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Cytotoxicity of Hydrogen Peroxide Produced by *Enterococcus faecium*

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Although the opportunistic bacterial pathogen *Enterococcus faecium* is a leading source of nosocomial infections, it appears to lack many of the overt virulence factors produced by other bacterial pathogens, and the underlying mechanism of pathogenesis is not clear. Using *E. faecium*-mediated killing of the nematode worm *Caenorhabditis elegans* as an indicator of toxicity, we determined that *E. faecium* produces hydrogen peroxide at levels that cause cellular damage. We identified *E. faecium* transposon insertion mutants with altered *C. elegans* killing activity, and these mutants were altered in hydrogen peroxide production. Mutation of an NADH oxidase-encoding gene eliminated nearly all NADH oxidase activity and reduced hydrogen peroxide production. Mutation of an NADH peroxidase-encoding gene resulted in the enhanced accumulation of hydrogen peroxide. *E. faecium* is able to produce hydrogen peroxide by using glycerol-3-phosphate oxidase, and addition of glycerol to the culture medium enhanced the killing of *C. elegans*. Conversely, addition of glucose, which leads to the down-regulation of glycerol metabolism, prevented both *C. elegans* killing and hydrogen peroxide production. Lastly, detoxification of hydrogen peroxide either by exogenously added catalase or by a *C. elegans* transgenic strain overproducing catalase prevented *E. faecium*-mediated killing. These results suggest that hydrogen peroxide produced by *E. faecium* has cytotoxic effects and highlight the utility of *C. elegans* pathogenicity models for identifying bacterial virulence factors.

Enterococci are gram-positive bacteria that usually reside in the gastrointestinal tract as commensal organisms, but they are also capable of causing severe infections (18). Enterococci are the third leading source of nosocomial infections, causing endocarditis, peritonitis, bacteremia, and urinary tract infections. Two enterococcal species are responsible for almost all of these infections. According to a 1997 survey, the majority (85 to 90%) of enterococcal infections are caused by *Enterococcus faecalis*, and the remaining infections are due to *Enterococcus faecium* (35). However, in certain settings, the frequency of infections due to *E. faecium* has been increasing in recent years (50). The acquisition of antibiotic resistance is believed to be the major contributing factor for the elevated incidence of *E. faecium* infections. Approximately one-half of *E. faecium* clinical isolates are now resistant to vancomycin, while only a small fraction of *E. faecalis* clinical isolates are vancomycin resistant (50).

The mechanism underlying *E. faecium* pathogenesis is obscure, due in part to the fact that few *E. faecium* virulence-related factors have been identified. The esp_{fm} and hyl_{Efm} genes, encoding a surface protein and hyaluronidase, respectively, are more likely to be present in pathogenic *E. faecium* strains than in strains isolated from healthy individuals (11, 45, 61). The esp_{fm} gene appears to be located on a pathogenicity island containing genes implicated in virulence, antibiotic resistance, and transcriptional regulation (29). Other *E. faecium* factors predicted to contribute to pathogenesis are the Acm and SagA proteins, which bind to extracellular matrix components (34, 58).

One of the limitations of studying *E. faecium* infections is the lack of a simple animal model. Our laboratory has pioneered the use of simple model hosts to identify microbial virulence factors. We have used the plant *Arabidopsis thaliana*, the wax moth caterpillar *Galleria mellonella*, and the nematode *Caenorhabditis elegans* as simple model hosts to study infections caused by *E. faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *Cryptococcus neoformans* (1, 17, 26, 31, 33, 44, 51, 57). Importantly, the majority of virulence factors required for infection of the simple model hosts are also required for infection in mouse infection models, indicating that the pathogenic mechanisms used in mammalian infections are also used in the simple model hosts (43).

The *C. elegans* model system has been used to study two different types of pathogenic mechanisms. *C. elegans* can be killed by pathogens either through an infection-like process or by diffusible toxins. To assay either mechanism, *C. elegans*, which feeds on bacteria, is transferred from its normal food source, lawns of an auxotrophic strain of *Escherichia coli*, to lawns of the pathogen. In the infection model system, the nematodes ingest the bacteria and then die after 2 to 5 days, depending on the pathogen. In contrast, killing by diffusible toxins occurs more quickly and does not require direct bacterial contact. *P. aeruginosa* strain PAO1 produces cyanide that paralyzes and kills *C. elegans* (9, 16). *P. aeruginosa* strain PA14 produces different toxins, including phenazines, when it is

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grown on high-osmolarity medium, which quickly kill but do not paralyze *C. elegans* (31).

Previously, our laboratory showed that *E. faecalis* kills *C. elegans* by an infectious process but that *E. faecium* does not (17). Here, however, we show that *E. faecium* is capable of killing *C. elegans* by a diffusible toxin after the bacteria are grown under anaerobic conditions. By identifying mutants altered in nematode killing, we found that *E. faecium* releases substantial amounts of the reactive oxygen species hydrogen peroxide and that hydrogen peroxide is responsible for nematode killing. Other bacterial pathogens, including *Streptococcus pneumoniae* and *Streptococcus pyogenes*, also produce hydrogen peroxide that kills *C. elegans* (5, 27, 53). Hydrogen peroxide produced by *S. pneumoniae* has been shown to contribute to virulence by damaging host tissue (6, 10, 22). An additional activity of hydrogen peroxide is the growth inhibition of competing bacterial species (20, 39). We suggest that the hydrogen peroxide produced by *E. faecium* causes damage to surrounding cells, and this may play a role in colonization or infection.

MATERIALS AND METHODS

Bacterial and nematode strains. Enterococcal strains were grown on brain heart infusion (BHI) media (Difco Becton Dickinson, Sparks, Md.) containing kanamycin (75 μg/ml) or nalidixic acid (10 μg/ml) at 37°C. Liquid cultures were typically grown without shaking. We used *E. faecium* strains GE-1 (= ATCC 51558) (12), E007 (17), E0158 (61), E0238 (61), E0318 (61), E0734 (61), SE34 (= TX1330) (59), and DO (= TX0016 = ATCC BAA-472) (2) and *E. faecalis* strains OG1RF (32) and VS583. The *vanHB* genes were deleted in strain V583 (49) to produce the vancomycin-sensitive strain VS583.

C. elegans strains were maintained by using standard practices (56). Wild-type Bristol strain N2 (7) was used for all experiments unless otherwise noted. We also used strains *daf-2*(*e1370*), *daf-16*(*mgDf47*) (37), and *daf-2*(*e1370*) *daf-16*(*mgDf47*).

Nematode killing assay. Bacterial cultures were grown in BHI medium at 37°C and diluted to an optical density at 600 nm (OD_{600}) of 0.1 to 0.2 in fresh medium, and then 10-μl portions were spread onto BHI agar containing kanamycin (75 μg/ml) or nalidixic acid (10 μg/ml) in 35-mm petri plates. The plates were incubated overnight at 37°C. GasPak Plus envelopes and containers (Becton Dickinson) were used to generate an anaerobic environment. Before the nematodes were transferred to the anaerobically grown lawns of *E. faecium*, the plates were cooled to room temperature in ambient air for 30 to 60 min. Nematodes at the L4 developmental stage were resuspended and washed two times in M9 buffer. Nematodes were then transferred to the bacterial lawns in 5- to 10-μl drops and incubated at 25°C. At the times indicated below, nematodes were scored for survival. Nematodes that did not respond to touch with a platinum wire pick were considered to be dead. Assays for each bacterial strain and condition were done at least twice, and each experiment was done in duplicate or triplicate.

In order to estimate the numbers of viable bacteria on the assay plates, the bacterial lawns were resuspended in 50 mM sodium phosphate (pH 7), diluted, plated, and counted to determine the number of CFU. Approximately $6.9 \times 10^9 \pm 2.3 \times 10^9$ CFU were recovered from an *E. faecium* lawn grown anaerobically on a 35-mm petri plate. For certain experiments, either catalase (catalogue no. C-1345; Sigma, St. Louis, Mo.) was mixed into unsolidified BHI agar at a concentration of 1,000 U/ml or 1,000 U of catalase in 100 μl was spread onto solidified BHI agar. Superoxide dismutase (catalogue no. S-2515; Sigma) was spread onto solidified BHI agar in 100 μl containing 1,000 U.

Screening for Tn*917* mutants. Tn*917* contained in the temperature-sensitive plasmid pTV1OK (19) was transformed into *E. faecium* SE34 by electroporation as described previously (15). Four independent overnight cultures were grown at 25°C in BHI medium containing 2 mg of kanamycin per ml and 0.05 μg of erythromycin per ml, plated onto BHI medium containing 2 μg of erythromycin per ml, and incubated at 47°C to select for transposon integrants. Colonies were picked by using a Q-bot (Genetix, Boston, Mass.) into 384-well plates, grown overnight in BHI medium containing 50 μg of erythromycin per ml and 15% glycerol, and frozen at −80°C. BHI medium containing 50 μg of erythromycin per ml was inoculated with the frozen strains and incubated at 37°C. Portions (20 μl) of the saturated cultures were spread onto 1 ml of BHI agar in duplicate 24-well plates and grown at 37°C. For each set of duplicate plates one plate was grown in a GasPak container overnight, and the other was grown in ambient air. The plates were cooled to room temperature, and the anaerobically grown plate was exposed to air. A mixture of starved L1-L2 stage and well-fed L4 stage nematodes was suspended in M9 buffer and pipetted onto the lawns of *E. faecium*. After 2 h, the anaerobically grown plates were examined for wells in which worms remained mobile. After 6 h, the aerobically grown plates were examined for wells in which worms became immobile.

A total of 3,120 erythromycin-resistant isolates were tested by both procedures, and 4 of these strains were found to have a reproducibly altered effect on nematode mobility. Mutants *2F22*, *3C23*, and *11M12*, identified on the anaerobically grown plates, exhibited diminished killing activity, whereas mutant *2C4*, identified on the aerobically grown plates, exhibited enhanced killing activity.

The transposon insertion sites of the *E. faecium* mutants were identified by using two rounds of arbitrary primed PCR followed by DNA sequencing (38). Two mutants (*2F22* and *3C23*) that were derived from the same liquid culture, which was grown under nonselective conditions, were found to have identical transposon insertion sites. These mutants are most likely siblings, and only the *2F22* mutant was characterized further. The genes surrounding the transposon insertion site of the *11M12* mutant were not found in the currently available genomic sequence of *E. faecium* strain DO/TX0016 (http://www.hgsc.bcm.tmc.edu/microbial/Efaecium/), and these genes were PCR amplified and sequenced.

Hydrogen peroxide measurement. To measure hydrogen peroxide accumulation upon aeration of saturated cultures grown under anaerobic conditions, 100-ml BHI medium cultures of the relevant *E. faecium* strains in 500-ml flasks were grown at 37°C without agitation in GasPak containers. Cultures were removed from the GasPak containers and aerated on an orbital shaker at 300 rpm. To measure bacterial growth and hydrogen peroxide accumulation in aerobic cultures, overnight cultures were grown without shaking, diluted into 100 ml of prewarmed BHI medium in 500-ml flasks, and aerated at 37°C by shaking at 300 rpm. Samples were filtered through 0.22-μm-pore-size filters (Costar Spin-x; Costar, Corning, N.Y.), and the hydrogen peroxide levels of the bacterium-free culture eluates were measured with a 907-015 Correlate assay kit by using a 1:10 dilution with buffer (Assay Designs, Ann Arbor, Mich.) or with an Amplex Red kit by using a 1:100 dilution (Molecular Probes, Eugene, Oreg.).

Northern blotting. Overnight saturated cultures were diluted 1:100 in prewarmed BHI medium and either shaken at 300 rpm or incubated without agitation in GasPak containers. Logarithmic-phase cultures were harvested at an OD_{600} of 0.2 to 0.3, and saturated cultures were harvested at an OD_{600} of ≥1.2. Cultures were treated with RNAprotect and were purified with RNeasy kits (QIAGEN, Valencia, Calif.). Five micrograms of RNA of each sample was run on 1.2% agarose–formaldehyde gels and transferred to positively charged nylon membranes. RNA blot hybridizations were carried out as previously described (3) by using 400-base probes labeled with [^{32}P]dCTP by random priming. To analyze the effect of heavy metal exposure, logarithmic-phase aerobic cultures were treated with heavy metals at a concentration of 1 mM for 10 min at 37°C before cells were harvested. One molar stock solutions of cadmium chloride, cobalt(II) chloride, sodium dichromate, chromium trioxide, copper(II) chloride, iron(II) chloride, iron(III) chloride, magnesium chloride, manganese(II) chloride, nickel(II) sulfate, silver nitrate, and zinc chloride were used.

NADH oxidase assay. Aerobic cultures were harvested at an OD_{600} of 0.2 to 0.3. Cells were washed with ice-cold buffer containing 50 mM potassium phosphate (pH 7.0) and 0.5 mM EDTA. Cells were disrupted by using glass beads (catalogue no. G-1145; Sigma) and a Mini Bead Beater (Biospec Products, Bartlesville, Okla.) and were microcentrifuged for 15 min at 4°C at 16,000 × *g*. The protein concentration was determined by the Pierce protein assay (catalogue no. 23200; Pierce, Rockford, Ill.) by using bovine serum albumin as a standard. Typically, 10 μg of extract was added to a 1-ml reaction mixture containing 150 μM NADH (catalogue no. N-8129; Sigma) in the buffer described above, and the absorbance at 340 nm was monitored for 2 to 3 min. For lysates from the *2F22* mutant, 100 μg of protein was added, and the reaction was monitored for 10 min. An extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ was used to calculate NADH concentrations. One unit was defined as 1 μmol of NADH oxidized per min at 25°C.

Nucleotide sequence accession number. The sequence of the genes surrounding the transposon insertion site of the *11M12* mutant has been deposited in the GenBank database under accession number AY527733.

RESULTS

***E. faecium*-mediated killing of *C. elegans*.** Our laboratory previously reported that several strains of *E. faecium* grown

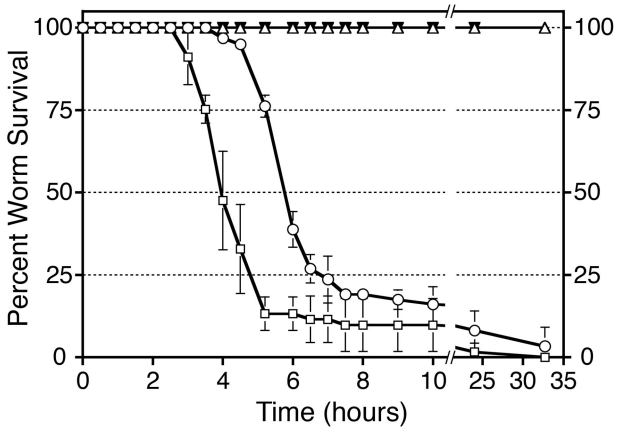

FIG. 1. *C. elegans* survival when it was fed enterococci. *E. faecium* and *E. faecalis* were grown on solid BHI medium under aerobic (solid symbols) or anaerobic (open symbols) conditions. L4-stage *C. elegans* was transferred onto the lawns of enterococci, incubated at 25°C, and scored for survival. Symbols: ○, *E. faecium* strain SE34 grown anaerobically; □, *E. faecium* strain E007 grown anaerobically; △, *E. faecalis* strain VS583 grown anaerobically; ▼, *E. faecium* strain SE34 grown aerobically.

aerobically on BHI medium do not have any apparent deleterious effect on the nematode *C. elegans* when the bacteria are provided to the worms as the sole source of food (17). However, when grown under anaerobic conditions on BHI medium and then exposed to aerobic conditions, a variety of different *E. faecium* strains appeared to produce a toxin that rapidly killed *C. elegans* (Fig. 1 and data not shown). Depending on the strain tested, the initial effect of the toxicity was observed in as little as 45 min as a decrease in nematode mobility. After 1 to 2 h of exposure to anaerobically grown *E. faecium*, the nematodes lost muscle tone and became immobile. Fifty percent of the nematodes died within 4 to 6 h after the initial exposure, and nearly all of the nematodes were dead after 24 h (Fig. 1). Eight assorted *E. faecium* strains (strains DO, E007, E0158, E0238, E0318, E0734, GE-1, and SE34) killed *C. elegans* with similar kinetics when they were grown under anaerobic conditions. Similar to previously reported results, no killing activity was observed when the eight strains were grown aerobically (Fig. 1 and data not shown). In contrast to *E. faecium*, *E. faecalis* (strain OG1RF or VS583), killed *C. elegans* relatively slowly over a 5-day period, irrespective of whether it was grown aerobically or anaerobically (Fig. 1, Table 1, and data not shown).

Identification of *E. faecium* mutants. In other *C. elegans* pathogenicity models developed in our laboratory and in other laboratories, relatively rapid killing of *C. elegans* is indicative of the production of a low-molecular-weight toxin (9, 16, 31). To identify the putative *E. faecium* fast-killing toxin, *E. faecium* was mutagenized with transposon Tn*917* as described in Materials and Methods, and 3,120 transposon-mutagenized clones were screened for mutants that exhibited aberrant *C. elegans* killing activity.

In order to identify mutants with diminished killing activity, mutant clones were grown anaerobically, returned to air, and then incubated with wild-type *C. elegans*. This screening procedure led to identification of two mutants (*2F22* and *11M12*) with diminished killing activity. Fifteen hours of exposure to anaerobically grown parental strain SE34 killed all of the nematodes. In contrast, 87 and 100% of the nematodes survived exposure to mutants *2F22* and *11M12*, respectively (Table 1). Some toxicity was retained in mutant *2F22* since it was able to impair nematode mobility, whereas no inhibition of nematode mobility was observed with mutant *11M12*.

TABLE 1. Worm survival on lawns of *Enterococcus*

Bacterial strain	Growth condition[a]	% Survival of nematodes	Mobility[b]
E007	Anaerobic	0.0 ± 0.0	−
VS583	Anaerobic	100 ± 0.0	+++
SE34	Anaerobic	0.0 ± 0.0	−
2C4	Anaerobic	0.0 ± 0.0	−
2F22	Anaerobic	86.6 ± 5.4	−/+
11M12	Anaerobic	100 ± 0.0	+++
SE34	Aerobic	100 ± 0.0	+++
2C4	Aerobic	23.1 ± 3.7	−/+

[a] Worm survival was scored after 15 h of incubation for anaerobic growth and after 18 h of incubation for aerobic growth.
[b] Average worm mobility was scored as follows: +++, normal movement; ++, slowed movement; +, movement restricted to the head and tail; −, no movement.

To identify *E. faecium* mutants with enhanced killing activity, we screened for mutants that acquired the ability to kill nematodes when they were grown aerobically. One such mutant, *2C4*, was identified. Under these assay conditions, mutant *2C4* killed 77% of the nematodes within 18 h, whereas no nematodes were killed by the aerobically grown parental strain SE34 (Table 1). The differences in *C. elegans* killing between the mutants and the parental strain could not be attributed to differences in the number of viable CFU on the assay plates (as described in Materials and Methods, the numbers of CFU of the mutant strains recovered from the assay plates were similar to the numbers of CFU of the parental strain).

Tn*917* is inserted just upstream of the *npr* gene encoding an NADH peroxidase in the hypervirulent mutant *2C4*. Molecular analysis of the enhanced-killing mutant *2C4* showed that Tn*917* had integrated into the *E. faecium* chromosome 16 bp upstream of the start codon of the *npr* gene, which encodes an NADH peroxidase (NPX) (Fig. 2A). The *E. faecium* NADH peroxidase exhibits 46% amino acid identity with the *E. faecalis* 10C1 NADH peroxidase (46) and is homologous over the entire length of the protein. In the parental strain SE34, *npr* is transcribed as a 1.6-kb monocistronic transcript that is expressed at low levels during aerobic growth and is highly induced during stationary-phase growth. However, the *npr* transcript was not detectable in the *2C4* mutant (Fig. 2B).

E. faecium is a facultative anaerobe that uses oxidases that reduce molecular oxygen to form hydrogen peroxide (23). *E. faecium* is catalase negative, and the major mechanism to scavenge hydrogen peroxide is NADH peroxidase. We hypothesized that the absence of NADH peroxidase allows increased amounts of hydrogen peroxide to accumulate in *2C4* cultures and that hydrogen peroxide might be the cause of *E. faecium*-mediated rapid killing of *C. elegans*.

In aerobic liquid cultures, both parental strain SE34 and mutant *2C4* produced hydrogen peroxide as the cultures entered the stationary phase (see below). To simulate the amount

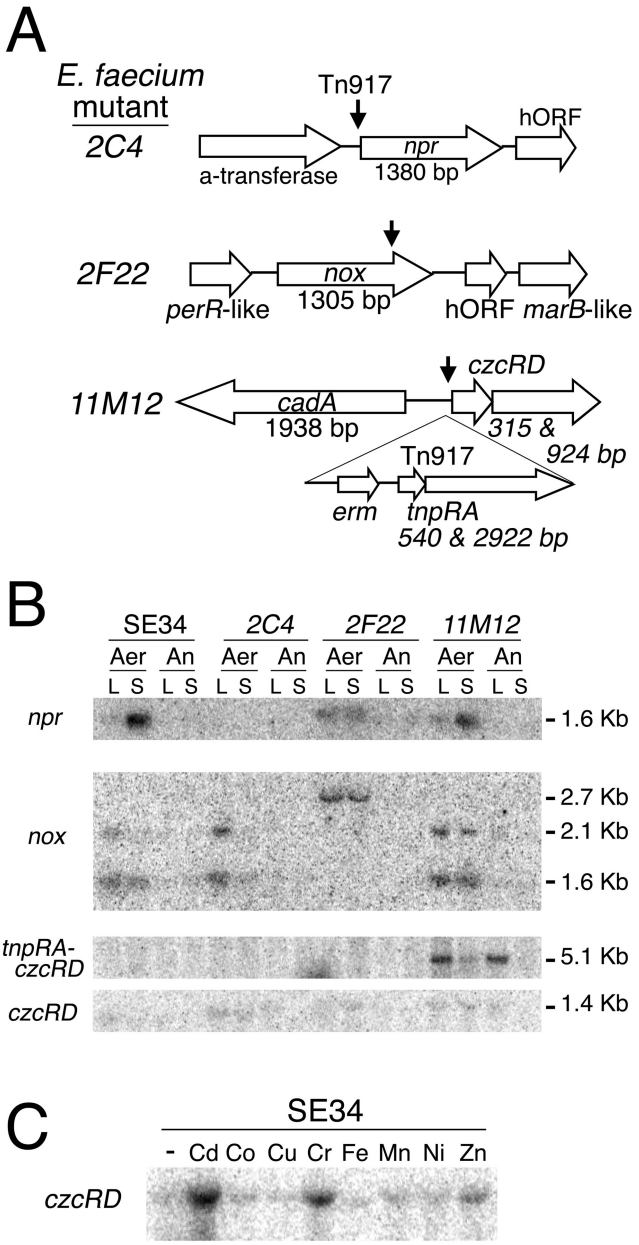

FIG. 2. (A) DNA map of transposon Tn*917* insertion sites in *E. faecium* mutants *2C4*, *2F22*, and *11M12*. The vertical arrows indicate Tn*917* insertion sites. (B) Northern blot of *E. faecium* strains SE34, *2C4*, *2F22*, and *11M12* hybridized against the *npr*, *nox*, and *czcRD* transcripts. Cultures were grown aerobically (Aer) or anaerobically (An) and harvested from logarithmic-phase (L) or saturated (S) cultures. (C) Northern blot of SE34 treated with various heavy metals hybridized against the *czcRD* transcript. Aerobic log-phase cultures were treated with cadmium, cobalt, copper, chromium, iron(II), manganese, nickel, or zinc at a concentration of 1 mM for 10 min. The first lane (−) contained a mock-treated culture.

of hydrogen peroxide putatively produced by *E. faecium* in the worm-killing assays, bacteria were grown to saturation in liquid cultures under anaerobic conditions and then aerated in the presence of atmospheric oxygen. No hydrogen peroxide was detectable in anaerobic cultures, but upon aeration, hydrogen peroxide accumulated at a rate of approximately 0.24 mM per h per 10^9 CFU/ml in the case of wild-type strain SE34 (Fig. 3A). Under the same experimental conditions, the *2C4* mutant culture accumulated hydrogen peroxide at a rate that was approximately two times the rate of the parental strain. The culture densities of SE34 and *2C4* were similar and remained constant during the 3 h of aeration.

Hydrogen peroxide produced by *E. faecium* is sufficient to kill *C. elegans*. Several lines of evidence are consistent with the hypothesis that hydrogen peroxide is the lethal toxin produced by *E. faecium*. First, nematodes were killed by hydrogen peroxide in a bacterium-free liquid assay at a rate similar to the rate of killing by lawns of *E. faecium* (Table 2). After 15 h of incubation with concentrations of hydrogen peroxide greater than 0.2 mM in M9 buffer, nematode mobility was decreased. Incubation with 0.3 to 0.4 mM hydrogen peroxide killed 50% of the nematodes, and 98% of the nematodes were killed by 1 to 2 mM hydrogen peroxide (Table 2).

Second, addition of exogenous catalase to the solid BHI medium completely rescued nematode killing by *E. faecium*. In addition, incorporation of catalase allowed the nematodes to remain mobile (Table 3). In contrast to catalase treatment, addition of superoxide dismutase to the medium did not have any effect on nematode killing (data not shown).

Third, *C. elegans daf-2* mutants, which are resistant to a variety of oxidative stresses, exhibit enhanced longevity, and constitutively overexpress catalase (60), were more resistant to killing by anaerobically grown *E. faecium* (Table 4). *daf-2* encodes an insulin-like receptor that negatively regulates the fork head transcription factor DAF-16 (37). Thus, *daf-16* mutations suppress the long-lived and stress-resistant phenotypes of *daf-2* mutations. As expected, a *daf-2 daf-16* double mutant was as susceptible to *E. faecium*-mediated killing as wild-type *C. elegans* (Table 4).

***E. faecium* mutants defective in nematode killing.** Based on the observation that *E. faecium* produces hydrogen peroxide, which kills *C. elegans*, we predicted that the *E. faecium* mutants identified in our screening analysis that are defective in nematode killing would produce less hydrogen peroxide. We confirmed this prediction for mutants *2F22* and *11M12* by measuring the hydrogen peroxide produced after aeration of stationary-phase cultures. When cultures of *2F22* and SE34 were grown under anaerobic conditions and then aerated for 3 h, the *2F22* culture accumulated 75% less hydrogen peroxide than the SE34 culture accumulated even though the culture densities were equivalent (Fig. 3A). Although cultures of *11M12* grown anaerobically to saturation had a lower number of viable CFU per milliliter than SE34 cultures had for unexplained reasons (data not shown), both *11M12* and SE34 grew to the same density in semiaerobic cultures (grown without agitation in the presence of atmospheric air). When semiaerobic saturated cultures of *11M12* and SE34 were aerated for 3 h, the *11M12* mutant, like *2F22*, accumulated 75% less hydrogen peroxide than SE34 accumulated (Fig. 3B).

In mutant *2F22*, Tn*917* integrated into the *nox* gene that apparently encodes the major NADH oxidase of *E. faecium* (Fig. 2A and see below). NADH oxidases use molecular oxygen as an electron acceptor to regenerate NAD^+ from NADH. There are two categories of NADH oxidases; one type produces water, whereas the second type produces hydrogen peroxide. In the parental SE34 strain under aerobic growth con-

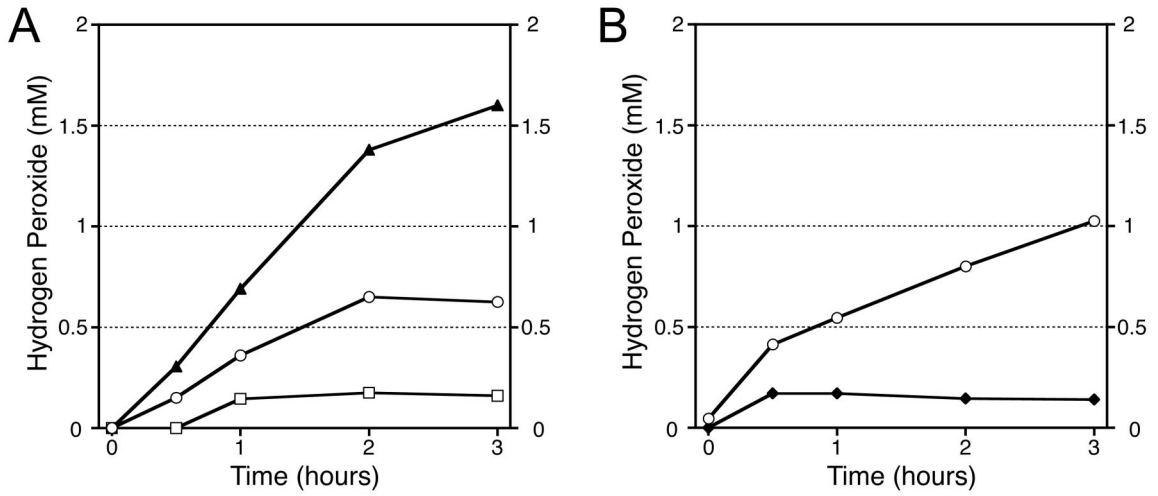

FIG. 3. (A) Hydrogen peroxide accumulation upon aeration of saturated cultures grown under anaerobic conditions. (B) Hydrogen peroxide accumulation upon aeration of saturated cultures grown under semiaerobic conditions. Symbols: ○, parental strain SE34; ▲, mutant *2C4*; □, mutant *2F22*; ♦, mutant *11M12*.

ditions, there are two *nox* gene transcripts, a 1.6-kb monocistronic transcript and a 2.1-kb transcript that also contains the adjacent 300-bp hypothetical open reading frame with an unknown function (Fig. 2B). In the *2F22* mutant, these two *nox* transcripts are replaced by a single 2.7-kb transcript (Fig. 2B). As measured by a spectroscopic assay, the total NADH oxidase activity was decreased 98% in the *2F22* mutant. The parental strain had an NADH oxidation activity of 0.882 ± 0.051 U per mg of total soluble protein, whereas the *2F22* mutant had an activity of only 0.019 ± 0.003 U per mg of protein. The partially sequenced *E. faecium* strain DO contains five other putative NADH oxidase genes, which may account for the remaining small amount of NADH oxidase activity in extracts of *2F22*.

Interestingly, even though *2F22* produces significantly less H_2O_2 than the wild type produces, the *nox* gene mutated in *2F22* appears to encode the water-forming type of NADH oxidase since no hydrogen peroxide was detectable in the NOX assay when wild-type extracts were used. Moreover, mutant *11M12*, which is also defective in hydrogen peroxide production, has the same NADH oxidase activity as the parental strain. The regeneration of NADH by NADH oxidases allows bacteria to use alternative mixed acid fermentation pathways when they are grown in the presence of molecular oxygen (21, 30). Mutation of *nox* constrains bacteria toward anaerobic metabolism, which in turn is predicted to lead to reduced activity of oxidases that produce hydrogen peroxide. Although this explains the phenotype of the *2F22* mutant, we have not ruled out the possibility that the decreased hydrogen peroxide production of the *2F22* mutant is due to a polar effect on downstream genes. Unfortunately, we were unable to transform plasmids into mutant *2F22* because the strain lysed upon centrifugation while the cells were being prepared for electroporation. Consequently, we were unable to express nox^+ in mutant *2F22*, and we were not able to confirm that the *nox* mutation is responsible for the phenotype of the *2F22* mutant.

In mutant *11M12*, Tn*917* is integrated in a locus involved in heavy metal regulation and is integrated between the *czcRD* operon and the *cadA* gene. Tn*917* is integrated approximately 50 bp upstream of the start codon of *czcR* and approximately 300 bp upstream of the start codon of *cadA* on the opposite DNA strand (Fig. 3A). *cadA* encodes a putative P-type cation efflux transmembrane pump that exhibits 58% identity with a predicted cadmium transporter from *E. faecalis* (GenBank accession no. AAO80575) (4) and 84% identity with the predicted cation transporter CopA from *Staphylococcus epidermidis* (GenBank accession no. AAO03659). *cadA* transcription is induced by cadmium, chromium, and zinc but is not altered by

TABLE 2. Worm survival after exposure to hydrogen peroxide[a]

H_2O_2 concn (mM)	% Survival	Mobility
0	100 ± 0.0	+++
0.1	100 ± 0.0	+++
0.2	100 ± 0.0	+/++
0.3	70.5 ± 23.7	−/+
0.4	30.3 ± 13.6	−
0.5	4.8 ± 1.1	−
1	1.3 ± 1.9	−
2	2.0 ± 1.4	−
5	0.0 ± 0.0	−

[a] Worm survival was scored after 15 h of incubation. Survival was assayed in M9 buffer containing hydrogen peroxide.

TABLE 3. Effect of catalase on worm survival[a]

Bacteria grown anaerobically	Untreated		Treated with catalase	
	% Survival	Mobility	% Survival	Mobility
SE34	6.3	−	100 ± 0.0	+++
2C4	0	−	100 ± 0.0	++/+++
2F22	83.3	−	100 ± 0.0	+++
11M12	100	+++	100 ± 0.0	+++
E007	0	−	100 ± 0.0	+++
VS583	100	+++	100 ± 0.0	+++

[a] Worm survival was scored after 15 h of incubation.

TABLE 4. Survival of worms mutated in *daf-2* or *daf-16*[a]

C. elegans strain	% Survival	Mobility
N2	0.0 ± 0.0	−
daf-2	100 ± 0.0	+
daf-16	2.0 ± 2.8	−
daf-2 daf-16	1.0 ± 1.4	−

[a] Worm survival was scored after 24 h of incubation with anaerobically grown SE34.

cobalt, copper, iron, manganese, or nickel (data not shown). *cadA* transcript levels are not altered in the *11M12* mutant (data not shown), making it unlikely that the phenotype of *11M12* is a consequence of aberrant *cadA* transcription.

CzcR belongs to the ArsR/SmtB family of metal-sensing transcriptional repressors, while CzcD belongs to the Czc (Co/Zn/Cd) family of heavy metal exporters (52). The two-gene operon organization of the *czcRD* genes is similar to the organization of the *czrAB* genes of *S. aureus*, and the protein sequences exhibit 43 and 27% identity, respectively (28). In response to the presence of specific heavy metal ions, ArsR-type proteins derepress transcription of heavy metal efflux transporters and stress response proteins (52). In the wild-type strain, the 1.4-kb *czcRD* operon is expressed at very low levels under normal growth conditions but is strongly induced in the presence of cadmium or chromium. Mild induction is seen upon exposure to zinc, whereas no induction is detectable upon exposure to cobalt, copper, iron, magnesium, manganese, nickel, or silver (Fig. 3C and data not shown). In mutant *11M12*, the *czcRD* operon is aberrantly expressed as a fusion to the Tn*917 tnpRA* operon, resulting in a 5.1-kb transcript (Fig. 2B). Toxicity from chromium and cadmium has been linked to oxidative stress (55), and the decreased hydrogen peroxide phenotype of *11M12* suggests a link between the regulation of genes involved in heavy metal detoxification and hydrogen peroxide production. Specifically, we hypothesize that the elevated levels of the transcriptional repressor CzcR in mutant *11M12* may result in the repression of oxidases, resulting in lowered hydrogen peroxide production. In an attempt to reproduce the phenotype of the *11M12* mutant, the *czcR* or *czcRD* genes were overexpressed from plasmids in SE34. Even though highly expressed transcripts were detected by Northern blotting, the strains overexpressing *czcR* or *czcRD* did not produce smaller amounts of hydrogen peroxide (data not shown). Thus, we cannot definitively conclude that the diminished hydrogen peroxide production phenotype of *11M12* is a consequence of the insertion of Tn*917* upstream of the *czcRD* operon or that another unlinked mutation is responsible.

Hydrogen peroxide production is modulated by glycerol and glucose. Enterococci synthesize glycerol-3-phosphate oxidase, which uses molecular oxygen as a reductant to form dihydroxyacetone and hydrogen peroxide (13). Addition of glycerol to liquid cultures of *E. faecium* that were incubated without agitation resulted in increased accumulation of hydrogen peroxide. After overnight culture, we determined that strain SE34 accumulated 232 ± 37.5 μM hydrogen peroxide in the absence of glycerol and 636 ± 48.5 μM hydrogen peroxide in the presence of 0.2% glycerol. We therefore tested whether nematode killing was enhanced when glycerol was added to the *E. faecium* solid growth medium. We found that addition of 0.2% glycerol to the medium enabled aerobically grown *E. faecium* to kill nematodes (Table 5).

The glycerol metabolic pathway is down-regulated by glycolytic intermediates, which inhibit glycerol kinase and glycerol-3-phosphate oxidase (8, 13, 23). Therefore, glucose addition should lead to a decrease in hydrogen peroxide production by reducing glycerol-3-phosphate oxidase activity and by possibly inhibiting other oxidases through catabolite repression. In liquid aerobic cultures, addition of 1% glucose to BHI medium completely eliminated hydrogen peroxide accumulation (data not shown) and nematode killing (Table 5) by all of the *E. faecium* strains tested.

Hydrogen peroxide produced in aerobic liquid cultures. We showed that *E. faecium* produces hydrogen peroxide after anaerobic cultures are exposed to aerobic conditions. We also determined, however, that growth under anaerobic conditions is not a prerequisite for hydrogen peroxide production in liquid cultures. When the parental strain SE34 was grown in a highly agitated and aerated culture, 1 to 2 mM hydrogen peroxide accumulated as the culture entered the stationary phase (Fig. 4). For the mutant strains, differences in hydrogen peroxide production did not correlate with changes in aerobic growth rates. Mutant *11M12* grew at the same rate as the wild type, but the *11M12* culture did not accumulate hydrogen peroxide (Fig. 4). Mutant *2F22* grew at a lower rate and entered the stationary phase at a density that was 90% less than that of the wild type. Because the amount of hydrogen peroxide that accumulated in aerobic cultures of *2F22* was also decreased by 90%, each viable cell of *2F22* was producing an amount of hydrogen

TABLE 5. Effects of glycerol and glucose on worm survival

Bacterial strain	Glycerol expt[a]				Glucose expt[b]			
	Untreated		Treated with 0.2% glycerol		Untreated		Treated with 1% glucose	
	% Survival	Mobility	% Survival	Mobility	% Survival	Mobility	% Survival	Mobility
SE34	100 ± 0.0	+++	0.0 ± 0.0	−	5.5 ± 3.5	−	99.8 ± 0.4	+++
2C4	34.0 ± 4.6	−	2.8 ± 2.0	−	2.0 ± 1.4	−	99.5 ± 0.6	+++
2F22	100 ± 0.0	+++	43.3 ± 18.5	+/−	78.1 ± 2.3	−	100 ± 0.0	+++
11M12	97.1 ± 0.7	+++	100 ± 0.0	+++	99.0 ± 1.4	+++	100 ± 0.0	+++
E007	97.4 ± 1.9	+++	2.9 ± 0.4	+/−	0.5 ± 0.7	−	100 ± 0.0	+++
VS583	95.5 ± 0.4	+++	100 ± 0.0	+++	98.1 ± 2.7	+++	99.5 ± 0.7	+++

[a] Worm survival was scored after 24 h of incubation with aerobically grown bacteria.
[b] Worm survival was scored after 15 h of incubation with anaerobically grown bacteria.

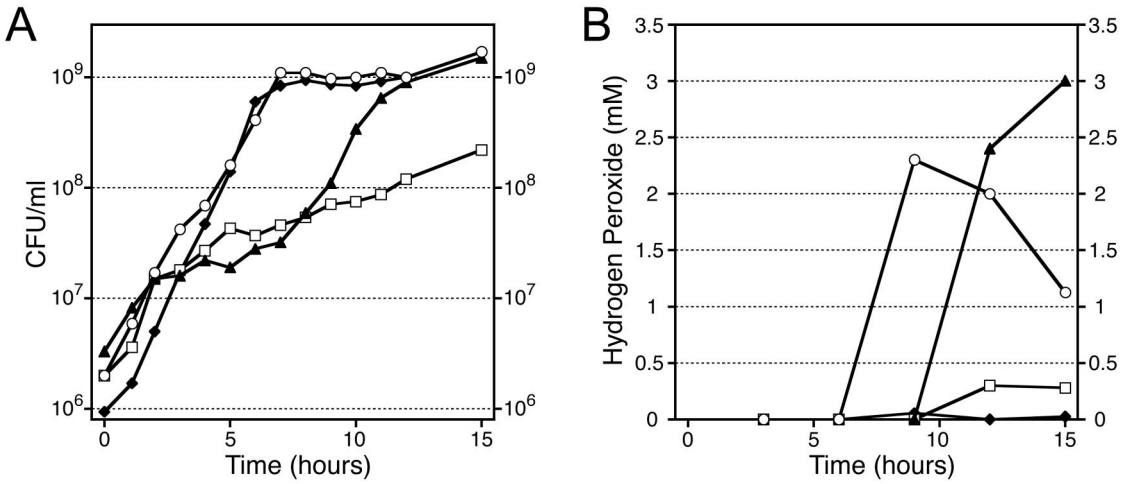

FIG. 4. (A) Growth of *E. faecium* mutants in aerobic cultures. (B) Hydrogen peroxide accumulation in aerobic cultures of the *E. faecium* mutants. Symbols: ○, parental strain SE34; ▲, mutant *2C4*; □, mutant *2F22*; ♦, mutant *11M12*.

peroxide that was equivalent to the amount produced by a wild-type cell under these culture conditions. The initial growth rate of the *2C4* mutant was similar to that of the wild type upon dilution of a semiaerobic saturated culture into fresh media (see Materials and Methods). Then the growth rate of the *2C4* mutant temporarily decreased, thereby delaying entry of the culture into the stationary phase (Fig. 4A). In the stationary phase, the *2C4* mutant accumulated up to 3 mM hydrogen peroxide (Fig. 4B).

We determined that the amount of hydrogen peroxide generated by wild-type *E. faecium* in an aerobic culture was sufficient to kill *C. elegans*. Bacterium-free supernatants taken from aerobic cultures were able to kill *C. elegans*, while supernatants treated with catalase were not able to kill *C. elegans* (data not shown).

DISCUSSION

In this study, we identified conditions in which *E. faecium* kills *C. elegans*, and in this report we present evidence that the killing is due to the production by *E. faecium* of hydrogen peroxide. First, we identified *E. faecium* mutants that exhibited either decreased or increased killing of *C. elegans*. The ability of the mutants to kill *C. elegans* was proportional to the amount of hydrogen peroxide produced. Second, we found that the amount of hydrogen peroxide produced by *E. faecium* could be modulated by glucose or glycerol, which in turn affected *C. elegans* killing. Third, hydrogen peroxide killed *C. elegans* at the same concentrations that were produced by *E. faecium*. Finally, nematode killing was prevented by addition of catalase to cultures of *E. faecium* that killed the worms.

When our laboratory originally developed the *Enterococcus-C. elegans* model system, we observed that *C. elegans* could use *E. faecium* as a food source and have a life span that is only slightly shorter than that of nematodes grown on the normal food source (*E. coli* on NGM medium). Because *E. faecium* is able to cause severe and potentially fatal infections, we hypothesized that the original *E. faecium-C. elegans* system lacked elements required for *E. faecium* pathogenicity. We tried growing *E. faecium* under a variety of different conditions, and we found that *E. faecium* acquires *C. elegans* killing activity after the bacteria are grown on solid BHI medium under anaerobic conditions. Initially, we hypothesized that *E. faecium* was producing a toxin during the anaerobic growth phase, but in actuality, the toxin hydrogen peroxide was not produced until the lawns of *E. faecium* were exposed to oxygen.

Hydrogen peroxide is scavenged by NADH peroxidase, which is expressed under aerobic conditions due to transcriptional regulation by OxyR (47). Under anaerobic conditions, *npr* is transcribed at very low levels in *E. faecium*. We speculate that during the *C. elegans* killing assay with anaerobically grown *E. faecium*, the abrupt exposure to atmospheric oxygen allows *E. faecium* to produce and accumulate large amounts of hydrogen peroxide because the low levels of NADH peroxidase in the cells cannot adequately scavenge hydrogen peroxide. On the other hand, anaerobic growth of *E. faecium* is not required for hydrogen peroxide production in liquid cultures. We have found that *E. faecium* produces high levels of hydrogen peroxide as aerobic cultures enter the stationary phase. Previous studies that only examined logarithmically growing cultures may have overlooked the production of hydrogen peroxide by *E. faecium* (39).

In contrast to *E. faecium*, *E. faecalis* does not kill *C. elegans* by a fast, toxin-mediated mechanism, and *E. faecalis* does not produce detectable amounts of hydrogen peroxide under our assay conditions. One difference between the two enterococcal species is that *E. faecium* is catalase negative and *E. faecalis* is catalase positive when the organisms are grown on media such as BHI medium containing hematin (14, 42). Another difference between *E. faecium* and *E. faecalis* is that *E. faecalis* is capable of respiration. Although *E. faecalis* possesses glycerol-3-phosphate oxidase and can produce hydrogen peroxide when glycerol is its sole carbon source (41), it appears that *E. faecalis* prefers to use respiration to meet its energetic needs. While *E. faecalis* did not produce detectable levels of hydrogen peroxide ($\leq$25 μM) under the conditions which we used in this study, *E. faecalis* produces extracellular superoxide when it is grown in the presence of molecular oxygen and in the absence of hema-

tin and fumurate (25). In rat intestines colonized by *E. faecalis*, there is sufficient molecular oxygen to allow the production of superoxide, and Hucyke et al. have postulated that *E. faecalis* is a potential source of oxidative stress for intestinal cells which may contribute to carcinogenesis (24, 25). Our data suggest that *E. faecium* may be another source of oxidative stress for intestinal cells.

E. faecium is a facultative anaerobe whose metabolism relies exclusively on glycolysis and fermentation (23). In anaerobic environments, lactate fermentation is used to regenerate NADH. In aerobic environments, NADH is regenerated by water-forming NADH oxidases, thereby allowing the use of mixed acid fermentation. Another type of NADH oxidase catalyzes the two-electron reduction of molecular oxygen, resulting in hydrogen peroxide formation (21, 40). The hydrogen peroxide-forming NADH oxidase works in concert with the AhpC protein to scavenge alky hydroperoxides (21, 40). In this study, we found an *E. faecium nox* mutant, *2F22*, that produces less hydrogen peroxide. Although our results indicate that a *nox* gene is mutated in *2F22*, the gene appears to encode a water-forming NADH oxidase, and we believe that the decreased hydrogen peroxide production in this mutant is a secondary phenotype due to alterations in energy metabolism. First, the amino acid sequence of the protein encoded by the mutated *nox* gene is more similar to the sequences of previously studied water-forming NADH oxidases than to sequences of hydrogen peroxide-forming NADH oxidases (48). Second, the growth defect phenotypes of mutant *2F22* are similar to the phenotypes of streptococcal strains mutated in the water-forming NADH oxidase (21, 62). Third, we were not able to detect hydrogen peroxide in the NADH oxidase assays of extracts of wild-type cells.

The release of hydrogen peroxide by bacteria has been shown to inhibit or kill other competing bacteria or host cells. This may be beneficial for both the host and the bacteria, as in the case of *Lactobacillus* species colonizing the vaginal tract, where hydrogen peroxide synthesized by *Lactobacillus* inhibits the growth of pathogenic species such as *S. aureus*, *Neisseria gonorrhoeae*, and *Gardnerella vaginalis* (20, 36, 54). Alternatively, hydrogen peroxide production can be detrimental to the host, as in the case of *S. pneumoniae*, in which hydrogen peroxide inhibits the growth of *Haemonphilus influenzae*, thereby allowing *S. pneumoniae* to colonize the respiratory tract without competition from other pathogens (39). The release of hydrogen peroxide by *S. pneumoniae* can also damage host cells and has been shown to contribute to the induction of apoptosis of neuronal cells in a meningitis infection model (6, 10, 22). Interestingly, similar to our results, Bolm et al. have shown that many streptococcal species, including *S. pneumoniae* and *S. pyogenes*, kill *C. elegans* by producing hydrogen peroxide (5, 27).

It appears that hydrogen peroxide generation is a common trait among catalase-negative streptococci and the related enterococci, and it seems likely that the hydrogen peroxide released by *E. faecium* damages nearby host cells. However, the relatively low levels of hydrogen peroxide released from *E. faecium* may be difficult to detect in vivo. Similarly, it may be difficult to determine whether hydrogen peroxide production plays an important role in *E. faecium* pathogenesis. To date, controlled genetic manipulation of pathogenic *E. faecium* strains has not been achieved, and no *E. faecium* mutants have been found to be attenuated in a mammalian infection model. The difficulties in studying *E. faecium* prompted the development of a *C. elegans* pathogenesis model as a system to discover potential virulence factors. Use of this simple model led us to the finding that *E. faecium* produces hydrogen peroxide at levels that are high enough to kill *C. elegans*. Additional studies are necessary to determine the significance of hydrogen peroxide in *E. faecium* pathogenesis in mammalian hosts.

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REFERENCES

1. **Aballay, A., P. Yorgey, and F. M. Ausubel.** 2000. *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. Curr. Biol. **10:**1539–1542.
2. **Arduino, R. C., K. Jacques-Palaz, B. E. Murray, and R. M. Rakita.** 1994. Resistance of *Enterococcus faecium* to neutrophil-mediated phagocytosis. Infect. Immun. **62:**5587–5594.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1996. Current protocols in molecular biology. John Wiley and Sons, New York, N.Y.
4. **Axelsen, K. B., and M. G. Palmgren.** 1998. Evolution of substrate specificities in the P-type ATPase superfamily. J. Mol. Evol. **46:**84–101.
5. **Bolm, M., W. T. Jansen, R. Schnabel, and G. S. Chhatwal.** 2004. Hydrogen peroxide-mediated killing of *Caenorhabditis elegans*: a common feature of different streptococcal species. Infect. Immun. **72:**1192–1194.
6. **Braun, J. S., J. E. Sublett, D. Freyer, T. J. Mitchell, J. L. Cleveland, E. I. Tuomanen, and J. R. Weber.** 2002. Pneumococcal pneumolysin and H_2O_2 mediate brain cell apoptosis during meningitis. J. Clin. Investig. **109:**19–27.
7. **Brenner, S.** 1974. The genetics of *Caenorhabditis elegans*. Genetics **77:**71–94.
8. **Charrier, V., E. Buckley, D. Parsonage, A. Galinier, E. Darbon, M. Jaquinod, E. Forest, J. Deutscher, and A. Claiborne.** 1997. Cloning and sequencing of two enterococcal *glpK* genes and regulation of the encoded glycerol kinases by phosphoenolpyruvate-dependent, phosphotransferase system-catalyzed phosphorylation of a single histidyl residue. J. Biol. Chem. **272:**14166–14174.
9. **Darby, C., C. L. Cosma, J. H. Thomas, and C. Manoil.** 1999. Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **96:**15202–15207.
10. **Duane, P. G., J. B. Rubins, H. R. Weisel, and E. N. Janoff.** 1993. Identification of hydrogen peroxide as a *Streptococcus pneumoniae* toxin for rat alveolar epithelial cells. Infect. Immun. **61:**4392–4397.
11. **Eaton, T. J., and M. J. Gasson.** 2002. A variant enterococcal surface protein Esp(fm) in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. FEMS Microbiol. Lett. **216:**269–275.
12. **Eliopoulos, G. M., C. Wennersten, S. Zighelboim-Daum, E. Reiszner, D. Goldmann, and R. C. Moellering, Jr.** 1988. High-level resistance to gentamicin in clinical isolates of *Streptococcus* (*Enterococcus*) *faecium*. Antimicrob. Agents Chemother. **32:**1528–1532.
13. **Esders, T. W., and C. A. Michrina.** 1979. Purification and properties of L-alpha-glycerophosphate oxidase from *Streptococcus faecium* ATCC 12755. J. Biol. Chem. **254:**2710–2715.
14. **Frankenberg, L., M. Brugna, and L. Hederstedt.** 2002 *Enterococcus faecalis* heme-dependent catalase. J. Bacteriol. **184:**6351–6356.
15. **Friesenegger, A., S. Fiedler, L. A. Devriese, and R. Wirth.** 1991. Genetic transformation of various species of *Enterococcus* by electroporation. FEMS Microbiol. Lett. **63:**323–327.
16. **Gallagher, L. A., and C. Manoil.** 2001. *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. J. Bacteriol. **183:**6207–6214.
17. **Garsin, D. A., C. D. Sifri, E. Mylonakis, X. Qin, K. V. Singh, B. E. Murray, S. B. Calderwood, and F. M. Ausubel.** 2001. A simple model host for identifying Gram-positive virulence factors. Proc. Natl. Acad. Sci. USA **98:**10892–10897. Epub 2001 Sep 4.
18. **Gilmore, M. S., P. S. Coburn, S. R. Nallapareddy, and B. E. Murray.** 2002. Enterococcal virulence, p. 301–354. *In* M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, and L. B. Rice (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, D.C.
19. **Gutierrez, J. A., P. J. Crowley, D. P. Brown, J. D. Hillman, P. Youngman,**

and A. S. Bleiweis. 1996. Insertional mutagenesis and recovery of interrupted genes of *Streptococcus mutans* by using transposon Tn*917*: preliminary characterization of mutants displaying acid sensitivity and nutritional requirements. J. Bacteriol. **178:**4166–4175.
20. **Hawes, S. E., S. L. Hillier, J. Benedetti, C. E. Stevens, L. A. Koutsky, P. Wølner-Hanssen, and K. K. Holmes.** 1996. Hydrogen peroxide-producing lactobacilli and acquisition of vaginal infections. J. Infect. Dis. **174:**1058–1063.
21. **Higuchi, M., Y. Yamamoto, L. B. Poole, M. Shimada, Y. Sato, N. Takahashi, and Y. Kamio.** 1999. Functions of two types of NADH oxidases in energy metabolism and oxidative stress of *Streptococcus mutans*. J. Bacteriol. **181:**5940–5947.
22. **Hirst, R. A., K. S. Sikand, A. Rutman, T. J. Mitchell, P. W. Andrew, and C. O'Callaghan.** 2000. Relative roles of pneumolysin and hydrogen peroxide from *Streptococcus pneumoniae* in inhibition of ependymal ciliary beat frequency. Infect. Immun. **68:**1557–1562.
23. **Huycke, M. M.** 2002. Physiology of enterococci, p. 133–175. *In* M. S. Gilmore, D. B. Clewell, P. Courvalin, G. M. Dunny, B. E. Murray, and L. B. Rice (ed.), The enterococci: pathogenesis, molecular biology, and antibiotic resistance. ASM Press, Washington, D.C.
24. **Huycke, M. M., V. Abrams, and D. R. Moore.** 2002. *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. Carcinogenesis **23:**529–536.
25. **Huycke, M. M., D. Moore, W. Joyce, P. Wise, L. Shepard, Y. Kotake, and M. S. Gilmore.** 2001. Extracellular superoxide production by *Enterococcus faecalis* requires demethylmenaquinone and is attenuated by functional terminal quinol oxidases. Mol. Microbiol. **42:**729–740.
26. **Jander, G., L. G. Rahme, and F. M. Ausubel.** 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol. **182:**3843–3845.
27. **Jansen, W. T., M. Bolm, R. Balling, G. S. Chhatwal, and R. Schnabel.** 2002. Hydrogen peroxide-mediated killing of *Caenorhabditis elegans* by *Streptococcus pyogenes*. Infect. Immun. **70:**5202–5207.
28. **Kuroda, M., H. Hayashi, and T. Ohta.** 1999. Chromosome-determined zinc-responsible operon *czr* in *Staphylococcus aureus* strain 912. Microbiol. Immunol. **43:**115–125.
29. **Leavis, H., J. Top, N. Shankar, K. Borgen, M. Bonten, J. van Embden, and R. J. Willems.** 2004. A novel putative enterococcal pathogenicity island linked to the *esp* virulence gene of *Enterococcus faecium* and associated with epidemicity. J. Bacteriol. **186:**672–682.
30. **Lopez de Felipe, F., M. Kleerebezem, W. M. de Vos, and J. Hugenholtz.** 1998. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. J. Bacteriol. **180:**3804–3808.
31. **Mahajan-Miklos, S., M. W. Tan, L. G. Rahme, and F. M. Ausubel.** 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. Cell **96:**47–56.
32. **Murray, B. E., K. V. Singh, R. P. Ross, J. D. Heath, G. M. Dunny, and G. M. Weinstock.** 1993. Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. J. Bacteriol. **175:**5216–5223.
33. **Mylonakis, E., F. M. Ausubel, J. R. Perfect, J. Heitman, and S. B. Calderwood.** 2002. Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. Proc. Natl. Acad. Sci. USA **99:**15675–15680.
34. **Nallapareddy, S. R., G. M. Weinstock, and B. E. Murray.** 2003. Clinical isolates of *Enterococcus faecium* exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. Mol. Microbiol. **47:**1733–1747.
35. **National Nosocomial Infections Surveillance System.** 1997. National Nosocomial Infections Surveillance (NNIS) report, data summary from October 1986-April 1997, issued May 1997. A report from the NNIS System. Am. J. Infect. Control **25:**477–487.
36. **Ocaña, V. S., A. A. de Ruiz Holgado, and M. E. Nader-Macías.** 1999. Growth inhibition of *Staphylococcus aureus* by H_2O_2-producing *Lactobacillus paracasei* subsp. *paracasei* isolated from the human vagina. FEMS Immunol. Med. Microbiol. **23:**87–92.
37. **Ogg, S., S. Paradis, S. Gottlieb, G. I. Patterson, L. Lee, H. A. Tissenbaum, and G. Ruvkun.** 1997. The fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans* Nature **389:**994–999.
38. **O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter.** 1999. Genetic approaches to study of biofilms. Methods Enzymol. **310:**91–109.
39. **Pericone, C. D., K. Overweg, P. W. Hermans, and J. N. Weiser.** 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect. Immun. **68:**3990–3997.
40. **Poole, L. B., M. Higuchi, M. Shimada, M. L. Calzi, and Y. Kamio.** 2000. *Streptococcus mutans* H_2O_2-forming NADH oxidase is an alkyl hydroperoxide reductase protein. Free Radic. Biol. Med. **28:**108–120.
41. **Pugh, S. Y., and C. J. Knowles.** 1982. Growth of *Streptococcus faecalis* var. *zymogenes* on glycerol: the effect of aerobic and anaerobic growth in the presence and absence of haematin on enzyme synthesis. J. Gen. Microbiol. **128:**1009–1017.
42. **Pugh, S. Y., and C. J. Knowles.** 1983. Synthesis of catalase by "*Streptococcus faecalis* subsp. *zymogenes*." Arch. Microbiol. **136:**60–63.
43. **Rahme, L. G., F. M. Ausubel, H. Cao, E. Drenkard, B. C. Goumnerov, G. W. Lau, S. Mahajan-Miklos, J. Plotnikova, M. W. Tan, J. Tsongalis, C. L. Walendziewicz, and R. G. Tompkins.** 2000. Plants and animals share functionally common bacterial virulence factors. Proc. Natl. Acad. Sci. USA **97:**8815–8821.
44. **Rahme, L. G., E. J. Stevens, S. F. Wolfort, J. Shao, R. G. Tompkins, and F. M. Ausubel.** 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science **268:**1899–1902.
45. **Rice, L. B., L. Carias, S. Rudin, C. Vael, H. Goossens, C. Konstabel, I. Klare, S. R. Nallapareddy, W. Huang, and B. E. Murray.** 2003. A potential virulence gene, *hylEfm*, predominates in *Enterococcus faecium* of clinical origin. J. Infect. Dis. **187:**508–512.
46. **Ross, R. P., and A. Claiborne.** 1991. Cloning, sequence and overexpression of NADH peroxidase from *Streptococcus faecalis* 10C1. Structural relationship with the flavoprotein disulfide reductases. J. Mol. Biol. **221:**857–871.
47. **Ross, R. P., and A. Claiborne.** 1997. Evidence for regulation of the NADH peroxidase gene (*npr*) from *Enterococcus faecalis* by OxyR. FEMS Microbiol. Lett. **151:**177–183.
48. **Ross, R. P., and A. Claiborne.** 1992. Molecular cloning and analysis of the gene encoding the NADH oxidase from *Streptococcus faecalis* 10C1. Comparison with NADH peroxidase and the flavoprotein disulfide reductases. J. Mol. Biol. **227:**658–671.
49. **Sahm, D. F., J. Kissinger, M. S. Gilmore, P. R. Murray, R. Mulder, J. Solliday, and B. Clarke.** 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **33:**1588–1591.
50. **Sahm, D. F., M. K. Marsilio, and G. Piazza.** 1999. Antimicrobial resistance in key bloodstream bacterial isolates: electronic surveillance with the Surveillance Network Database—USA. Clin. Infect. Dis. **29:**259–263.
51. **Sifri, C. D., J. Begun, F. M. Ausubel, and S. B. Calderwood.** 2003. *Caenorhabditis elegans* as a model host for *Staphylococcus aureus* pathogenesis. Infect. Immun. **71:**2208–2217.
52. **Silver, S., and M. Walderhaug.** 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. **56:**195–228.
53. **Spellerberg, B., D. R. Cundell, J. Sandros, B. J. Pearce, I. Idänpään-Heikkilä, C. Rosenow, and H. R. Masure.** 1996. Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. Mol. Microbiol. **19:**803–813.
54. **St. Amant, D. C., I. E. Valentin-Bon, and A. E. Jerse.** 2002. Inhibition of *Neisseria gonorrhoeae* by *Lactobacillus* species that are commonly isolated from the female genital tract. Infect. Immun. **70:**7169–7171.
55. **Stohs, S. J., and D. Bagchi.** 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. **18:**321–336.
56. **Sulston, J., and J. Hodgkin** 1988. Methods, p. 587–606. *In* W. B. Wood (ed.), The nematode *Caenorhabditis elegans.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
57. **Tan, M. W., S. Mahajan-Miklos, and F. M. Ausubel.** 1999. Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc. Natl. Acad. Sci. USA **96:**715–720.
58. **Teng, F., M. Kawalec, G. M. Weinstock, W. Hryniewicz, and B. E. Murray.** 2003. An *Enterococcus faecium* secreted antigen, SagA, exhibits broad-spectrum binding to extracellular matrix proteins and appears essential for *E. faecium* growth. Infect. Immun. **71:**5033–5041.
59. **Teng, F., B. E. Murray, and G. M. Weinstock.** 1998. Conjugal transfer of plasmid DNA from *Escherichia coli* to enterococci: a method to make insertion mutations. Plasmid **39:**182–186.
60. **Vanfleteren, J. R., and A. De Vreese.** 1995. The gerontogenes age-1 and daf-2 determine metabolic rate potential in aging *Caenorhabditis elegans*. FASEB J. **9:**1355–1361.
61. **Willems, R. J., W. Homan, J. Top, M. van Santen-Verheuvel, D. Tribe, X. Manzioros, C. Gaillard, C. M. Vandenbroucke-Grauls, E. M. Mascini, E. van Kregten, J. D. van Embden, and M. J. Bonten.** 2001. Variant *esp* gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. Lancet **357:**853–855.
62. **Yu, J., A. P. Bryant, A. Marra, M. A. Lonetto, K. A. Ingraham, A. F. Chalker, D. J. Holmes, D. Holden, M. Rosenberg, and D. McDevitt.** 2001. Characterization of the *Streptococcus pneumoniae* NADH oxidase that is required for infection. Microbiology **147:**431–438.

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