Augmented Production of Extracellular Superoxide by Blood Isolates of Enterococcus faecalis

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To assess the frequency of extracellular superoxide (O$_2^-$) production by enterococci, multiple species were surveyed in a whole organism assay for their ability to reduce ferricytochrome c in a superoxide dismutase–inhibitable fashion. For stool and clinical enterococcal isolates and 12 type strains, only Enterococcus faecalis (87/91 isolates and ATCC 19443), Enterococcus faecium (5/13 isolates), Enterococcus casseliflavus (ATCC 25788), and Enterococcus gallinarum (ATCC 35038) produced extracellular O$_2^-$. Among 106 strains comprising 13 species of enteric gram-negative bacilli and gram-positive cocci, only Lactococcus lactis subspecies lactis produced extracellular O$_2^-$. Mean (±SE) rates of extracellular O$_2^-$ production in vitro by E. faecalis for isolates associated with bacteremia and endocarditis and for isolates from stool were 2.4 ± 0.2, 1.9 ± 0.2, and 1.5 ± 0.3 nmol of O$_2^-$/min·10$^9$ cfu, respectively (P = .025, analysis of variance), suggesting an association between invasiveness and extracellular O$_2^-$ production.

Enterococcus faecalis is a common nosocomial pathogen that causes 9% of the >100,000 annual hospital-acquired bacteremias in the United States [1]. Case/fatality ratios for enterococcal bacteremia range from 12% to 68%, with death due to enterococcal sepsis in 4%–50% of these cases [2]. Despite the frequency with which enterococci cause infection, little is known of enterococcal determinants important to intestinal colonization or translocation, tissue adherence, evasion of host immunity, or modulation of inflammatory responses [2]. Understanding these events has become a pressing concern because the number of serious infections due to vancomycin- and methicillin-resistant strains that are less effectively treated by standard therapies has greatly increased [3].

Enterococci secrete few known extracellular products [2]. Some are potential virulence factors, such as chemoattractant pheromone peptides, gelatinase, and a membrane-lytic, bi-component cytolysin. Other characteristics potentially important to infection, however, remain poorly defined. For example, the potent extracellular reducing capacity of enterococci, although long recognized by microbiologists [4], has not been investigated as a potential virulence trait. Recent reports have described enterococcal cell extracts and whole organisms as capable of generating superoxide (O$_2^-$) [5–7], a free radical anion formed by the single electron reduction of molecular oxygen. Under appropriate conditions, O$_2^-$ can lead to powerful oxidants such as hydrogen peroxide and hydroxyl radical [8]. Whether O$_2^-$ production by enterococci augments the pathogenicity of these organisms remains to be determined. Using a whole organism assay, we sought to determine the frequency of extracellular O$_2^-$ by enterococci and selected facultative and aerobic intestinal bacteria. Then, to assess whether this trait was associated with invasive infection, the O$_2^-$ production was quantified in vitro and compared among clinical and commensal enterococcal isolates.

Clinical isolates included Escherichia coli (n = 11), Serratia marcescens (n = 6), Pseudomonas aeruginosa (n = 10), Enterobacter aerogenes (n = 7), Enterobacter cloacae (n = 8), Klebsiella pneumoniae (n = 10), S. salivarius (n = 8), S. bovis (n = 4), S. sanguis (n = 10), Streptococcus anginosus (n = 3), S. aureus (n = 10), and Staphylococcus epidermidis (n = 10) (Clinical Microbiology Laboratories, University Hospital, and Department of Veterans Affairs Medical Center, Oklahoma City) and S. pyogenes (n...
Results

To determine the frequency of extracellular O$_2$ production among intestinal organisms and common gram-positive pathogens, enterococci and selected facultative or aerobic bacteria were assayed for O$_2^-$ production using reduction of ferricytochrome c in the absence and presence of manganese-superoxide dismutase (MnSOD). Representative spectrophotometric assays are shown in figure 1. Among stool and blood isolates and type strains of enterococci, only E. faecalis (87/91 isolates), E. faecium (5/13 isolates), E. casseliflavus, and E. gallinarum produced detectable extracellular O$_2^-$.

The latter 2 species are more enterococci and uncommon causes of human infection [3]. Two noncytolytic, plasmid-free laboratory strains of E. faecalis (JH2-2 and OG1RF) also produced extracellular O$_2^-$, indicating the phenotype was not necessarily a plasmid-encoded trait. Among other gram-positive organisms, only L. lactis subspecies lactis generated extracellular O$_2^-$. None of the gram-negative organisms reduced ferricytochrome c in a superoxide dismutase–inhibitable fashion.

To determine whether extracellular O$_2^-$ production by E. faecalis was greater for strains causing bloodstream infection than intestinal commensals, the rate of production was mea-

Three sets of enterococcal isolates, two from blood and one from stool, were used in this study. One set of blood isolates (n = 36; provided by W. W. Wilson and J. M. Steckelberg, Mayo Clinic, Rochester, MN) was collected between 1973 and 1991 from patients with endocarditis. Four strains were E. faecium, and the rest were E. faecalis. The second set of blood isolates (n = 43) consisted of nonduplicate strains from patients with bacteremia at the University of Wisconsin Hospital and Clinics (Madison) between June 1985 and April 1987 [9]. All strains in this set were E. faecalis. In the original cohort, ~45% of the strains were closely related or identical by contour-clamped homogeneous electric field electrophoresis; only 1 of these strains was used. Among Wisconsin isolates, 3 were from patients with endocarditis and 2 were associated with burn wounds, 4 with catheter-related infections, 10 with urinary tract infections, and 9 with abscess, wound, or soft tissue infections; 15 had no identifiable source. The third set of strains (n = 23) were isolated on bile-esculin-azide agar (Difco, Detroit) in 1994 from swabs of stool passed by healthy persons, 3–42 years old, who had not been recent inpatients and did not work in health-related areas. Of these strains, 14 were E. faecalis, 8 E. faecium, and 1 E. avium. All enterococci were identified using the API 20S kit (Analytab Products, Plainview, NY).

Extracellular superoxide assay. Bacteria were grown in brain-heart infusion broth (Difco) overnight, washed twice with PBS, and resuspended in ice-cold Hanks’ balanced salt solution (GIBCO, Grand Island, NY) to an approximate optical density (OD) of 0.2 absorbance unit (AU). Before each assay, the OD of bacteria was determined at 550 nm using a spectrophotometer (DU 640; Beckman Instruments, Irvine, CA). After being warmed to room temperature, a stock solution of ferricytochrome c (Sigma, St. Louis) was added to bacteria in 1-cm cuvettes to give a final concentration of 20 μM, and the reduction of ferricytochrome c was measured over 3 min at 550 nm in the presence or absence of manganese-superoxide dismutase (25 μg mL$^{-1}$; Sigma). As a 12-kDa protein, ferricytochrome c is not likely to diffuse across intact cellular membranes. In a whole organism assay, its reduction is consistent with an extracellular redox species. The rate of ferricytochrome c reduction (dAU min$^{-1}$ OD$^{-1}$) was computed using linear regression.

For strains that reduced ferricytochrome c, the portion due to O$_2^-$ was determined by subtracting the rate of reduction in the presence of manganese–superoxide dismutase (Sigma) from the rate measured in its absence. O$_2^-$ production (nmol min$^{-1}$ OD$^{-1}$) was calculated using an extinction coefficient of 21.5 mM$^{-1}$ cm$^{-1}$ for reduced cytochrome c [10]. Since 1 OD U for enterococci corresponds to 10$^{9}$ cfu mL$^{-1}$ (data not shown), results of O$_2^-$ production are reported in nmol min$^{-1}$ 10$^9$ cfu$^{-1}$. For each enterococcal strain, ferricytochrome c reduction was the average of three or more independent assays of separately picked colonies. Differences in O$_2^-$ production among sets of enterococci were analyzed by analysis of variance. P < .05 was considered significant.

Figure 1. Reduction of ferricytochrome c by E. faecalis 19443, Enterococcus faecium 19434, and a clinical blood isolate of Pseudomonas aeruginosa with and without manganese-superoxide dismutase (MnSOD).
Enterococcus faecalis

Figure 2. Extracellular O2 production by invasive and commensal Enterococcus faecalis isolates.

...asured for the sets of blood and stool isolates (figure 2). Mean rates (±SE) for isolates associated with bacteremia and endocarditis and for isolates from stool were 2.4 ± 0.2, 1.9 ± 0.2, and 1.5 ± 0.3 nmol O2 min⁻¹ 10⁹ cfu⁻¹, respectively (P = .025 by analysis of variance). By post hoc testing, extracellular O2 production was significantly higher for bacteremia- and endocarditis-associated strains than for stool isolates (P = .017). E. faecium strains, which comprised 35% of enterococci recovered from stool (8/23) but only 5% of blood isolates (4/75; P < .001, Fisher’s exact test), typically produced little or no extracellular O2. Five E. faecium strains reduced ferricytochrome c, and of these, only 1.0 ± 0.3 nmol O2 min⁻¹ 10⁹ cfu⁻¹ was produced on average.

Discussion

This study demonstrates that extracellular O2 production is a trait frequently expressed by several enterococcal species and L. lactis subspecies lactis but not by some common commensal streptococci, staphylococci, and aerobic gram-negative bacilli. Furthermore, among enterococci, extracellular O2 production is more common for strains of E. faecalis and motile enterococci than E. faecium. As E. faecalis is most commonly associated with invasive infection among enterococci, this suggests a potential role for extracellular O2 as a virulence factor.

Reactive oxygen species produced by Mycoplasma pneumoniae, S. pneumoniae, and P. aeruginosa have been implicated as virulence factors [11–13]. To begin assessment of what role, if any, extracellular O2 might play in the pathogenesis of E. faecalis infections, we surveyed isolates from the bloodstream of patients and stool of healthy volunteers. E. faecalis strains associated with bacteremia produced O2 at a 60% higher rate than strains from stool. Although this difference was statistically significant, its biologic relevance is currently unknown. Perhaps extracellular O2 (or hydrogen peroxide and hydroxyl radical arising from O2⁻ under appropriate in vivo conditions [8]) confers subtle growth advantages to enterococci in the intestinal milieu so as to facilitate colonization or overgrowth, or perhaps it heightens the ability of invasive strains to evade host defenses, augment inflammatory responses, or damage eukaryotic cells. These observations also fail to explain why enterococci evolved the ability to generate extracellular O2. The trait does not appear essential for intestinal colonization or bloodstream infection, since strains that do not produce extracellular O2 were isolated from these sites, and motile strains of enterococci that produce extracellular O2 are rarely isolated from humans. Further work is needed in this area.

The mechanism by which these organisms generate extracellular O2 is unknown. Cytochrome c reductase activity was reported 30 years ago by Dolin [14] in membrane preparations of E. faecalis 10C1. This finding is consistent with a membrane location for O2⁻ production that has also been found by our laboratories [7]. The nature of this enzyme activity, however, has yet to be elucidated. Additional studies are in progress to clone genes involved in extracellular O2 production and determine whether this free radical promotes the virulence of E. faecalis.

References