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# Multilocus Sequence Typing of *Enterococcus faecalis* from Vegetable Foods Reveals Two New Sequence Types

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## Abstract

A collection of 16 isolates of *Enterococcus faecalis* from different vegetable foods were characterized by multilocus sequence typing (MLST). One isolate belonged to sequence type (ST) 9 of the previously described clonal complex 9, which is frequently associated with hospital environments. The rest of the isolates were grouped into two new STs named 168 and 169. ST168 represented a singleton clone that included 14 isolates and seemed to be the predominant type among *E. faecalis* from vegetable samples. ST168 was closely related to ST72, differing only by one allele type. Singleton ST169 was not related to any of the previously described STs.

## Introduction

*Enterococcus* species are ubiquitous, commensal inhabitants of the gastrointestinal tract of humans and animals (Tannock and Cook, 2002). They can also be found in soil, on plants, in water, and in several food products. Enterococci are also important pathogens responsible for serious illnesses and remain among the top three most common pathogens (together with *Staphylococcus aureus* and *Escherichia coli*) that cause nosocomial infections, involving urinary tract infections, bloodstream infections, and wound infections (Murray and Weinstock, 1999; Richards *et al.*, 2000; Kayser, 2003; Tendolkar *et al.*, 2003). Nosocomial enterococcal infections typically occur in very ill, debilitated patients who have been exposed to broad-spectrum antibiotics.

While virulence of enterococci can be enhanced by the production of several proteins known as virulence factors, at present there is no clear definition of virulent and nonvirulent strains, and epidemiological studies to trace hospital clones must rely on typing methods such as pulsed-field gel electrophoresis and multilocus sequence typing (MLST). MLST is considered the gold standard procedure for characterizing isolates of bacterial species using the sequences of internal fragments of selected housekeeping genes (Urwin and Maiden, 2003; Aanensen and Spratt, 2005). MLST provides an unambiguous nomenclature for genotypes, and clones and data are easily stored in databases that can be exchanged between different laboratories via the Internet. In the case of *E. faecalis*, a MLST scheme based on seven housekeeping genes has been established (Ruiz-Garbajosa *et al.*, 2006). Approximately 450–500 bp internal fragments of each gene are used, since these can be accurately sequenced on both strands using an automated DNA sequencer. For each

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housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). STs can be grouped in a clonal complex (CC). The *E. faecalis* MLST database (http://efaecalis.mlst.net) becomes an increasingly useful resource for studies on the epidemiology of *E. faecalis*. Compared to *E. faecium* typing scheme, the amount of information available for *E. faecalis* sequence typing is much more limited, and this includes mostly isolates of clinical origin.

Two major concerns about enterococci are the dissemination of virulent, clinical isolates through external routes such as food and water, and their differentiation from nonvirulent isolates naturally adapted to nonclinical environments. In a previous study based on random amplification of genes located in the *E. faecalis* pathogenicity island region, we showed that enterococcal isolates from vegetable foods and water clustered in separate groups compared to clinical isolates (Abriouel *et al.*, 2008). The purpose of the present study was to determine the relatedness of enterococci from vegetable foods with existing STs and CCs established by MLST.

## Materials and Methods

### *Isolation and characterization of bacterial strains*

A collection of 16 *E. faecalis* isolates from raw vegetable foods (Abriouel *et al.*, 2008) were used for the present study (Table 1). Isolates were kept in 20% glycerol at −80°C and cultivated routinely on brain heart infusion (BHI) broth (Scharlab, Barcelona, Spain) at 37°C. Determination of antibiotic resistance and virulence traits was described previously (Abriouel *et al.*, 2008), and the unpublished data on the per strain incidence of traits are reported in Table 1. Ampicillin (10 μg; Difco, Madrid, Spain) and penicillin (10 U; bioMérieux, Marcy-l'Etoile, France) resistance were corroborated by the Kirby-Bauer disk diffusion assay. All the antimicrobial resistance assays and interpretation of results conformed to the National Committee on Clinical Laboratory Standards (NCCLS) guidelines (CLSI, 2006a, 2006b; NCCLS, 2003). Beta-lactamase production was tested with nitrocefin disks (Oxoid, Barcelona, Spain) following the manufacturer's instructions. The presence of beta-lactamase genes was determined by polymerase chain reaction (PCR) amplification with Bla-forward and Bla-reverse primers according to Hummel *et al.* (2007). PCR amplification with primers targeting *ddl* gene was used to confirm species identity (Dutka-Malen *et al.*, 1995).

### *MLST analysis*

The isolates were analyzed by MLST for *E. faecalis* (http://efaecalis.mlst.net) (Ruiz-Garbajosa *et al.*, 2006). The allelic profiles of isolates were obtained by sequencing internal fragments of seven housekeeping genes, *gdh* (glucose-6-phosphate dehydrogenase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *pstS* (phosphate ATP binding cassette transporter), *gki* (putative glucokinase), *aroE* (shikimate 5-dehydrogenase), *xpt* (shikimate 5-dehydrogenase), and *yqil* (acetyl-coenzyme A acetyltransferase).

Internal fragments from the final set of seven genes were amplified by PCR as described by Ruiz-Garbajosa *et al.* (2006). For each isolate, each gene was amplified a minimum of two times and sequenced with the specific forward or reverse primer a minimum of three times. STs of isolates are defined by the allelic profile at these seven loci, with each unique combination of alleles assigned to a distinct ST number. The STs of all isolates were determined. PCR products were purified with Exo-SAP-IT® (USB Europe GmbH), and sequenced with the respective primers in a CEQ 2000 XL DNA Analysis System (Beckman Coulter, CA).

### *Computer analysis of MLST data*

The relatedness between the different STs was investigated using eBURST (http://www.mlst.net). Clusters of related STs differing in not more than two of the seven loci that were thought to be descendents from a common ancestor were grouped into CCs. A singleton was defined as an ST that is not grouped into a CC, and a singleton clone was defined as a singleton represented by more than one isolate. The minimum spanning tree was used to infer patterns of evolutionary descent under the principle of parsimony, in which identical alleles in

Table 1. Food Source, Sequence Type, Virulence Determinants, and Antibiotic Resistance of *E. faecalis* Isolates

| | | | | *Virulence factors*[b] | | | | | | | *Antimicrobial resistance* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Isolates* | *Source*[a] | *ST* | *CC* | *agg* | *gelE* | *cylB* | *esp* | *efaAfs* | *cpd* | *cob* | Pen | Amp | Ery | Tet | Cmp | Rfa | Cip | Lvx | Fur | Gen | Str | Qda |
| *E. faecalis* V48c1 | Lettuce | 9 | 9 | X | X | | | X | X | | | | | R | R | | | | | R | | R |
| *E. faecalis* V8c1 | Green table olives | 168 | NC | X | X | | X | X | X | X | | | R | | | R | | | | | | R |
| *E. faecalis* V20c1 | Celery | 168 | NC | X | X | | | X | X | | | | | | | | R | R | | | | R |
| *E. faecalis* V29c1 | Tender onion | 168 | NC | X | X | | | X | X | X | | | | | R | R | R | | | | | R |
| *E. faecalis* V30c2 | Tender onion | 168 | NC | X | X | | | X | X | | | | R | | | | R | R | | | | R |
| *E. faecalis* V31c5 | Tender onion | 168 | NC | X | | | | X | X | X | | | R | | | R | | R | | R | R | R |
| *E. faecalis* V33c1 | Cherries | 168 | NC | X | X | | | X | X | | | | R | | | R | | | | | | R |
| *E. faecalis* V39c2 | Packed Medit. salad | 168 | NC | X | | X | X | | X | X | R | R | R | R | | | R | R | | | | R |
| *E. faecalis* V41c1 | Packed salad | 168 | NC | X | X | X | | X | X | | | | | | | | R | R | | | | R |
| *E. faecalis* V43c1 | Green asparagus | 168 | NC | X | X | X | | X | X | | | | R | | | R | | | | | | R |
| *E. faecalis* V46c1 | Strawberries | 168 | NC | X | X | X | X | X | X | | | | | | | R | | | | | | R |
| *E. faecalis* V54c2 | Lettuce | 168 | NC | X | X | X | X | X | X | | | | R | | | R | | | | | | R |
| *E. faecalis* V64c2 | Green pepper | 168 | NC | X | X | X | | X | X | | | | | | | R | | | | | | R |
| *E. faecalis* V72c3 | Tomato | 168 | NC | | | X | | | X | | | | R | | | R | | | | | | R |
| *E. faecalis* V75c1 | Carrot | 168 | NC | | X | | | | | | | | | | | | | | | | | |
| *E. faecalis* V68c1 | Radish | 169 | NC | X | X | X | | X | X | | | | R | | | R | R | R | R | | | R |

[a]Abriouel *et al.*, 2008.

[b]Unpublished data from Abriouel *et al.* (2008).

Allele types of the new ST 168: *gdh* (20), *gyd* (1), *pstS* (7), *gki* (25), *aroE* (23), *xpt* (2), and *yqil* (2). Allele types of the new ST 169: *gdh* (9), *gyd* (5), *pstS* (41), *gki* (37), *aroE* (7), *xpt* (2), and *yqil* (2).

X, positive amplification by PCR. *cyl*, cytolysin genes; *gelE*, gelatinase; *agg*, aggregation substance; *esp*, enterococcal surface protein; *efaA*, enterococal antigen; *cpd*, *cob*, *ccf*, sex pheromones. All isolates were negative for *cylM*, *cylA*, *ace*, and *ccf*.

R, resistance to antimicrobial; MIC, minimum inhibitory concentration; Pen, penicillin (MIC > 8 μg/mL); Amp, ampicillin (MIC > 8 μg/mL); Ery, erythromycin (MIC > 4 μg/mL); Tet, tetracycline (MIC > 8 μg/mL); Cmp, chloramphenicol (MIC > 16 μg/mL); Rfa, rifampicin (MIC > 2 μg/mL); Cip, ciprofloxacin (MIC > 2 μg/mL); Lvx, levofloxacin (MIC > 4 μg/mL); Fur, nitrofurantoin (MIC > 64 μg/mL); Gen, gentamicin (MIC > 500 μg/mL); Str, streptomycin (MIC > 1000 μg/mL); Qda, quinupristin/dalfopristin (MIC > 2 μg/mL). All isolates were sensitive to vancomycin and teicoplanin.

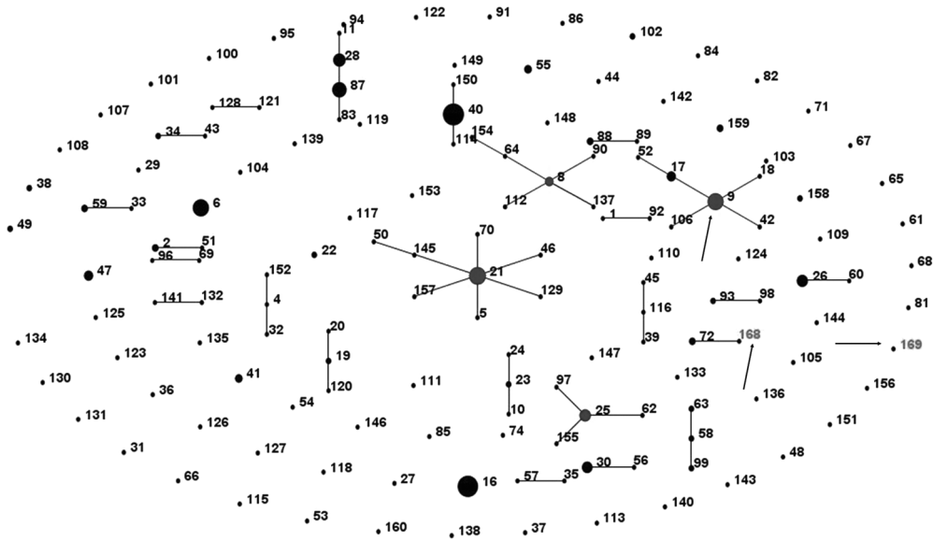


**FIG. 1.** Distribution of isolates from vegetables within the *E. faecalis* multilocus sequence typing scheme, by using eBURST algorithm (arrows). The new sequence types are marked in gray.

different genotypes are thought to have evolved from a common ancestor instead of by convergent evolution.

## Results and Discussion

Comparison of allele profiles with the *E. faecalis* MLST database revealed that enterococcal isolates from the vegetable foods studied represented three different groups, one belonging to the already-described ST9 and two new STs that were assigned the numbers 168 and 169 by the database curator (Table 1, Fig. 1). Isolate *E. faecalis* V48c1 (from lettuce) belonged to ST9 of the previously described CC9. This CC includes many isolates from hospitalized patients and is considered to be particularly fit to hospital environments (Ruiz-Garbajosa *et al.*, 2006). Similar to CC, ST9 also comprises mainly human isolates from Spain (Ruiz-Garbajosa *et al.*, 2006), the beta-lactamase (Bla+) ACB (Argentina-Connecticut-Bla+) clone involved in small nosocomial outbreaks (Nallapareddy *et al.*, 2005), and hospital isolates from Poland (Kawalec *et al.*, 2007). Strain V48c1 had a similar low content of virulence determinants as the rest of the isolates from vegetables, although it showed a more uncommon antibiotic resistance profile compared to the rest of the isolates for tetracycline, chloramphenicol, and gentamicin (Abriouel *et al.*, 2008; Table 1). Strikingly, gentamicin resistance was also found in most CC9 clinical isolates (Ruiz-Garbajosa *et al.*, 2006). However, random typing with primers targeting the pathogenicity island region clearly indicated that isolate V48c1 clustered in a group including other enterococci from vegetable foods, which was well differentiated from groups that included hospital isolates (Abriouel *et al.*, 2008). These results emphasize the necessity to explore the clonal diversity of enterococci from nonclinical environments in order to better understand the data derived from hospital settings.

The new singleton ST169 included a single isolate obtained from radish (*E. faecalis* V68c1) and was not related to any other STs after setting the database search down to four common alleles. In contrast, ST168 was highly represented and included 14 isolates from various vegetable food sources. This singleton clone was linked to ST72 by eBURST analysis (Fig. 1). Both STs showed identical allele types for *gdh*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL*, and differed only for *gyd* allele type. ST72 includes the hemolytic enterococcal strains F1 (ATCC 376, L36[4]) isolated by S. Orla-Jensen from milk in the early 1900s and SS-7 (one original strain from the Lancefield Collection isolated from

cheese in 1918) (McBride *et al.*, 2007), and two strains from hospitalized patients in Poland (strain A1284, from a cervical swab, and strain A1285, from blood) (Kawalec *et al.*, 2007). A comparative analysis carried out of ST168 allele profile with eBURST program for STs with at least four identical alleles identified STs 72, 162, 196, and 199 as the closest, but still no information was available for isolates from STs 196 and 199 in the MLST database. A neighbor joining tree constructed with the allele profiles for these STs suggests a common lineage of descent for STs 72, 162, 168, and 196 (Fig. 2). According to these results it is tempting to suggest that hospital ST72 isolates may have probably evolved from ancestors transmitted through the oral–fecal route from vegetable foods to human and animal digestive tracts, animal food products, and finally to feces and water. The capacity of enterococci to be involved in opportunistic infections may have strengthened in recent decades due to the increase of hospital interventions, antibiotic use, and the more susceptible population (such as the elderly, immunocompromised, and hospitalized patients). Although isolates from vegetable foods belonging to ST168 had in general lower incidence of virulence traits and antibiotic resistance compared to clinical isolates (Abriouel *et al.*, 2008), resistance to penicillin and ampicillin found in one isolate and to gentamicin and streptomycin found in another suggest that these two isolates from vegetable foods could have resided previously in antibiotic-treated hosts. Since ampicillin and penicillin resistance are uncommon in *E. faecalis*, the identity of strain V39c2 was confirmed by PCR amplification and antibiotic resistance was confirmed by the Kirby-Bauer disk diffusion method. However, phenotypic and genetic tests clearly indicated that this strain does not produce beta-lactamase activity and lacks the corresponding *bla* gene. These results suggest that the observed beta-lactam resistance in strain V39c2 is mediated by penicillin-binding proteins of lower affinity, as has been previously described in *E. faecium* and also in other *E. faecalis* strains (McAlister *et al.*, 1999).

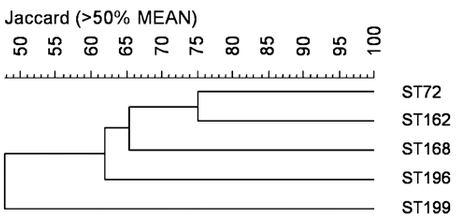


**FIG. 2.** Dendrogram showing the relatedness of sequence type (ST) 168 to other STs with closest allelic profiles differing by one, two, or three alleles.

Previous studies have reported the detection of enterococci on plants (Mundt, 1961, 1963; Ulrich and Müller, 1998; Müller *et al.*, 2001; Ott *et al.*, 2001) as well as in fresh produce (Johnston and Jaykus, 2004; Johnston *et al.*, 2005; McGowan *et al.*, 2006; Abriouel *et al.*, 2008), although their MLST profiles need to be studied. While *E. faecalis* is the predominant enterococcal species involved in nosocomial infections, a greater variety of enterococcal species has been reported for plant-associated as well as soil enterococci (Müller *et al.*, 2001; Petersen and Dalsgaard, 2003; Johnston and Jaykus, 2004; Abriouel *et al.*, 2008). The great differences existing between the ecological niches in which enterococci must thrive (including a lower and more heterogeneous temperature, irregular nutrient availability, competition with other enterococcal species, and other types of environmental stress that may occur in vegetables, water, and soil) would suggest that clinical isolates might have a lower possibility of survival in such environments. In a previous study, virulence factors most frequently found in clinical environments such as the collagen adhesin (Ace) and enterococcal surface protein (Esp) were not detected or had a very low incidence among isolates from vegetables (Abriouel *et al.*, 2008). Unlike *E. faecium*, the CCs described in *E. faecalis* do not suggest a well-defined origin or host specificity. In agreement with data from the present study, this lack of specificity is probably the result of a more versatile capacity for adaptation of *E. faecalis* to different ecological niches. Further work needs to be done with nonclinical *E. faecalis* isolates in order to better understand the population structure of this bacterial species.

## Conclusions

Results from the present study revealed two new sequence types in *E. faecalis*. The new

sequence type ST168 represents a singleton that was predominant among the enterococcal isolates from vegetable foods studied. Although one isolate belonged to the previously described CC9 associated to hospital environments, the lowest incidence of virulence factors and antibiotic resistance of this isolate suggest it is not a strain of clinical origin. The CCs described in *E. faecalis* do not suggest a well-defined origin or host specificity, and further work needs to be done with nonclinical *E. faecalis* isolates in order to better understand the population structure of this bacterial species.

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## Disclosure Statement

No competing financial interests exist.

## References

Aanensen DM and Spratt BG. The multilocus sequence typing network: mlst.net. Nucleic Acids Res 2005;33: W728–W733.

Abriouel H, Ben Omar N, Cobo Molinos A, *et al.* Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical samples. Int J Food Microbiol 2008;123:38–49.

[CLSI] Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*. Approved standard. Seventh edition. M7-A7. Villanova, PA: CLSI, 2006a.

[CLSI] Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing.* Sixteenth informational supplement. M100-S16. Villanova, PA: CLSI, 2006b.

Dutka-Malen S, Evers, S, and Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant Enterococci by PCR. J Clin Microb 1995;33:24–27.

Hummel AS, Hertel C, Holzapfel WH, *et al.* Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. Appl Environ Microbiol 2007;73:730–739.

Johnston LM and Jaykus LA. Antimicrobial resistance of *Enterococcus* species isolated from produce. Appl Environ Microbiol 2004;70:3133–3137.

Johnston LM, Jaykus LA, Moll D, *et al.* A field study of the microbiological quality of fresh produce. J Food Protect 2005;68:1840–1847.

Kawalec M, Pietras Z, Daniłowicz E, *et al.* Clonal structure of *Enterococcus faecalis* isolated from Polish hospitals: characterization of epidemic clones. J Clin Microbiol 2007;45:147–153.

Kayser FH. Safety aspects of enterococci from the medical point of view. Int J Food Microbiol 2003;88:255–262.

McAlister T, George N, Faoagali J, *et al.* Isolation of a β-lactamase positive vancomycin resistant *Enterococcus faecalis;* first case in Australia. Commun Dis Intell 1999; 23:237–239.

McBride SM, Fischetti VA, Leblanc DJ, *et al.* Genetic diversity among *Enterococcus faecalis*. PLoS ONE 2007;2: e582.

McGowan LL, Jackson CR, Barrett JB, *et al.* Prevalence and antimicrobial resistance of enterococci isolated from retail fruits, vegetables, and meats. J Food Prot 2006; 69:2976–2982.

Müller T, Ulrich A, Ott EM, *et al.* Identification of plant-associated enterococci. J Appl Microbiol 2001;91:268–278.

Mundt JO. Occurrence of the enterococci: bud, blossom, and soil studies. Appl Microbiol 1961;9:541–544.

Mundt JO. Occurrence of enterococci on plants in a wild environment. Appl Microbiol 1963;11:141–144.

Murray BE and Weinstock GM. Enterococci: new aspects of an old organism. Proc Assoc Am Physicians 1999;111: 328–334.

Nallapareddy SR, Wenxiang H, Weinstock GM, *et al.* Molecular characterization of a widespread, pathogenic, and antibiotic resistance-receptive *Enterococcus faecalis* lineage and dissemination of its putative pathogenicity island. J Bacteriol 2005;187:5709–5718.

[NCCLS] National Committee on Clinical Laboratory Standards. *Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard.* Eighth edition. M2-A8. Wayne, PA: NCCLS, 2003.

Ott EM, Müller T, Müller M, *et al.* Population dynamics and antagonistic potential of enterococci colonizing the phyllosphere of grasses. J Appl Microbiol 2001;91:54–66.

Petersen A and Dalsgaard A. Species composition and antimicrobial resistance genes of *Enterococcus* spp., isolated from integrated and traditional fish farms in Thailand. Environ Microbiol 2003;5:395–402.

Richards MJ, Edwards JR, Culver DH, *et al.* Nosocomial infections in combined medical-surgical intensive care units in the United States. Infect Control Hosp Epidemiol 2000;21:510–515.

Ruiz-Garbajosa P, Bonten MJ, Robinson DA, *et al.* Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol 2006;44:2220–2228.

Tannock GW and Cook G. Enterococci as members of the intestinal microflora of humans. In: *The Enterococci:*

*Pathogenesis, Molecular Biology and Antibiotic Resistance.* Gilmore MS (ed). Washington, DC: ASM Press, 2002, pp. 101–132.

Tendolkar PM, Baghdayan AS, and Shankar N. Pathogenic enterococci: new developments in the 21st century. Cell Mol Life Sci 2003;60:2622–2636.

Ulrich A and Müller T. Heterogeneity of plant-associated streptococci as characterised by phenotypic features and restriction analysis of PCR-amplified 16S rDNA. J Appl Microbiol 1998;84:293–303.

Urwin R and Maiden MC. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol 2003;11: 479–487.

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