage, as described above, this diagnostic testing may not always be performed and, consequently, physicians might improvise by prescribing last line (more potent) antibiotics that may or may not be efficacious in curing patient illness or eliminating the *Enterococcus* species or strain if the bacteria are resistant to the antibiotics or if patients are not monitored for appropriate administration of the medicines (Carvalho, 2011).

In this study, we sought to discover a set of rapid, cost effective, and technically simple procedures that could more easily differentiate strains and clinical isolates of *Enterococcus faecalis* from *Enterococcus faecium* that were acquired from various sources. In addition, we sought to determine whether or not our library of *Enterococcus* strains and clinical isolates revealed a trend in antibiotic susceptibility, which could have relevance for patient treatment.

Materials and methods

Bacterial strains and clinical isolates, reagents, and media

The bacterial strains and clinical isolates used in this study are included in Tables 1 and 2. Gram-positive bacteria were grown at 35°C in Luria Broth. Plate incubations of bacteria were grown in a candle jar or 5% CO_2. Antibiotics in the antimicrobial battery/panel were purchased from Fischer Scientific (Tustin, CA) and used at the standard following concentrations on antibiotic disks for susceptibility testing: vancomycin (5μg), ampicillin (10μg), tetracycline (30μg), amikacin (30μg), penicillin (10 units), erythromycin (15μg), streptomycin (10μg), bacitracin (10 units), polymyxin B (300 units). Sheep blood agar plates were obtained from Hardy Diagnostics (Santa Maria, CA). Mannitol salt agar, for MSA plates, and all other growth media were acquired from Fischer Scientific (Tustin, CA).

Antibiogram analysis

For antibiotic susceptibility testing, all experiments were performed in triplicate to confirm results and prevent sampling error of resistant colonies. The 0.5 McFarland standard was adhered to in preparation of liquid cultures (Forbes et al., 2007). Sterile swabs were dipped in cultures and streaked in three directions to cover the surface of sheep blood agar plates with bacteria. Using sterile forceps and antibiotic disk dispensers, antibiotic disks were placed on the

Table 1 *Enterococcus faecalis* strains or clinical isolates used in this study

Bacteria	Special characteristics	Strain or clinical isolate
Enterococcus faecalis (18)		Strain[a]
Enterococcus faecalis (9)		Strain[b]
Enterococcus faecalis (10)		Strain[b]
Enterococcus faecalis (11)		Strain[b]
Enterococcus faecalis (14)		Strain[b]
Enterococcus faecalis (310)	Weakened strain	Strain[b]
Enterococcus faecalis (313 VA-R)	Vancomycin-resistant	Strain[b]
Enterococcus faecalis (300A)		Strain[b]
Enterococcus faecalis (411)		Clinical isolate[c]
Enterococcus faecalis (412)		Clinical isolate[c]
Enterococcus faecalis (413)		Clinical isolate[c]
Enterococcus faecalis (414)		Clinical isolate[c]
Enterococcus faecalis (415)		Clinical isolate[c]
Enterococcus faecalis (416)		Clinical isolate[c]
Enterococcus faecalis (417)		Clinical isolate[c]
Enterococcus faecalis (418)		Clinical isolate[c]
Enterococcus faecalis (419)		Clinical isolate[c]
Enterococcus faecalis (420)		Clinical isolate[c]

[a] California State University Dominguez Hills (Carson, CA), [b] Microbiologics (St. Cloud, MN), [c] Focus Diagnostics (Cypress, CA).

Table 2 *Enterococcus faecium* strains or clinical isolates used in this study

Bacteria	Strain or Clinical Isolate
Enterococcus faecium (5)	Strain[a]
Enterococcus faecium (8)	Strain[a]
Enterococcus faecium (316)	Clinical isolate[b]
Enterococcus faecium (152A)	Clinical isolate[b]
Enterococcus faecium (401)	Clinical isolate[c]
Enterococcus faecium (402)	Clinical isolate[c]
Enterococcus faecium (403)	Clinical isolate[c]
Enterococcus faecium (404)	Clinical isolate[c]
Enterococcus faecium (405)	Clinical isolate[c]
Enterococcus faecium (406)	Clinical isolate[c]
Enterococcus faecium (407)	Clinical isolate[c]
Enterococcus faecium (408)	Clinical isolate[c]
Enterococcus faecium (409)	Clinical isolate[c]
Enterococcus faecium (410)	Clinical isolate[c]
Enterococcus faecium (421)	Clinical isolate[c]
Enterococcus faecium (422)	Clinical isolate[c]

[a] Microbiologics (St. Cloud, MN), [b] Clinical Diagnostics Laboratory at Los Angeles Harbor General Hospital (Torrance, CA), [c] Patient clinical isolates from Focus Diagnostics (Cypress, CA).

plates, but were not placed closer than 15mm from the edge of the plates or closer than 24mm from each other (center to center). Plates were incubated at 35°C for no longer than 24 h. Zones of inhibition were measured around each disk in mm and susceptibility values were interpreted by comparing these measurements to the zone diameters published in the *Manual of Clinical Microbiology* or the *National Committee for Clinical Laboratory Standards (NCCLS)* (Jorgensen and Turnridge, 2007).

Mannitol salt agar

Using sterile loops, bacteria were streaked in six quadrants on mannitol salt agar (MSA) plates. Plates were incubated at 35°C for 24 h and growth was observed and recorded. Fermentation was recorded where the plate revealed a yellow quadrant. (The organic acids produced by mannitol lower the pH of the medium around the bacterial colony, so the indicator, phenol red, changes from red to yellow) (Leboffe and Pierce, 2005).

Results

Bacterial strains and clinical isolates used in this study

The bacterial strains and clinical isolates used in this study are listed in Tables 1 and 2. We sought to compare our medical microbiology laboratory strains at California State University Dominguez Hills (CSUDH) with clinical isolates from the Clinical Diagnostics Laboratory at Los Angeles Harbor Hospital (Torrance, CA), clinical isolates from Focus Diagnostics (Cypress, CA), and with strains of enterococci from Microbiologics (St. Cloud, MN). A highly susceptible *Enterococcus faecalis* strain and one that is vancomycin-resistant were also acquired from Microbiologics and were used as controls for antibiotic susceptibility testing (see below). We also tested *Enterococcus faecium* strains acquired from the same company. *Staphylococcus aureus* was used as a positive control and *Staphylococcus epidermidis* was employed as a negative control in the mannitol salt agar assays (see below) (Harrison, 2007). Both

of the staphylococci were acquired from our medical bacteriology laboratory at CSUDH.

Antibiotic susceptibility testing of enterococci

We hypothesized that if *E. faecalis* and *E. faecium* were susceptible to different antibiotics, then we could use antibiogram analysis to discover one or more antibiotics that could be used to distinguish *E. faecalis* from *E. faecium* in the laboratory setting. Toward this end, we performed susceptibility tests on all our enterococci using a range of antibiotics commonly employed as bacteriostatic/bactericidal agents for Gram-positive microbes. It is important to note that Mueller-Hinton agar is the standard agar base medium for testing most bacterial genera in antibiotic susceptibility testing given its batch-to-batch uniformity in clinical laboratories around the country (Forbes et al., 2007). However, enterococci are fastidious organisms and they prefer a blood based medium for quality growth (Murray et al., 2009; Teixeira et al., 2011) and, as a result, all of our tests were conducted on sheep blood agar, much like what would be done in a clinical laboratory. It is true that an extension of these tests could be conducted on Mueller-Hinton agar as a follow-up study, but one would suspect that the results would not be different. Rather, the zones of inhibition might be greater, but still within the appropriate ranges, given the weaker growth of the enterococci on Mueller-Hinton agar. Furthermore, clinical laboratory scientists are aware of the fastidious nature of enterococci and virtually never use Mueller-Hinton agar as the sole medium of choice for their antibiotic susceptibility testing.

Results of the antibiotic disk diffusion susceptibility tests are shown in Table 3. As examples, zones of growth inhibition at 24 h for some of the enterococci are shown in Fig. 1 (*E. faecalis* 11 and 14, and *E. faecium* 5 and 8). Our two control strains behaved as expected in the antibiograms: *E.*

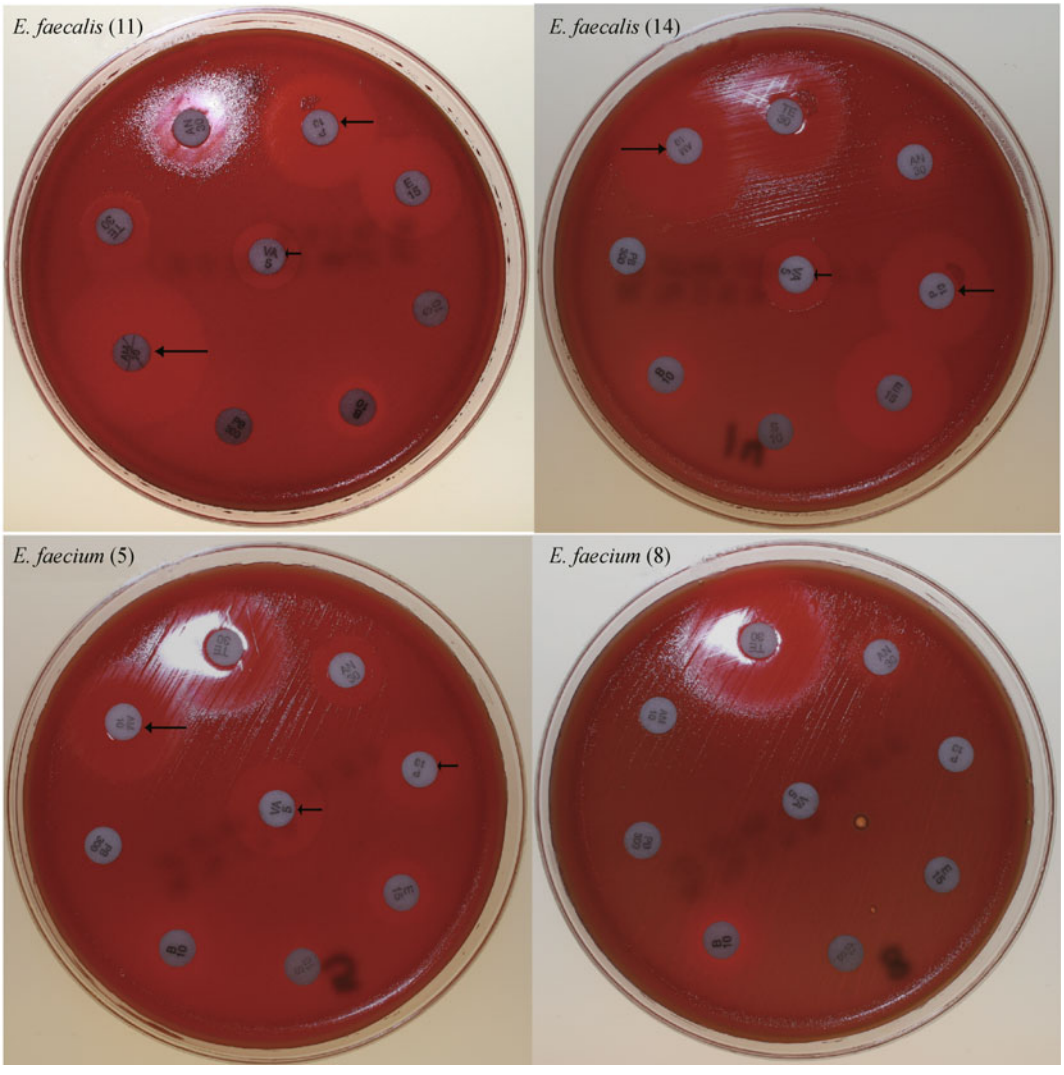

Figure 1 Zones of growth inhibition after 24 h for four representative enterococci. The arrows indicate the zone of inhibition following 24 h of incubation. The measurements of the arrows are found in Tables 3 and 4.

Table 3 Antibiotic disk diffusion susceptibility testing for *E. faecalis* and *E. faecium*

	Average disk diffusion sensitivities, zone diameter (mm)								
	VA 5*	AM 10*	TE 30	AN 30	P 10*	E 15	S 10	B 10	PB 300
E. faecalis (18)	12.5*	20.8*	14.8	3.3	18.3*	17.0	0.0	9.0	0.0
E. faecalis (9)	10.0*	24.0*	20.0	12.0	18.0*	16.0	0.0	10.0	10.0
E. faecalis (10)	12.0*	26.0*	20.0	10.0	18.0*	20.0	0.0	16.0	12.0
E. faecalis (11)	10.0	24.0*	8.0	8.0	18.0*	16.0	0.0	10.0	10.0
E. faecalis (14)	12.0*	24.0*	18.0	8.0	18.0*	20.0	0.0	14.0	12.0
E. faecalis (300A)	12.3*	25.0*	7.5	4.0	18.5*	13.3	0.0	8.3	0.0
***E. faecalis* (310)**	**11.7***	**22.3***	**22.0**	**25.3**	**17.0***	**22.7**	**15.3**	**22.0**	**7.3**
E. faecalis (313-VA-R)	0.0*	21.3*	16.7	0.0	10.7*	0.0	0.0	10.3	0.0
E. faecalis (411)	11.3*	24.0*	0.0	8.0	18.0*	0.0	0.0	16.0	0.0
E. faecalis (412)	11.3*	24.0*	6.0	10.0	18.0*	20.0	0.0	14.0	0.0
E. faecalis (413)	11.3*	24.0*	6.0	8.0	18.0*	20.0	0.0	0.0	0.0
E. faecalis (414)	11.3*	24.0*	6.0	10.0	18.0*	0.0	0.0	12.0	0.0
E. faecalis (415)	12.0*	24.0*	8.0	8.0	16.0*	0.0	0.0	0.0	0.0
E. faecalis (416)	12.0*	30.0*	10.0	20.0	20.0*	22.0	10.0	16.0	0.0
E. faecalis (417)	12.0*	30.0*	8.0	0.0	22.0*	0.0	0.0	14.0	0.0
E. faecalis (418)	10.0*	24.0*	20.0	12.0	20.0*	24.0	0.0	10.0	0.0
E. faecalis (419)	10.0*	24.0*	0.0	12.0	18.0*	0.0	0.0	8.0	0.0
E. faecalis (420)	10.0*	24.0*	0.0	12.0	18.0*	16.0	0.0	10.0	0.0
E. faecium (5)	14.0*	20.0*	20.0	10.0	8.0*	10.0	0.0	10.0	0.0
E. faecium (8)	0.0*	0.0*	20.0	8.0	0.0*	0.0	0.0	14.0	0.0
E. faecium (316)	13.7*	23.0*	18.3	4.0	8.0*	15.0	0.0	17.0	0.0
E. faecium (152A)	0.0*	0.0*	6.8	7.3	0.0*	0.0	0.0	10.0	0.0
E. faecium (401)	0.0*	0.0*	0.0	0.0	0.0*	0.0	0.0	10.0	0.0
E. faecium (402)	0.0*	0.0*	22.0	0.0	0.0*	0.0	0.0	10.0	0.0
E. faecium (403)	0.0*	0.0*	0.0	0.0	0.0*	12.0	0.0	10.0	0.0
E. faecium (404)	0.0*	0.0*	0.0	8.0	0.0*	0.0	0.0	18.0	0.0
E. faecium (405)	0.0*	0.0*	0.0	0.0	0.0*	0.0	0.0	10.0	0.0
E. faecium (406)	14.0*	0.0*	0.0	0.0	0.0*	0.0	0.0	12.7	0.0
E. faecium (407)	0.0*	0.0*	0.0	0.0	0.0*	0.0	0.0	10.0	0.0
E. faecium (408)	0.0*	0.0*	0.0	0.0	0.0*	0.0	0.0	8.0	0.0
E. faecium (409)	0.0*	0.0*	6.0	0.0	0.0*	0.0	0.0	16.0	0.0
E. faecium (410)	0.0*	0.0*	6.0	0.0	0.0*	0.0	0.0	14.0	0.0
E. faecium (421)	14.0*	0.0*	0.0	0.0	0.0*	0.0	0.0	0.0	0.0
E. faecium (422)	0.0*	0.0*	0.0	0.0	0.0*	0.0	0.0	10.0	0.0

Shown are the averages of three independent trials of disk diffusion susceptibility tests for strains or clinical isolates of *E. faecalis* and *E. faecium*. Vancomycin, ampicillin and penicillin are highlighted with "*" to indicate that they revealed either the most dramatic susceptibility differences between the bacteria to differentiate species or could be relevant for drug treatment of patients. *E. faecalis* (310) is a modified weakened control strain for college laboratories.

faecalis (310) was susceptible to virtually all of the antibiotics, including bacitracin, and *E. faecalis* (313-VA-R) was vancomycin-resistant (Table 3). Concurrent with the literature on the emergence of vancomycin-resistant enterococci (Clark et al., 1993; Cetinkaya et al., 2000; Huycke et al., 1998; Courvalin, 2006; Teixeira et al., 2011), we discovered that all of our strains and clinical isolates fall into the vancomycin-resistant range as determined by comparison of the zones of growth inhibition to the database in the *Clinical Microbiology Manual* or the *National Committee for Clinical Laboratory Standards* (Jorgensen and Turnridge, 2007), including the weaker *E. faecalis* (310) (Table 3, Fig. 1). In fact, *E. faecium* (152A) was just as strongly resistant as the vancomycin-resistant positive control *E. faecalis* (313-VA-R) (Table 3). These data corroborate with an alarming trend that has been observed in hospital and community enterococci isolated from sick patients around Southern California, the United States and around the world (see Discussion), and suggest that vancomycin-resistance shows no sign of abating in the near future and is even penetrating many laboratory stocks. The fact that even our weakest *E. faecalis* (310) strain, which was the sole bacterium susceptible to streptomycin and amikacin (Table 3), is also vancomycin-resistant is especially disturbing.

Comparison of antibiotic susceptibility of enterococci for vancomycin, ampicillin, and penicillin

Our data indicate that two antibiotics, ampicillin and penicillin, stand out in the antibiogram analysis as additionally interesting with respect to either (1) patient treatment of enterococcal infections in hospitals or (2) bacterial identification in clinical laboratories. A close comparison of these antibiotics to vancomycin is shown in Table 3. As already stated, all of the strains and clinical isolates in our library are vancomycin-resistant (see above). Strikingly, we discovered that all of our *E. faecalis*, including our vancomycin-resistant control *E. faecalis* (313-VA-R), were susceptible to ampicillin (Table 3; Jorgensen and Turnridge, 2007), while all of our *E. faecium*, with the exception of *E. faeciums* (5) and (316), were resistant (Table 3). These data are encouraging for treatment of patients infected with *E. faecalis* and, possibly, some *E. faecium* (see Discussion). These data also reveal that speciation of the enterococci is critical in choosing an effective antibiotic. Ampicillin, for example, would not be helpful against most *E. faecium* strains or clinical isolates but would be helpful against *E. faecalis*.

Supporting our hypothesis that we could identify antibiotics for the differentiation of *Enterococcus* species within the *Enterococcus* genus, all strains and clinical isolates of *E. faecalis*, including our weaker laboratory control *E. faecalis* (310), were strongly susceptible to penicillin, while all our *E. faecium* strains and clinical isolates were strongly resistant (Table 3; Jorgensen and Turnridge, 2007). The other antibiotic that revealed a partial susceptibility difference between the species within the *Enterococcus* genus was, of course, ampicillin (Table 3) given that all *E. faecalis* were susceptible, while all *E. faecium*, with the exception of *E. faeciums* (5) and (316), were resistant. These data have importance for bacterial identification in clinical microbiology laboratories and for patient treatment (see Discussion).

Growth and fermentation characterization of enterococci on mannitol salt agar

It is known that enterococci can grow in high concentrations (6.5%) NaCl (Devriese et al., 2006; Fisher and Phillips, 2009; Teixeira et al., 2011). It is also known that *Staphylococcus aureus*, which recently received a plasmid from *Enterococcus faecalis* encoding gene products that provide vancomycin-resistance (Noble et al., 1992; Sung and Lindsay, 2007), and *Staphylococcus epidermidis* have each shown differences in physiologic fermentation patterns with respect to each other in the mannitol salt agar (MSA) assay (Blair et al., 1967; Leboffe and Pierce, 2005; Kateete et al., 2010). We hypothesized that if the enterococci behave like the staphylococci with respect to mannitol carbon source utilization, then we could observe differences in growth characteristics and/or fermentation phenotypes for the enterococci in the MSA test as another way of differentiating one *Enterococcus* species from another. The MSA test would therefore provide us with a second method that could be used to corroborate any speciation performed on enterococci via antibiotic susceptibility testing. Toward this end, we cultured our bacterial strains and clinical isolates on MSA plates and observed for differences in both growth and fermentation phenotypes in three separate experimental trials. MSA plates contain 7.5% salt and, as a result, select for staphylococci and enterococci, but not most other bacteria that would not survive at the high level of salinity (Leboffe and Pierce, 2005). As expected, our positive control for mannitol fermentation, *S. aureus*, grew on MSA and fermented mannitol (Table 4, Fig. 2), as is evidenced by the yellow colonies (Leboffe and Pierce, 2005). Conversely, as expected, our negative control, *S. epidermidis*, grew on MSA but did not utilize mannitol as a carbon source since its colonies appeared red in the assay (Table 4, Fig. 2; Leboffe and Pierce, 2005). Intriguingly, of the enterococci, only the *E. faecalis* strains and clinical isolates grew on mannitol (Table 4, Fig. 2), though at a reduced level in comparison to *S. aureus* or *S. epidermidis*, which might be expected given the slightly higher salinity of MSA plates over liquid NaCL broth. By contrast, the *E. faecium* strains and clinical isolates grew extremely poorly or not at all during our trials (Table 4, Fig. 2), and any slight growth did not reveal a change of the pH indicator (Fig. 2). All *E. faecalis*, however, were capable of fermenting mannitol in multiple trials as was evidenced by the yellow colonies (Table 4, Fig. 2). Though *Enterococcus faecalis* does not grow as well as *Staphylococcus aureus* on mannitol salt agar at 7.5% NaCl, it does show enough growth on MSA plates that MSA can still be employed as a differentiation assay. In light of these results, *Enterococcus faecalis* can be differentiated from *Enterococcus faecium* by differences in growth characteristics and fermentation phenotypes in the MSA test.

Discussion

In this study, we sought to discover a set of rapid, cost effective, and technically simple procedures that could more easily differentiate strains and clinical isolates of *Enterococcus faecalis* from *Enterococcus faecium*. This was important for us given that molecular technology such as nucleic acid sequencing is presently required to differentiate *Enterococcus* species within the genus—a costly, time consuming, and technically challenging procedure for laboratory technicians (Domig et al. 2003; Lehman et al., 2007; Murray et al., 2009) that is presently unfeasible in developing world laboratories and our medical bacteriology laboratories at CSUDH because of the expense of this procedure and the time constraints within the laboratory agenda. Toward this end, we hypothesized two things: (1) if *E. faecalis* and *E. faecium* were susceptible to different antibiotics, then we could use antibiogram analysis to discover one or more antibiotics

Table 4 Growth and fermentation characteristics of different enterococci on mannitol salt agar

Bacteria	Growth	Fermentation	Bacteria	Growth	Fermentation
E. faecalis (18)	+ +	+	*E. faecium* (5)	+	+
E. faecalis (9)	+ +	+	*E. faecium* (8)	+	−
E. faecalis (10)	+ +	+	*E. faecium* (316)	+/ −	−
E. faecalis (11)	+ +	+	*E. faecium* (152A)	+/ −	−
E. faecalis (14)	+ +	+	*E. faecium* (401)	+	−
E. faecalis (300A)	+ +	+	*E. faecium* (402)	+	−
E. faecalis (310)	+ +	+	*E. faecium* (403)	+	−
E. faecalis (313-VA-R)	+ +	+	*E. faecium* (404)	+	−
E. faecalis (411)	+ +	+	*E. faecium* (405)	+	−
E. faecalis (412)	+ +	+	*E. faecium* (406)	+	−
E. faecalis (413)	+ +	+	*E. faecium* (407)	+	−
E. faecalis (414)	+ +	+	*E. faecium* (408)	+	−
E. faecalis (415)	+ +	+	*E. faecium* (409)	+	−
E. faecalis (416)	+ +	+	*E. faecium* (410)	+	−
E. faecalis (417)	+ +	+	*E. faecium* (421)	+	−
E. faecalis (418)	+ +	+	*E. faecium* (422)	+	−
E. faecalis (419)	+ +	+	*S. aureus*	+ + +	+
E. faecalis (420)	+ +	+	*S. epidermidis*	+ + +	−

Shown is the presence of growth and fermentation on mannitol salt agar of the different enterococci. Growth (+); Lack of growth (−); Ferments mannitol (+); Does not ferment mannitol (−). *S. aureus* (positive control); *S. epidermidis* (negative control).

Figure 2 Growth and fermentation patterns on mannitol salt agar. Plate shows the differing growth and fermentation patterns of selected enterococci on Mannitol Salt Agar, where fermentation is indicated by a change of the phenol red indicator from a red to yellow color. *Staphylococcus aureus* was used as a positive control for growth and fermentation while *Staphylococcus epidermis* was used as a negative control. (1) *S. epidermidis*, (2) *S. aureus*, (3) *E. faecium* (401), (4) *E. faecium* (402), (5) *E. faecalis* (411), (6) *E. faecalis* (412).

that could be used to distinguish *E. faecalis* from *E. faecium* in a rapid manner in the laboratory setting, and (2) if the enterococci behave like the staphylococci with respect to mannitol carbon source utilization, then we could observe differences in growth characteristics and/or fermentation phenotypes for the enterococci in the MSA test as another way of differentiating one *Enterococcus* species from another. In both cases, our hypotheses were proven correct. Comparison of *Enterococcus* strains and clinical isolates from various sources on sheep blood agar revealed that penicillin was able to differentiate *E. faecalis* from *E. faecium* (Table 3, Fig. 1) and that we could differentiate the genera partly with ampicillin (Table 3, Fig. 1). Similarly, *E. faecalis* could be differentiated from *E. faecium* on mannitol salt agar via the difference in its growth and fermentation phenotypes (Table 4, Fig. 2).

The confirmation of our two hypotheses has relevance for both our teaching laboratory at CSUDH and for other laboratories around the world, especially those in developing nations. Again, Medical Bacteriology Laboratory (Bio 435L) at CSUDH presently only employs procedures to differentiate *Enterococcus* from other bacterial genera, but not species within the *Enterococcus* genus. Now students in the laboratory can use antibiogram analysis (especially with respect to penicillin) to identify *Enterococcus* species in a rapid, high-throughput manner when they are doing diagnostics for their class assignments. Indeed, we have discovered that our methods are accurate with respect to our laboratory strains and clinical isolates in our tests (Tables 3 and 4, Figs. 1 and 2) and rapid (a result can be acquired within 24 h as opposed to days, unlike sequencing procedures that normally need to be outsourced to a sequencing laboratory). In addition, our procedures are cost effective for our

university budget (i.e. the antibiogram and mannitol salt plates are cheaper than the sequencing procedure), easy to perform, and easy for quality control management during laboratory preparation. These methods, therefore, can be applied to any medical bacteriology teaching or hospital laboratory within the United States, where cost of laboratories is a concern in times of shrinking budgets. In addition, these methods are appropriate for clinical diagnostic laboratories in the developing world, where laboratories are not as well funded as those in the United States, and where molecular probing and nucleic acid sequencing technology is not readily available (Stetler et al., 1997; Wilkinson et al., 1997; Archibald and Reller, 2001; Pang and Peeling, 2007; Usdin et al., 2010; Vu and Carvalho, 2011). In fact, the mannitol salt agar approach is already being used in developing nations for the speciation of the staphylococci because of its cost effectiveness (Kateete et al., 2010).

Our results are a qualitative study of the enterococci based on strains and clinical isolates from California State University Dominguez Hills, the Clinical Diagnostics Laboratory at Los Angeles Harbor Hospital, Focus Diagnostics, and the biologic supply company Microbiologics. Using more bacterial isolates from around the country, a further quantitative study can be performed to confirm these findings. However, it must be recognized that our bacteria were already derived from *geographically separate* regions of the country (different areas of California and St. Cloud, Minnesota) and further screening will most likely corroborate with our findings.

Our approach using antibiogram analysis or mannnitol salt agar to differentiate bacteria is not without precedent for the identification of other microorganisms in clinical laboratories. As already stated, *Staphylococcus* species can be differentiated in the MSA test (Leboffe and Pierce, 2005; Kateete et al., 2010), and *Bacillus subtilis* ferments mannitol while other *Bacillus* species do not (Forbes et al., 2007). Similarly, the genus *Pasteurella*, like *Enterococcus*, grows well on blood agar plates (Murray et al., 2009), and *Pasteurella multocida* and *Pasteurella canis*, two of the species associated with human infections, are sensitive to low concentrations of penicillin (2 units/disc), which can be used to differentiate them from the genera *Hemophilus* and *Bordetella* (Murray et al., 2009). Similarly, *Morexella catarrhalis*, a Gram-negative diplococcus, can be distinguished from other Gram-negative diplococci via penicillin sensitivity (Murray et al., 2009). *Bacillus anthracis*, the causative agent of anthrax, is sensitive to penicillin, while *Bacillus cereus*, a cause of food poisoning and ocular disease, is resistant (Lightfoot et al., 1990; Murray et al., 2009). Consequently, the possibility that penicillin, either alone or in combination with other antibiotics (such as ampicillin), can differentiate enterococci in antibiograms is not necessarily surprising. Similarly unsurprising is the possibility that the MSA test can be used to differentiate the enterococci. Further research on the enterococci will determine the molecular mechanism by which growth and fermentation characteristics are possible and thus fortify the significance of our screening data. For example, antibiotic susceptibility differences could be linked to specific nucleotide sequences, like with genes that code for ampicillin or penicillin resistance, and these sequences can be amplified in the polymerase chain reaction. Our strains and clinical isolates of *E. faecalis* and *E. faecium* could therefore be classified via PCR to confirm the antibiotic susceptibility profile (Teixeira et al., 2011). Similarly, pulse field gel electrophoresis (PFGE) may be used to identify clonal complexes that relate to antibiotic resistance (Teixeira et al., 2011). Or, multilocus sequencing typing (MLST) can be used to develop an allelic profile of our strains and clinical isolates to see if they corroborate with the antibiotic susceptibility profile (Teixeira et al., 2011). Similarly, the PCR approach can be used to confirm the significance of the different phenotypes between *E. faecalis* and *E. faecium* in the MSA test. It is known that mannitol is fermented with the help of key enzymes such as mannitol dehydrogenase and fructokinase (Leboffe and Pierce, 2005). PCR confirmation of the presence or absence of these enzymes would provide molecular evidence for the significance of our MSA screen. Alternatively, a dysfunctional enzyme may be the result of a single nucleotide mutation and single nucleotide polymorphism (SNP) genotyping of possible genes would reveal these differences with our strain and clinical isolate stocks.

In addition to our two hypotheses in this study, we wanted to determine whether our laboratory strains and clinical isolates of enterococci revealed a trend in antibiotic susceptibility. As was already stated, we showed that all our enterococci were resistant to vancomycin (Table 3) and that all *E. faecalis* strains were susceptible to ampicillin (Table 3), while all E. *faecium*, with the exception of *E. faeciums* (5) and (316), were resistant (Table 3). With regards to vancomycin resistance, our data corroborate with an alarming trend that has been observed with patient isolates from hospitals and communities within Southern California and globally (Noble et al., 1992; Clark et al., 1993; Woodford et al., 1995; Courvalin, 2006; Carvalho, 2011; Vu and Carvalho, 2011), and suggest that vancomycin-resistance shows no sign of abating in the near future. Indeed, the fact that even our weakest *E. faecalis* (310) strain, which was the sole bacterium susceptible to streptomycin and amikacin (Table 3), is also vancomycin-resistant is especially disturbing because it indicates that vancomycin-resistance has penetrated laboratory stocks around the United States. These vancomycin-resistant enterococci are a source of the plasmid or transposon that provides the resistance, and they can transfer this plasmid to other species and strains within the *Enterococcus* genus (Clark et al., 1993; Woodford et al., 1995). In addition, researchers already discovered that vancomycin-resistant *Enterococcus faecalis* transferred episomal DNA to previously vancomycin-susceptible *Staphylococcus aureus* (Noble et al., 1992; Sung and Lindsay, 2007), revealing that antibiotic resistance can cross *both* bacterial species *and*

genus barriers. Thus, there is a possibility of further vancomycin-resistance transfer to other bacterial genera and this needs to be monitored in hospital and community surveillance programs.

Our results further support the need for a robust clinical microbiology workforce for diagnostics and surveillance in US hospitals (Carvalho, 2011). Without proper diagnosis and surveillance, physicians are left with no other choice but to prescribe broad-spectrum antibiotics and, many times, last line drugs, like vancomycin, to cure patients rapidly (Carvalho, 2011). Though for some patients this method may offer desired relief in a timely manner, the prospect of evolutionary emergence of antibiotic resistant bacteria becomes all the more probable, as is already seen in hospital and community surveillance (Courvalin, 2006; Carvalho, 2011) as well as in our study (Table 3, Fig. 1). Our data suggest that, with respect to enterococci, first line drugs such as ampicillin (or even the less broad-spectrum penicillin that works with *E. faecalis* but not *E. faecium*) may be administered to patients singularly or in combination with other drugs and will have at least an equal, if not better, bactericidal/bacteriostatic effect than last-line drugs (such as vancomycin). Of course, the unwillingness of sick patients to take their medicines appropriately is well documented (Elzinga et al., 2004; Farmer, 2005; Chan and Iseman, 2008; Carvalho, 2011), and this notoriously leads to evolutionary emergence of antibiotic resistant bacteria. By prescribing first line drugs that are efficacious for patient recovery, physicians would be preserving the efficacy of last line drugs (Carvalho, 2011). In addition, surveillance of what precise species is the causative agent of disease is critical, since, as is stated above, our antibiotics (like penicillin and ampicillin) may work for one species but not another. This would be critical in handling the health crisis that is sure to arise by microbial infections in the near future.

Some may argue that there is a lack of correlation between antibiotic susceptibility testing *in vitro* versus the activity of the antibiotic *in vivo* (in patients) and that antibiotic susceptibility results should not be used as a therapeutic outcome. For example, the laboratory test conditions may not mimic the *in vivo* environment in terms of temperature, pH, bacterial inoculum size, oxygen environment, etc. at different sites of infection. It must be noted that the antibiotics that are of interest here—penicillin and ampicillin—have been around for decades and extensive research has been done on their mode of action and the dosage that is necessary to relieve patient symptoms or cure patients of a bacterial infection. Physicians are well acquainted with this scientific and clinical information. Consequently, the tests in our study suggest that these antibiotics can indeed be used in patient treatment efficaciously in spite of the misconception that all bacteria are resistant to them. A newly discovered antibiotic or chemotherapeutic agent would, by contrast, need extensive clinical trials.

It is important to note that, over the years, traditional therapies for enterococcal infections centered on the use of a cell wall active agent, such as a β-lactam, an aminoglycoside, or vancomycin (a last line drug for combating many bacterial infections) (Teixeira et al., 2011). Our study conclusively reveals a number of important points with regards to traditional antimicrobial treatment. First, we discovered that all of our strains and clinical isolates of *Enterococcus* are resistant to vancomycin, regardless of species (Table 3, Fig. 1). Our data strongly suggest that vancomycin, when used singularly, is unlikely to cure any enterococcal infection caused by the strains presently circulating the globe. Our data corroborates with findings in other laboratories (Teixeira et al., 2011), and, as stated above, reveal that vancomycin resistance shows no sign of abating in the near future, though we still argue for continual surveillance. Secondly, all of our *E. faecalis* were susceptible to penicillin and ampicillin, though there is a common misconception that virtually all *E. faecalis* are resistant to both. Our experiments used strains and clinical isolates from separate regions of the country and we reveal that the common belief is clearly in error. Though resistance can occur, it must be supported by surveillance studies within the respective region of the world. A blanket dismissal of possible therapeutic antibiotics is inadvisable. This is encouraging because it reveals that penicillin and/or ampicillin used singularly or in combination with other antibiotics is likely still efficacious at curbing *E. faecalis* infections. We recommend that the more severe enterococcal infections be treated with combination therapy that is based on surveillance data. Thirdly, and perhaps most importantly, we conclusively show that all of our *E. faecium* is resistant to penicillin, ampicillin, vancomycin, and even streptomycin (which has been used in some combinatorial therapies for *E. faecium*). Our data suggest that traditional therapies employing these drugs singularly are unlikely to be efficacious in curing patients. Furthermore, we are suspicious that even combinations of these drugs for treatment of *E. faecium* infections may begin to yield poor results as the more pathogenic varieties of *E. faecium* circulate. Our findings parallel the fact that some laboratories are isolating more *E. faecium* from patients rather than the usually more common *E. faecalis*. These two trends are extremely troubling. Once again, we are advocating for extensive surveillance of the enterococci. The traditional singular or combinatorial methods for treatment of *E. faecium* infections likely will need to be revised in the future. If the trend for *E. faecium* continues, laboratories around the world may witness the isolation of *E. faecium* bacteria that are resistant to any known drug. In fact, our laboratory is now testing gentamycin, which has been used for treating infections with *E. faecium*, to determine if resistance is developing to this drug as well.

Given that many bacteria developed resistance to penicillin soon after its discovery, one may ask "why can we differentiate *E. faecalis* from *E. faecium* using penicillin"? And, for that matter, "why can we still employ penicillin as a drug for patient treatment"? The likely answer: enterococci

that are not under evolutionary *selective pressure* are free to *revert* to susceptibility to penicillin or other weaker line drugs. By contrast, last line drugs, which are prescribed but may not be taken appropriately by patients under treatment, leads to the presence of evolutionary pressure to select for antibiotic-resistant bacteria (Elzinga et al., 2004; Farmer, 2005; Chan and Iseman, 2008; Carvalho, 2011). Given that doctors are constantly in the habit of prescribing the last-line, broad-spectrum drugs, they are not keeping bacteria under the selective pressure for the weaker line drugs in their patients. In essence, the resistance to penicillin becomes a neutral mutation that may be lost in evolutionary time. Like for vancomycin resistance, as was noted above, the enterococci also carry a plasmid that codes for "penicillinase," which is responsible for causing the resistance to penicillin (Murray et al., 2009). Similar to what happens with bacteria that lose plasmids encoding ampicillin resistance genes when they are not continually selected for in liquid broth medium containing ampicillin or other important growth conditions, or with those bacteria that have a plasmid size that enhances plasmid loss (Godwin and Slater, 1979; Seo and Bailey, 1985; Gerdes et. al, 1986; Boe et. al, 1987; Lenski and Bouma, 1987; Caldwell et. al, 1989; Modi and Adams, 1991; Rhee et al., 1994; Kües and Stalh, 1989; Rhode, 1995; Valenzuela et. al, 1996; Guerrier-Takada et al., 1997; Smith and Bidochka, 1998), those enterococci tested that are not under continual penicillin selection may have lost their plasmid-based resistance. A similar situation appears to be occurring with ampicillin, given that all of our *E. faecalis* strains and some *E. faecium* strains are sensitive to this antibiotic (Table 3, Fig. 1), even though there is a common misconception in the clinical community that virtually all enterococci are also resistant to ampicillin (and aminoglycosides, for that matter). This is not to say that genes conferring antibiotic resistance cannot be found within the bacterial chromosome. Quite the contrary, though in our study the antibiotics of interest can only be overcome by plasmid presence in the enterococci of interest. Nevertheless, reversion mutations are also quite common within the internal genomes of bacteria, much like what we see for antiviral resistance in viruses (Flint et al., 2000; Andersson, 2003). As a result, enterococci derived from communities and hospitals within the US and, most likely, elsewhere around the world, could be experiencing a reversion to susceptibility to some of the first-line antibiotics by either plasmid loss or nucleotide reversion within the chromosome. Certainly, our study suggests this. Such information will be critical for patient treatment in the near future because the cost of healthcare is rising, most last-line drugs are expensive, and many bacterial genera and species are revealing antibiotic resistance to last-line medicines.

In conclusion, the mannitol salt test and antibiotic susceptibility testing can distinguish enterococci at the species level. However, continual surveillance needs to be performed on the enterococci, and, indeed, all bacterial genera, that are derived from hospitals or communities. Our study shows how surveillance can bring into question some common misconceptions about antibiotic resistance of the enterococci with regards to penicillin and ampicillin, and suggests that this new information is relevant for patient treatment, especially in a time when the world is witnessing extensive antibiotic resistant microbes. Unfortunately, it is the clinical diagnostic laboratory workforce that is required for such surveillance and the United States, like all nations, is experiencing a shortage of such a workforce (Archibald and Reller, 2001; Carvalho, 2011).

Acknowledgements

We would like to thank Anthony Cipriano for technical assistance and Gaby Gomez for administrative assistance for this project. This work was supported by a California State University Sally Casanova Memorial RSCAAP research grant, a research grant from the Lois Stokes Alliance for Minority Participation (LS-AMP), and a research grant from The Faculty Legacy Fund of the CSUDH Emeritus Faculty Association.

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