1-1-2011

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JOURNAL OF BACTERIOLOGY, Apr. 2011, p. 1643–1652 Vol. 193, No. 7
0021-9193/11/$12.00 doi:10.1128/JB.01063-10

A Selenium-Dependent Xanthine Dehydrogenase Triggers Biofilm Proliferation in *Enterococcus faecalis* through Oxidant Production▽‡

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Received 7 September 2010/Accepted 8 January 2011

Selenium has been shown to be present as a labile cofactor in a small class of molybdenum hydroxylase enzymes in several species of clostridia that specialize in the fermentation of purines and pyrimidines. This labile cofactor is poorly understood, yet recent bioinformatic studies have suggested that *Enterococcus faecalis* could serve as a model system to better understand the way in which this enzyme cofactor is built and the role of these metalloenzymes in the physiology of the organism. An mRNA that encodes a predicted selenium-dependent molybdenum hydroxylase (SDMH) has also been shown to be specifically increased during the transition from planktonic growth to biofilm growth. Based on these studies, we examined whether this organism produces an SDMH and probed whether selenoproteins may play a role in biofilm physiology. We observed a substantial increase in biofilm density upon the addition of uric acid to cells grown in a defined culture medium, but only when molybdate (Mo) and selenite (Se) were also added. We also observed a significant increase in biofilm density in cells cultured in tryptic soy broth with 1% glucose (TSBG) when selenite was added. In-frame deletion of *selD*, which encodes selenophosphate synthetase, also blocked biofilm formation that occurred upon addition of selenium. Moreover, mutation in the gene encoding the molybdoenzyme (*xdh*) prevented the induction of biofilm proliferation upon supplementation with selenium. Tungstate or auranofin addition also blocked this enhanced biofilm density, likely through inhibition of molybdenum or selenium cofactor synthesis. A large protein complex labeled with ^{75}Se is present in higher concentrations in biofilms than in planktonic cells, and the same complex is formed in TSBG. Xanthine dehydrogenase activity correlates with the presence of this labile selenoprotein complex and is absent in a *selD* or an *xdh* mutant. Enhanced biofilm density correlates strongly with higher levels of extracellular peroxide, which is produced upon the addition of selenite to TSBG. Peroxide levels are not increased in either the *selD* or the *xdh* mutant upon addition of selenite. Extracellular superoxide production, a phenomenon well established to be linked to clinical isolates, is abolished in both mutant strains. Taken together, these data provide evidence that an SDMH is involved in biofilm formation in *Enterococcus faecalis*, contributing to oxidant production either directly or alternatively through its involvement in redox-dependent processes linked to oxidant production.

Selenium is a required micronutrient in some prokaryotes and many eukaryotes (including humans). Basic research of the formate dehydrogenase (FDH_H) enzyme of *Escherichia coli* became a model system for elucidation of the incorporation of selenium in the form of selenocysteine (51–53). These studies, along with biochemical analysis of selenoenzymes from clostridia, built the foundation for essentially the entire field of selenium biology. Although the form of selenium in most selenoproteins has been identified as selenocysteine, selenium has also been shown to occur in enzymes as a labile cofactor (Fig. 1). Three enzymes have been shown to require selenium in a labile form: xanthine dehydrogenase (XDH) from *Clostridium acidurici* (15, 58, 59), *Clostridium cylindrosporum* (12, 59), *Clostridium purinilyticum* (16, 49, 50), and *Eubacterium barkeri* (43); nicotinic acid hydroxylase (NAH) from *E. barkeri* (13, 14, 17, 18, 25, 38, 57); and purine hydroxylase (PH) from *C. purinilyticum* (49, 50). The strictly anaerobic bacteria which express these labile selenoenzymes were originally isolated in enrichment cultures using uric acid (*C. acidurici*, *C. cylindrosporum*), nicotinic acid (*E. barkeri*), or adenine (*C. purinilyticum*) as a primary source of carbon, nitrogen, and energy. Each of these selenoenzymes is a complex metalloenzyme that contains an organic molybdopterin cofactor, FeS centers, and flavin adenine dinucleotide (FAD). These enzymes are a subfamily of the larger class of molybdoenzymes known as molybdenum hydroxylases, enzymes that are produced by bacteria, plants, and mammals (23).

Molybdenum hydroxylases are a well-studied group of metalloenzymes, including the bovine xanthine oxidoreductase (XOR), which has been the subject of biochemical analysis for over 100 years (36). In the absence of an electron acceptor, XOR can reduce oxygen with one electron to form a superoxide anion radical through a reaction with FAD semiquinone and produce hydrogen peroxide through other redox active cofactors (21, 26). Based on the primary assumption that selenium must first be activated prior to incorporation into a selenium-dependent molybdenum hydroxylase (SDMH) (a presumption not yet tested experimentally), our group and Gladyshev's both identified a gene cluster in eubacteria that linked the genes encoding the enzyme for activation of selenium (selenophosphate synthetase [SPS], or SelD) with a gene

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‡ Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 21 January 2011.

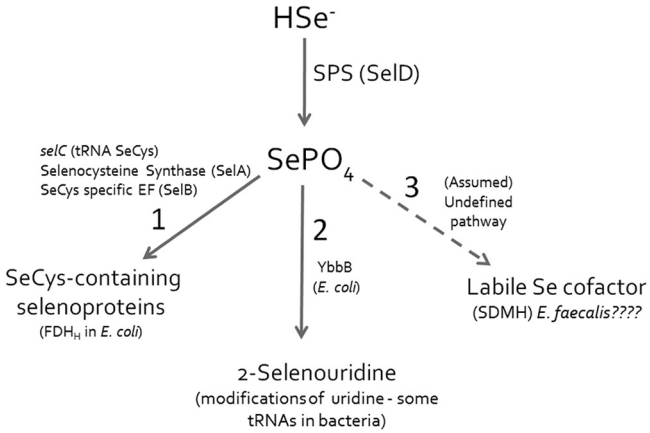

FIG. 1. Three pathways for the specific incorporation of selenium into biological macromolecules. Selenophosphate is required for synthesis of selenocysteine (SeCys)-containing selenoproteins via cotranslational insertion of SeCys via *selC* tRNA that is first charged with serine. A specific elongation factor (SelB) interacts with the selenocysteine insertion sequence mRNA element located 3′ to the UGA codon (encoding the 21st amino acid, SeCys). This pathway was best defined in *E. coli* using the FDH_H enzyme as the model (35, 42, 53). Selenophosphate is also required as a metabolic precursor for modification of uridine residues in several tRNAs (not SeCys tRNAs) to form 2-selenouridine. Both selenophosphate synthetase (SelD) and YbbB proteins have been shown to be required for this production of the selenouridine residue (63). In the third pathway, the incorporation of selenium into SDMHs in a labile form is not understood, and the dependence of this pathway on selenophosphate has not yet been established, although this labile form of selenium has been shown to occur in several molybdoenzymes isolated from clostridial species (14, 17, 18, 43–45, 49, 50). It has been proposed that this pathway exists in *E. faecalis*, based on two computational studies (20, 64), and that the first and second pathways are absent.

encoding a molybdenum hydroxylase (20, 64). Other genes also colocalized with this group in an operon structure in a number of anaerobes and facultative organisms (20). These putative gene products are also presumed to be involved in the metabolism of selenium and synthesis of the molybdenum cofactor. Importantly, only two organisms were found to possess this cluster but lack the other genetic determinants of selenocysteine utilization (*selABC*) or modification of tRNA (*ybbB*): *Enterococcus faecalis* and *Haloarcula marismortui*. Since *E. faecalis* is a well-studied model organism and since an established genetic model system exists for this pathogen, we chose this as a potential model system to elucidate the pathway for labile selenoenzyme biosynthesis.

E. faecalis is an opportunistic pathogen that has been shown to produce biofilms during infection, often in the bladder, where the uric acid content is high (62). *E. faecalis* also produces vegetative growths on heart tissue, and this formation is likely related to the ability to form robust biofilms. Recent studies have focused on determining the genes encoding proteins that are critical for biofilm formation and involved in proliferation and production of extracellular DNA in biofilms (7, 55, 56). In a recombinase *in vivo* expression technology (RIVET) genetic screen, the gene encoding a putative xanthine dehydrogenase (EF2570) was identified as a locus that is upregulated in biofilms versus planktonic cells (7). In the wake of our computational work and this published finding linking this gene product to biofilm physiology, we undertook this work.

To date, selenoproteins in bacteria have been shown to be involved in intermediary metabolism and bioenergetics but not pathogenesis. In this work, we first determine whether the growth of *E. faecalis* is altered in the presence of various purines in the presence of added Se and Mo in a defined cultured medium. The rationale for these experiments is based upon several decades of work showing the catabolic degradation of purines in several species of clostridia (1, 14, 16, 17, 45, 49, 51). We then test the hypothesis that selenium must first be activated prior to insertion into an SDMH by analyzing both *selD* and *xdh* mutant strains. Finally, we analyze the contribution of the SDMH to biofilm physiology under growth conditions that have been shown to lead to increased biofilm density in the context of quorum sensing and response to external stimuli, such as glucose (40, 54).

MATERIALS AND METHODS

Materials. The standard 20 amino acids, pantothenic acid, sodium selenite, sodium molybdate, and sodium tungstate were purchased from ICN Biomedicals Inc. (Aurora, OH). Thiamine and biotin were purchased from Sigma-Aldrich (St. Louis, MO). Riboflavin and nicotinic acid were purchased from Arcos Organic (Geel, Belgium). Magnesium sulfate was purchased from Alfa Aesar (Ward Hill, MA). Tryptic soy broth (TSB) minus glucose was purchased from Difco (Sparks, MD). Pyridoxine, KH_2PO_4, K_2HPO_4, sodium citrate, $(NH_4)_2SO_4$, and glucose were purchased from Fisher Scientific (Fair Lawn, NJ). Uric acid was purchased from Avocado Research Chemicals (Lancashire, England). Auranofin was purchased from Alexis Biochemicals (San Diego, CA). All restriction enzymes and thermostable polymerases were purchased from New England BioLabs (Ipswich, MA).

Bacterial strains and culture media. The bacterial strains used in this study are derivatives of *Enterococcus faecalis* V583. Defined medium used for growth of *E. faecalis* strains was composed of the 20 standard amino acids (20 mg/liter), pantothenic acid (20 mg/liter), folic acid (200 μg/liter), thiamine (100 mg/liter), biotin (20 mg/liter), nicotinic acid (2 mg/liter), riboflavin (2 mg/liter), pyridoxine (20 mg/liter), KH_2PO_4 (2 g/liter), K_2HPO_4 (7 g/liter), sodium citrate (0.5 g/liter), $(NH_4)_2SO_4$ (1 g/liter), glucose (2 g/liter), and $MgSO_4$ (120 mg/liter). Tryptic soy broth (TSB) or tryptic soy broth plus glucose (1% glucose) (TSBG) was utilized as a rich medium. Additives to the growth medium consisted of uric acid (1 mM), sodium selenite (50 nM), sodium molybdate (1 μM), sodium tungstate (1 μM), or auranofin (1 μM). Modified Luria broth (tryptone, 10 g/liter; yeast extract, 5 g/liter; and NaCl, 5 g/liter) was used for the culture of *E. coli*.

Isolation of in-frame *selD* mutant. In-frame deletion mutants were obtained using the suicide vector pLT06 as previously described (56). Two PCR products were engineered to fuse an in-frame mutation of the *selD* gene (EF2567) or the *xdh* gene (EF2570) from *E. faecalis* V583 into the multiple cloning site of pLT06. A region 5′ of either gene that encompasses roughly one quarter of the coding sequence of the gene and flanking DNA was amplified using the following oligonucleotides: to target *selD*, 5′ TATGAATTCTATGCAGCGACTTCCTAT 3′ and 5′ ATGCTCGAGTCCGGTTACCGCTAGACC 3′, and to target *xdh*, 5′ AGAGAATTCGTTTCCGCCGAAGGTAGA 3′ and 5′ TAACTCGAGACAT GCACCGCAAGAGCC 3′. This fragment was cleaved with EcoRI (5′ end) and XhoI (3′ end) after amplification. A second region that encompasses roughly one quarter of the 3′ coding sequence of either gene and an additional downstream region to give roughly 1 kb was amplified using the following oligonucleotides: to target *selD*, 5′ CTGCTCGAGAGTGTCGCTGCTGACGAA 3′ and 5′ CAATC TAGAGACGCGAACAGCCACCTT 3′, and to target *xdh*, 5′ GGACTCGAG AACGCCACTCAAGTACCA 3′ and 5′ TGTTCTAGATCGCCAAGCTCGCT GAAT 3′. This fragment was cleaved with XhoI (5′ end) and XbaI (3′ end) after amplification. These cleaved products were then subsequently ligated to pLT06 that had been digested with EcoRI and XbaI to select for a plasmid that carries an in-frame deletion mutant allele with substantial (over 750 bp) flanking regions. These constructs were confirmed by sequencing and used to obtain several independent isogenic *selD* and *xdh* mutants and corresponding wild-type revertants upon selection as previously described (56). Primers SelDUp (5′ AGATG CTGAGAATAGCGACTC 3′) and SelDDown (5′ TTTAAGGACTCCACAG TTGGT 3′) and XdhUp (5′ CGATTGGTACAAGTTCAGGAT 3′) and

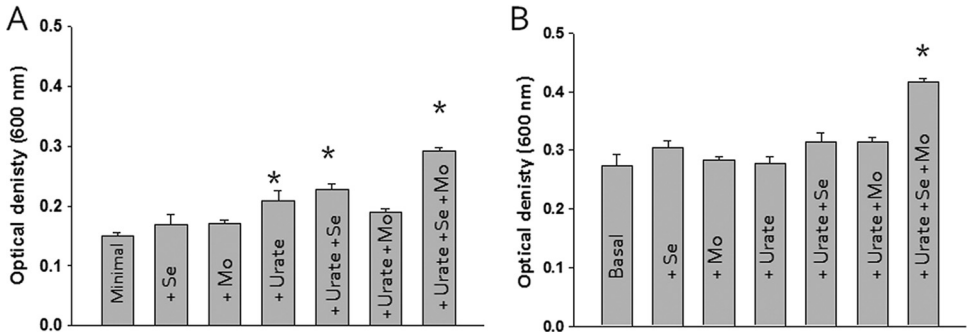

FIG. 2. *E. faecalis* V583 growth yield is increased in minimal medium when supplemented with uric acid, but only in the presence of Se and Mo. Cultures were grown aerobically in basal medium (BM) or minimal medium (M16) with and without uric acid (1 mM), selenite (Se, 1 μM), or molybdate (Mo, 1 μM). Cultures (5 ml) were shaken at 225 rpm in 125-ml Erlenmeyer flasks. Optical density was measured after 24 h of growth. An asterisk (*) denotes significant differences ($P < 0.05$) determined using Student's *t* test. Error bars indicate standard deviations.

XdhDown (5′ TAAGACATTGGATGCGTGTGT 3′), which flank the cloned region to delete these genes, were used in colony PCR to confirm the presence of either mutants or wild-type revertants.

Xanthine dehydrogenase enzyme assay. Xanthine-dependent reduction of 2,6-dichloroindophenol (DCPIP) was carried out essentially as previously described (49). Cells were harvested by centrifugation at 7,500 × *g*, washed with lysis buffer (50 mM tricine [pH 8.0], 1 mM EDTA, 0.05 mM benzamidine), and subsequently resuspended in lysis buffer. Rupture of cells was accomplished by sonication (model 100 sonifier; Fisher) at a power setting of 4 W. Cell extracts were clarified by centrifugation at 20,000 × *g* for 30 min at 4°C. Protein concentration was determined using the method of Bradford (11). XDH activity was determined by following the xanthine-dependent reduction of DCPIP (0.2 mM) at 600 nm in 50 mM sodium phosphate buffer, pH 8.0, using a UV-visible spectrophotometer (8350; Agilent). Xanthine was present at a concentration of 200 μM. The rate of DCPIP reduction was calculated using a molar extinction of 22,000 for reduced DCPIP (49).

Assay for biofilm density. *E. faecalis* was incubated in defined medium with or without Se, uric acid, or Mo in a 96-well polystyrene plate for 48 h at 37°C. Alternatively, cells were cultured in tryptic soy broth with or without 1% glucose. Adherent biofilms were washed with sterile water (three times) to remove loosely attached cells and dried at 37°C for 20 min. Dried biofilms were stained with 0.1% safranine at room temperature for 20 min. Excess safranine was removed by washing biofilms with water three times, and cells were subsequently redried for 20 min. Safranine was dissolved in an ethanol and acetone mix (80:20, vol/vol). Biofilm density was measured at 490 nm in a UV-visible multiplate spectrophotometer (Spectra Max 190; Molecular Devices).

Specific labeling of selenoproteins. *E. faecalis* was cultivated in defined medium as described above in the presence of 5 nM Na_2SeO_3 (0.25 μCi/μl ^{75}Se), with or without tungsten (1 μM) and with or without auranofin (1 μM). Alternatively, cultures were grown in TSB with and without glucose (1%) in the presence of 5 nM Na_2SeO_3 (0.25 μCi/μl ^{75}Se). Planktonic cells were removed, and cells present in biofilms were harvested by dispersion of the biofilm with trypsin at 37°C (10-min incubation), subsequent centrifugation, and washing of the biofilm twice in lysis buffer to remove excess trypsin. Cell extracts were prepared via sonication as described above, and clarified cell extracts (25 μg of protein) were analyzed via reducing 12% SDS-polyacrylamide gel or native 4 to 20% gradient gels (Bio-Rad). The presence of small-molecule forms of selenium and/or native selenoprotein complex was detected using a phosphorimager (Storm; Molecular Dynamics).

Real-time reverse transcriptase PCR (RT-PCR) analysis of mRNA levels. *E. faecalis* was cultured in defined medium with or without selenite, uric acid, or molybdate at 37°C for 24 h. Cells were harvested by centrifugation (5,000 × *g*), and the enzymatic and chemical lysis of bacteria was performed in the presence of RNAprotect reagent (Qiagen). Cell walls were disrupted using a bead beater (Disruptor Genie; Scientific Industries), and total RNA was extracted using an RNeasy minisystem (Qiagen). Total RNA was quantified by a UV-visible spectrophotometer (Nanodrop; Thermo Scientific, Wilmington, DE). cDNA was generated starting with 0.5 μg of RNA using an Iscript cDNA kit (Bio-Rad). Real-time PCR was carried out on a Bio-Rad iQ5 multicolor real-time PCR system. IQ SYBR green supermix (Bio-Rad) was utilized to follow real-time amplification, and primers were present at a concentration of 200 nM each. cDNA was diluted 1:100 in sterile distilled water (dH_2O) before addition to the reaction mix. Reaction conditions were as follows: a single cycle at 95°C for 3 min, and 40 amplification cycles (95°C for 10 s, 55°C for 45 s). Oligonucleotides used were as follows: *xdh* forward, 5′ GAACTTTTGCGCGAGGATAG 3′, and *xdh* reverse, 5′ AAGCGATTCCAGAAGAA 3′; *selD* forward, 5′ ATTATTCA AGCGGCTGTTCG 3′, and *selD* reverse, 5′ CCAACGCTCCTGTAATGGTT 3′; *rpoB* forward, 5′ GCGATATGGACGATGACGATGATG 3′, and *rpoB* reverse, 5′ TTAGTCTCTGTCTTCGGCTGTTTC 3′. Melt curve analysis was performed to confirm the presence of a single product. Efficiency of amplification for each target gene was calculated using the Pfaffl method, described previously (39).

Hydrogen peroxide determination. Peroxide levels were assessed essentially as previously described (10). Cultures (5 ml) were grown in TSBG with or without 50 nM selenite in a 6-well culture dish for 48 h at 37°C. Extracellular hydrogen peroxide levels were determined using an Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen, Carlsbad, CA). Culture-free medium was diluted in reaction buffer until the level of peroxide obtained was within the standard curve (0 μM to 2.5 μM hydrogen peroxide). Fluorescence of resorufin dye was determined using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA), with excitation at 545 nm, emission at 590 nm, and a detector voltage of 400 V.

Detection of extracellular superoxide production. Extracellular superoxide production was analyzed as previously described (29). Briefly, 0.2 ml of an overnight culture (brain heart infusion [BHI]; Oxoid) was used to inoculate 5 ml shaking cultures (150 rpm) that were incubated for 48 h. Cells were isolated by centrifugation (5,000 × *g*), washed twice in phosphate-buffered saline, and resuspended in ice cold Hanks' balanced salt solution (HBSS). Cell density was adjusted to an optical density (absorbance at 600 nm) of 0.2 and stored on ice. A reaction mixture containing 2 mM ferricytochrome C in HBSS (200 μl total volume) was used to follow superoxide production. To start the reaction, 4 μl of cells was added, and the absorbance of reduced ferricytochrome C was followed at 550 nm using a UV-visible multiplate reader (Molecular Dynamics) for 3 min.

RESULTS

Uric acid stimulates growth in defined medium that is dependent on Se and Mo. Our initial hypothesis focused on the use of uric acid as an alternate nitrogen source for biosynthesis. Since *E. faecalis* is auxotrophic, this made limiting nitrogen difficult. We tested growth in defined medium containing various purines (hypoxanthine, xanthine, adenine, guanine, and nicotinic acid) and found no benefit to growth in either the presence or the absence of selenite or molybdate (data not shown). However, the growth yield of *E. faecalis* V583 was significantly increased in the presence of uric acid and increased further when selenite (1 μM) and molybdate (1 μM) were added to the medium (Fig. 2A), suggesting some benefit to growth. This effect was also seen in complex culture medium (termed basal medium, containing tryptone and yeast extract)

(Fig. 2B). These studies suggested that a selenium-dependent molybdenum hydroxylase was involved in metabolism of uric acid and that the catabolism of uric acid had some benefit to either bioenergetics or biosynthesis in this organism. It should be noted that a lower level of molybdate or selenite (50 nM) was sufficient to yield this increase (data not shown). These results suggest that *E. faecalis* utilizes only uric acid as a purine source from the environment, and this is corroborated by the presence of a putative uric acid transporter in the SDMH gene cluster (20). We also observed increased xanthine-dependent reduction of DCPIP in cells supplemented with Se, Mo, and uric acid, with the highest enzyme activity observed when all were present in the culture medium (data not shown). The lack of induction by xanthine suggests that this pathway is specific to utilizing uric acid as a source of purines for DNA synthesis or alternatively to extract electrons from uric acid through its conversion to xanthine to another metabolic process. Indeed, the biological reversal of this pathway is not unprecedented. Bradshaw and Barker demonstrated that a xanthine dehydrogenase was induced and active in metabolism in several species of clostridia when cells were cultured in the presence of uric acid as a purine source (12), presumably through its potential reverse catalysis to derive xanthine and hypoxanthine from uric acid as a source of purines for DNA synthesis. The xanthine dehydrogenase studied by Barker several decades ago was expressed when cells were cultured with uric acid in several clostridium species that likely contained an SDMH (8, 15, 41).

Biofilm formation is stimulated in the presence of uric acid, Se, and Mo and inhibited by auranofin or tungstate. Since the gene cluster that carries a putative SDMH has also recently been shown to be upregulated in a RIVET screen for genes upregulated in biofilms (7), we designed experiments to test whether the presence of uric acid, Se, or Mo affected formation of biofilms. Culturing strain V583 in either 96-well or 6-well culture dishes, we determined biofilm density using a well-established safranine staining method (34). We indeed observed stimulation of biofilm formation in cultures grown in the presence of selenite and uric acid (Fig. 3A). We observed a concomitant reduction in planktonic cell density (Fig. 3B), which suggests that the population shifted from planktonic to biofilm growth. Optimal production of the biofilm was observed in the presence of selenite, molybdate, and uric acid (Fig. 3A). Indeed, the addition of selenite alone increased biofilm density (Fig. 3A), and the addition of uric acid with molybdate also increased biofilm density, suggesting that each of these components is somehow sensed to trigger the induction of this pathway. Experimental conditions that led to more-dense biofilms also reduced the growth of cells in the planktonic phase of the culture (Fig. 3B). This suggests that the induction of the catabolic use of uric acid is optimal when both Se and Mo are present and that the induction of this enzyme correlates with a shift from planktonic cell growth to biofilm growth.

The addition of tungstate to cultures has been shown to inhibit the transport and metabolism of molybdenum into the molybdenum cofactor in a variety of model systems (33). To probe whether tungsten could inhibit biofilm formation, we added tungstate at 1 μM to the culture in which induction of biofilms was optimal due to the presence of molybdate, selenite, and uric acid (Fig. 4A). Indeed, tungstate did inhibit

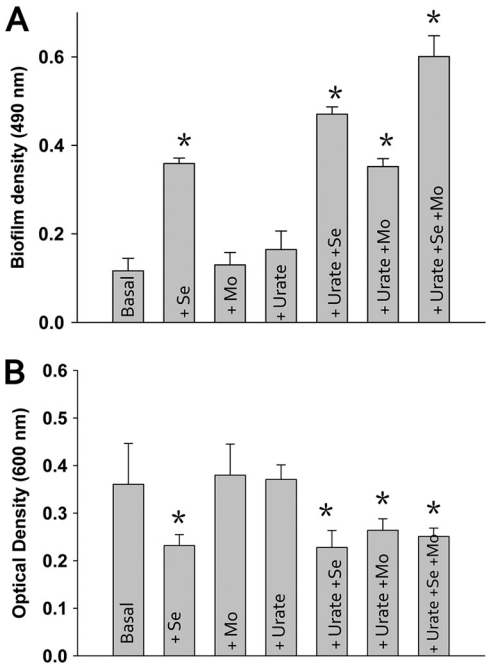

FIG. 3. Biofilm formation is stimulated by growth in defined or rich medium with uric acid in a Se- and Mo-dependent manner. (A) *E. faecalis* (V583) biofilms were established in defined medium with supplements as indicated in the text after growth in a 96-well plate for 48 h at 37°C. Biofilm density was measured using a safranine staining method as described in Materials and Methods. (B) Optical density of the corresponding planktonic cells is plotted. An asterisk (*) denotes a significant increase in biofilm density or a significant decrease in optical density compared to that with basal medium alone, determined using Student's *t* test ($P < 0.05$). Error bars indicate standard deviations.

biofilm formation, suggesting that inactivation of the biosynthesis of the Mo cofactor does lead to the inability of the cell to form a biofilm. We have recently shown that the rheumatoid drug auranofin can block the metabolism of selenium to selenophosphate by forming an adduct to hydrogen selenide in *Clostridium difficile*, *E. coli*, and *Treponema denticola* (31, 32). Exposure of *E. faecalis* to auranofin also inhibited biofilm formation, suggesting strongly that selenium is required for biofilm formation in this organism. Neither tungstate nor auranofin reduced growth of planktonic cultures (Fig. 4B), and thus these were not general inhibitors of cell proliferation. These exciting results strongly suggest that induction of an SDMH is sufficient to trigger increased biofilm density in *E. faecalis*. To date, no other study has shown a role for Se- or Mo-dependent enzymes in biofilms or any other area of microbial pathogenesis.

Transcription of genes encoding an SDMH and genes within the same apparent operon is upregulated in the presence of uric acid, selenium, and molybdenum. To begin to understand how the increased growth yield correlates with increased expression of genes within the SDMH operon, we cultured *E. faecalis* in the presence and absence of uric acid, selenium, and molybdenum and utilized quantitative RT-PCR to analyze changes in mRNA levels in cells isolated from biofilms as well as planktonic cells (Fig. 5; also see Fig. S1 in the supplemental material) in response to these additions. A clear and substan-

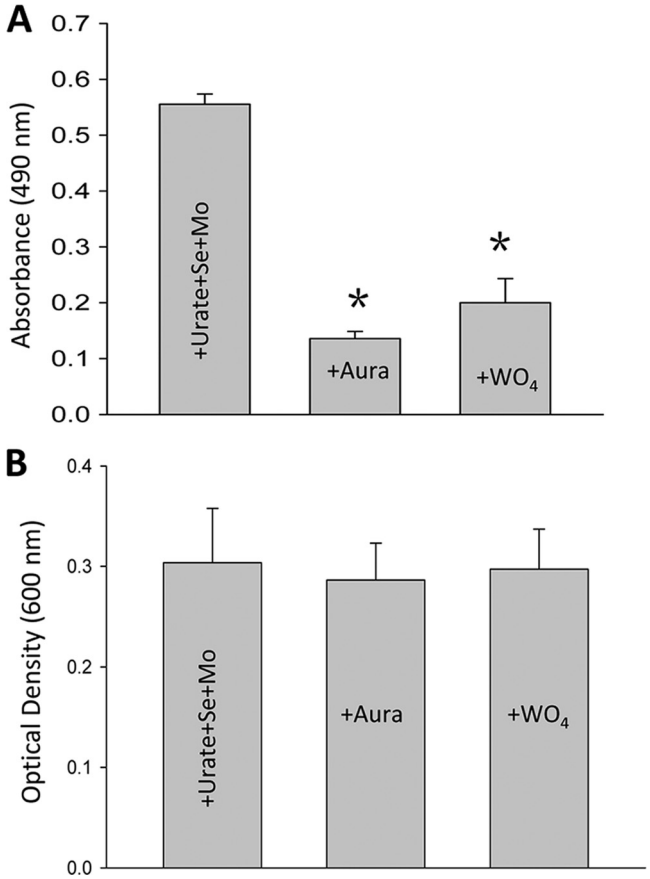

FIG. 4. Inhibition of the metabolism of selenium or molybdenum results in decreased biofilm density but does not alter planktonic cell yield. *E. faecalis* (V583) biofilms were established in defined medium with supplements as indicated in the text. (A) Addition of auranofin (Aura, 1 μM) or tungstate (1 μM) inhibits formation of biofilm. (B) Optical density of the corresponding planktonic culture is plotted. An asterisk (*) denotes a significant decrease in biofilm density compared to that with basal medium with urate, selenite, and molybdate alone, determined using Student's *t* test ($P < 0.05$). Error bars indicate standard deviations.

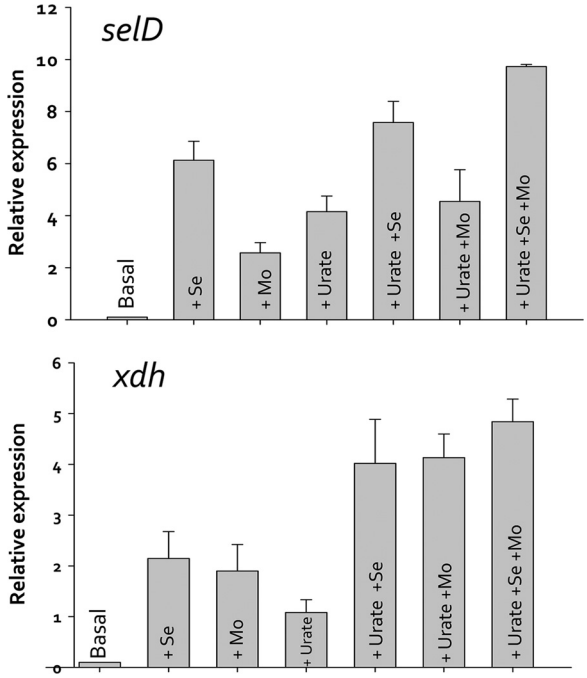

FIG. 5. Addition of uric acid, selenite, or molybdate results in increased expression of *selD* and *xdh* mRNA. mRNA was isolated from biofilms after cells were harvested with trypsin as described in Materials and Methods. Two-step real-time RT-PCR was carried as described in Materials and Methods using two oligonucleotide primer sets specific for *selD* or *xdh* cDNA. Fold induction versus that for control cultures is plotted as determined by semiquantitative real-time RT-PCR and calculation of fold changes as per the Pfaffl method (39). The mRNA levels were standardized by using *rpoB* as an internal control. Error bars indicate standard deviations.

tial increase in mRNA levels (using RNA polymerase *rpoB* as an internal standard) was observed for *selD* and *xdh* (EF2567 and EF2570, respectively) (Fig. 5) mRNA isolated from biofilms. Each mRNA was increased in the presence of Se, Mo, or uric acid to some extent (Fig. 5). These data suggest that some transcriptional control of this operon is able to sense the availability of each of these components in the culture medium. Overall, the pattern in general correlates with the response in growth yield observed in Fig. 2 and suggests that the presence of each of these required elements/substrates is necessary for upregulation of the putative gene cluster. We observed similar results for other targets in this gene cluster (data not shown), suggesting that this region is coregulated. We also determined changes in mRNA levels in cells isolated from the planktonic phase of these cultures (see Fig. S1 in the supplemental material). We did observe a small increase in response to Se, Mo, or uric acid but not to the levels seen in biofilms. The level of 23S rRNA was also measured from biofilms and planktonic cultures, and no significant changes occurred upon addition of Se, Mo, or uric acid (see Fig. S2 in the supplemental material). This eliminated the possibility that a general metabolic change was occurring and producing artifacts in our gene expression profile.

These data suggest that once a biofilm is established, an amplified response for this gene cluster apparently takes place. Future studies of in-depth gene expression changes over time are certainly warranted but are beyond the primary scope of this work. This induction by substrate (uric acid) and molybdenum is reminiscent of work carried out in the *E. coli* model system on transcriptional regulation of the molybdoenyzmes formate dehydrogenase and nitrate reductase in their response to formate, nitrate, or molybdate (19, 22, 45–48). A molybdate-responsive regulatory protein (ModE) is also encoded in the SDMH gene cluster, and this protein has been shown to be responsible for molybdate-dependent gene expression of nitrate reductase and components of the formate hydrogenlyase (46).

A labile selenoenzyme complex is produced in biofilms in the presence of uric acid and molybdenum. To determine whether a labile selenoenzyme was being produced under our established growth conditions and specifically enriched in biofilms, we cultured strain V583 in the presence of radiolabeled ^{75}Se (as selenite) to follow the metabolism of selenium under the growth conditions described above. We analyzed crude cell extracts from planktonic cells and biofilms by both native

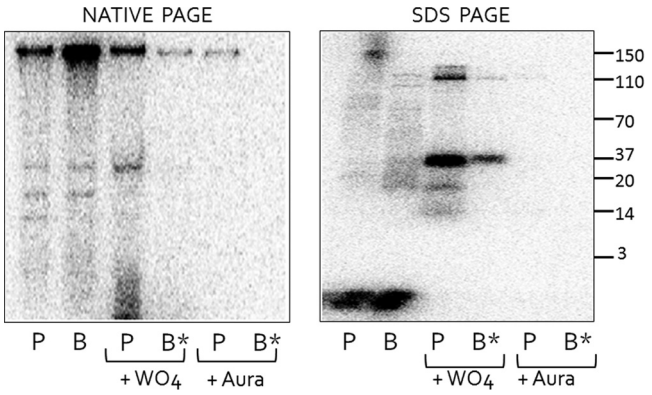

FIG. 6. Addition of tungstate or auranofin suppresses incorporation of ^{75}Se into a labile selenoprotein in *E. faecalis*. Planktonic (P) and biofilm (B) cell extracts of cells cultured with and without tungstate or auranofin (1 μM each) were prepared following growth in the presence of 5 nM selenite (0.25 μCi/μl ^{75}Se). Crude cell extracts (S30, 25 μg of protein [see below]) were analyzed via native gel (nonreducing) or 12% denaturing/reducing (SDS) gel electrophoresis, and the presence of selenoproteins was determined using a phosphorimager. For lanes labeled with an asterisk, due to insufficient protein levels, the entire extract was loaded in the biofilm lanes treated with tungstate or auranofin and as such was not equal to 25 μg of protein. This lack of protein yield directly correlates with the inhibition of biofilm density observed in Fig. 3. Values at right are molecular size markers in kilodaltons.

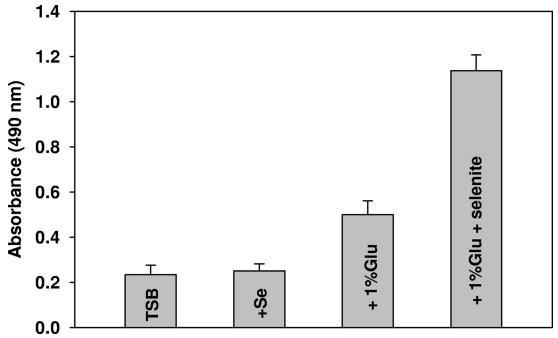

FIG. 7. Selenite triggers enhanced biofilm formation only in the presence of glucose when cultures are grown in tryptic soy broth (TSB). Biofilm density was assessed after 48 h of growth in TSB with and without glucose (1%) or selenite (50 nM). Error bars indicate standard deviations.

PAGE and reducing SDS-PAGE (Fig. 6). A large-molecular-mass complex was resolved on native polyacrylamide gels that contained selenium, and this complex was significantly more abundant in extracts from biofilms than in those from planktonic cells (Fig. 6; also see Fig. S3A in the supplemental material). Multiple cultures were labeled, and the production of this labeled complex in biofilm cell extracts relative to that in planktonic cell extracts was quantified in native gels to confirm the increase using densitometry (see Fig. S3B in the supplemental material). The selenium present in this complex was labile, as seen by the presence of ^{75}Se in the dye front of the SDS-polyacrylamide gel (Fig. 6B). This is similar to the behavior of the labile selenium cofactor found in the NAH enzyme (18). The addition of tungstate or auranofin reduced the synthesis of this labeled complex in biofilms; however, only auranofin treatment prevented synthesis in planktonic cells (Fig. 6A). Most intriguing is the accumulation of radioisotope selenium on smaller proteins in SDS-PAGE extracts from cells cultured in the presence of tungstate. Although the results are somewhat speculative, one can imagine that these stable Se-protein complexes are likely precursor intermediates for insertion of the selenium into the SDMH, such as NifS-Se complexes that have been recently described as they relate to selenium metabolism with SelD (37). These results strongly suggest that inhibiting the incorporation of Mo into the Mo cofactor directly affects the incorporation of Se into the SDMH active site. These results also suggest that this model system will be amenable to developing a complete understanding of the synthesis of the active site of SDMHs, analogous to the substantial work done in the past on Mo cofactor biosynthesis for nitrogenase and nitrate reductase (24).

Selenium addition to tryptic soy broth triggers biofilm formation, but only in the presence of glucose. Studies that have focused on biofilm physiology of *E. faecalis* have reported that the addition of glucose to 0.75% or 1.0% in tryptic soy broth can increase biofilm density at least in part due through quorum sensing (40, 54). We determined the effect of adding Se, Mo, or uric acid to TSB with and without glucose. The addition of uric acid or molybdate did not alter the biofilm density (data not shown), but the addition of selenite (50 nM) was sufficient to enhance the density of biofilms in TSBG (Fig. 7). This required that glucose be present in the culture medium, as no effect was observed when selenite was added to TSB alone (Fig. 7). Auranofin and tungstate addition still prevented biofilm formation under these growth conditions (data not shown), consistent with our other findings (Fig. 4). This is good evidence that the SDMH plays an important role in biofilm formation and that this biofilm yield is notably higher than that which occurs when glucose alone is added to TSB.

Isolation of *selD* and *xdh* in-frame deletion mutants with null phenotype for biofilm formation. Given the enhanced biofilm density upon addition of selenite to TSBG, we assessed whether an in-frame deletion mutation in the gene encoding the first step in the specific use of selenium in biology, *selD* (Fig. 1), would alter this phenomenon. This is in fact the first true test of the hypothesis presented in our computational work (20). We compared biofilm formation between V583 and the *selD* mutant strain and found that the mutant exhibited a null phenotype for Se-dependent biofilm production (Fig. 8A). Likewise, we isolated a strain carrying an in-frame deletion in the *xdh* gene to support our assumption that the selenium is present as a labile cofactor in a molybdenum hydroxylase enzyme (SDMH). As with the *selD* mutant, the *xdh* mutant strain did not respond to the addition of selenite for increased biofilm density (Fig. 8A). The baseline TSBG biofilm appeared slightly reduced, suggesting that the SDMH-dependent pathway is responsible for increasing biofilm yield through an as yet undetermined mechanism and perhaps that this pathway may play a role in glucose-dependent biofilm induction in the wild-type strain as well.

We have identified a labile selenoprotein complex that is produced in larger amounts in biofilms than in planktonic cells (Fig. 6). To confirm that this labile selenoprotein complex is not produced in either the *selD* or the *xdh* mutant, we isolated cell extracts from biofilms cultivated in TSBG (Fig. 8B) with

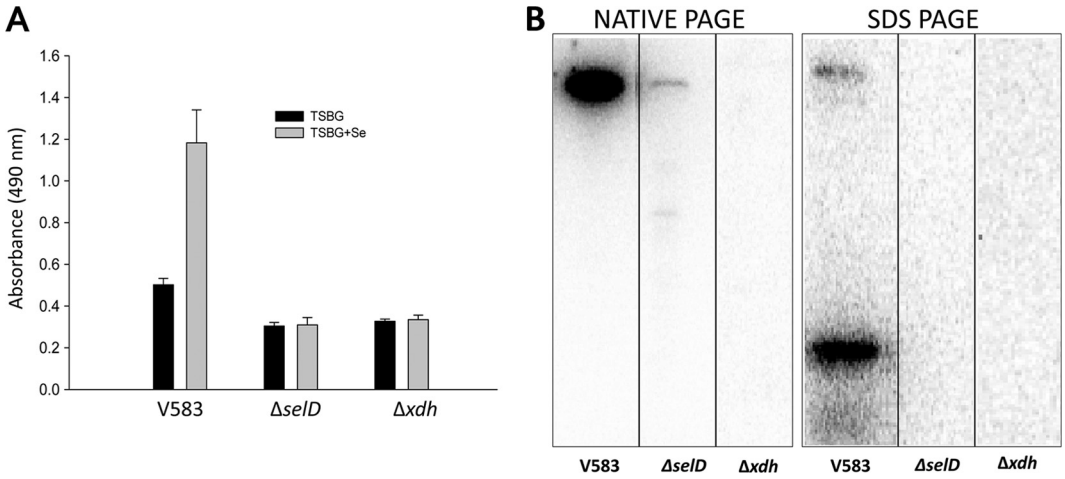

FIG. 8. Mutation in *selD* or *xdh* prevents selenium-dependent biofilm formation. (A) Wild-type (V583) and *selD* and *xdh* mutant strains were cultured for 48 h at 37°C in a 96-well plate in tryptic soy broth plus 1% glucose (TSBG) with and without selenite (50 nM). Biofilm density was determined using the safranine staining method as described in Materials and Methods. Error bars indicate standard deviations. (B) Native and reducing SDS-PAGE of wild-type and *selD* and *xdh* mutant extracts cultured in TSGB and labeled with ^{75}Se. Clarified cell extracts were analyzed as described in the legend for Fig. 6.

radioisotope selenium. This labile selenoprotein does not appear to be produced in either mutant strain, and the small-molecule selenium that is released upon denaturation of the complex (SDS-PAGE) is also absent. Further, we observed xanthine-dependent reduction of DCPIP in cell extracts grown in TSBG with selenite (Table 1), but this xanthine dehydrogenase activity was absent in both of the mutant strains (Table 1). These data demonstrate that the labile selenoprotein complex is not produced in the absence of selenophosphate or in the absence of the putative molybdoenzyme. There appears to be some slight labeling of the complex in the *selD* mutant. Since this mutant does not exhibit significant levels of xanthine dehydrogenase in cell extracts (Table 1), this may be nonspecific binding of selenium to other large-molecular-mass proteins. These results, when taken together, strongly suggest that the *xdh* gene encodes a selenium-dependent xanthine dehydrogenase.

Evidence that a selenium-dependent xanthine dehydrogenase is necessary for extracellular superoxide and hydrogen peroxide production by *E. faecalis*. Huycke has shown in several studies that superoxide production by *E. faecalis* is tied to virulence and that this reactive oxygen species (ROS) production is linked with carcinogenesis *in vivo* (27–29, 61). Since SDMH enzymes contain several redox active cofactors and most of the enzymes in biology that produce superoxide contain a flavin cofactor, we determined the levels of extracellular hydrogen peroxide in culture medium after growth in TSBG with and without selenium. We also tested whether a mutation in the *selD* or *xdh* gene would affect peroxide levels. As shown in Fig. 9, a clear increase in hydrogen peroxide levels was observed when cells were cultured in TSBG in the presence of selenite (50 nM). However, this peroxide production was clearly lost in an in-frame *selD* mutant. Thus, biofilm produc-

TABLE 1. Xanthine dehydrogenase activity in cell extracts cultured in TSBG

Strain	Culture medium	Sp act (±SD) (nmol min^{-1} mg^{-1})
V583	TSBG	1.4 ± 0.01
	TSBG + Se	11.5 ± 0.75
selD mutant	TSBG	1.5 ± 0.08
	TSBG + Se	2.0 ± 0.07
xdh mutant	TSBG	1.7 ± 0.04
	TSBG + Se	2.2 ± 0.13

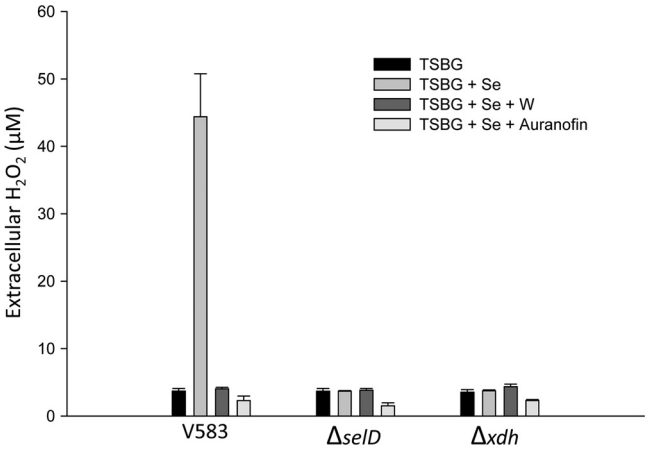

FIG. 9. Both activation of selenium and production of a xanthine dehydrogenase are required for increased hydrogen peroxide production observed in the presence of TSBG with selenite. Wild-type (V583) and *selD* and *xdh* mutant strains were incubated at 37°C for 48 h in a 6-well plate with tryptic soy broth plus 1% glucose (TSBG). Selenite was added to a final concentration of 50 nM. Tungstate (W) or auranofin was added to give 1 μM. Amplex red-horseradish peroxidase (Invitrogen) was used to determine the concentration of hydrogen peroxide in the culture medium. The medium was diluted to achieve peroxide levels that fell within a standard curve of peroxide between 0.1 and 2.5 μM. Tungstate and auranofin do not affect peroxide levels in cultures that lack selenite (data not shown). Error bars indicate standard deviations.

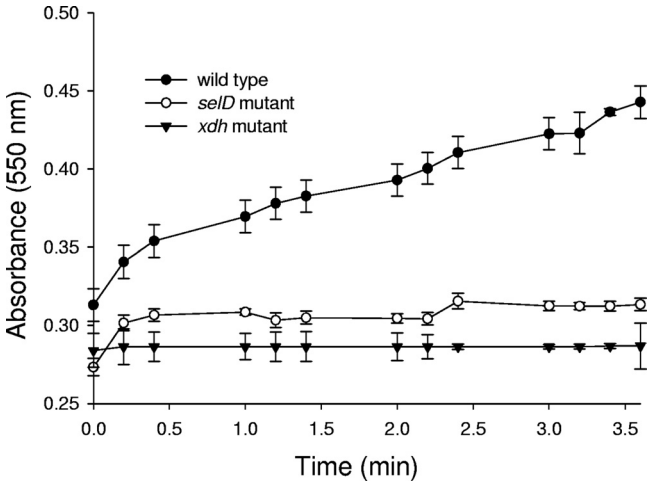

FIG. 10. Mutation in either *selD* or *xdh* leads to decreased extracellular superoxide production. Cultures were incubated at 37°C for 48 h in brain heart infusion (BHI) medium with shaking at 150 rpm. Wild-type strain V583 is compared to an in-frame *selD* or *xdh* mutant. Superoxide production was measured using ferricytochrome C in the presence of washed whole cells. The means from several independent cultures are plotted, with standard deviations shown as error bars.

tion/density correlates well with peroxide levels, and by use of site-directed mutagenesis, it is clear this pathway (SDMH enzyme biosynthesis and catalytic activity) is linked.

Since hydrogen peroxide is a stable by-product of a superoxide radical anion, we further studied whether a mutation in *selD* or *xdh* would alter the superoxide production that has been known as a hallmark of *E. faecalis* physiology. It should be noted that the established protocol to measure superoxide production uses culture conditions where biofilms are not allowed to form (shaking flasks). We first tested wild-type (V583) *selD* and *xdh* mutant strains grown in TSBG with and without selenite to be consistent with other experiments in this study. Although there were slight differences between the two strains as assessed by the ferricytochrome C assay, the level of superoxide production was nearly undetectable when cells were cultured under these conditions (data not shown). However, when BHI was used as a medium (the medium used in previous reports), we observed significant superoxide production that was in line with the previous studies (Fig. 10). The wild-type strain produced significant levels of superoxide, whereas the *selD* and *xdh* mutants did not exhibit superoxide production. When combined, our results strongly suggest that the genes in the SDMH operon encode a selenoenzyme that is integral to the production of superoxide by *E. faecalis*. Moreover, this ties our work into the significant literature established by Huycke and Gilmore on the relationship between superoxide production and clinical isolates, work that now links this production to colon cancer (28–30, 60, 61).

DISCUSSION

The selenium-dependent molybdenum hydroxylases that have been described from strict anaerobes (*Clostridium purinilyticum*, *Eubacterium barkeri*) have a turnover rate that is nearly 2 orders of magnitude faster than those of the non-selenium-dependent enzymes of this family (18, 43, 49, 50). The biological rationale for this improved catalytic efficiency relates to the use of purines/pyrimidines as primary carbon, nitrogen, and energy sources (15, 16, 25, 49, 50). Xanthine dehydrogenase is a critical early step in the metabolism of purines (15, 43, 44), and nicotinic acid hydroxylase is a parallel catalyst in *E. barkeri*, since this organism was isolated through batch culture with NAD^+ as a primary carbon source (25, 38, 57). In some instances, selenium has been proposed to improve the catalytic efficiency of a biochemical reaction compared to its sulfur-dependent counterpart (3, 4), but this concept has been challenged more recently to focus more broadly on the chemistry of selenium versus that of sulfur (2).

In this work, we tested the hypothesis that external purines might be metabolized in a manner similar to that seen in purinolytic clostridia, i.e., through a selenium-dependent molybdoenzyme. We found evidence that *E. faecalis* prefers uric acid and that this catabolism of uric acid depends on both Se and Mo. Moreover, this use of uric acid somehow triggered a shift from planktonic cell growth to biofilms. Given the abundance of uric acid in blood and urine, two fluids in which *E. faecalis* thrives in the host, this suggests that uric acid may be a key metabolite in the decision to produce biofilms during infection.

Further, we tested the hypothesis that selenium must first be activated to selenophosphate to become incorporated into a selenium-dependent molybdenum hydroxylase (SDMH). We found that in the absence of selenophosphate synthetase (SelD) no labile selenoprotein complex was produced. We confirmed that the labile selenoprotein complex observed was also not made when the gene encoding the molybdoenzyme was not made. These results confirm the previous suggestion that SelD is needed for synthesis of SDMH enzymes, a concept that allowed us to use computational biology to lead us to the model organism of *E. faecalis*.

The rationale for a selenoenzyme (SDMH) to be involved in biofilm formation is not at all clear. The observed increase in hydrogen peroxide production suggests that oxidant production could be at least part of the rationale for having a catalytically superior xanthine oxidase type of enzyme in *E. faecalis*. The strong correlation with extracellular superoxide and hydrogen peroxide production upon addition of selenite, combined with the lack of oxidant production in the *selD* mutant, points to oxidant production as the enzyme's primary role, although this remains to be tested upon characterization of the native enzyme isolated from the wild-type strain. It should be noted that metalloenzymes are at the heart of all biological production of both reactive nitrogen and reactive oxygen species, whether they occur in the electron transport chain or in a specialized compartment, such as the phagolysosome (6). Some have also argued that the human XOR plays a role in oxidative stress during ischemia injury and also in metabolism of reactive nitrogen species (5, 9).

The production of an oxidant as a potential signal for development of a biofilm appears to be novel based on our survey of the literature. It should be noted that we could not mimic the increase in biofilm density by treating cells in TSBG (without selenium) with 50 μM or even 500 μM hydrogen peroxide in wild-type V583. This suggests that internal production of this oxidant, or channeling of electrons to a membrane-asso-

ciated cofactor that generates peroxide, is required to trigger the increased density of biofilms. It is possible that this triggers a physiological change in the population of cells that promotes growth in the biofilm community by an as yet undetermined mechanism. Nonetheless, we are encouraged by our findings that suggest that this model system will be fruitful in defining the pathway for SDMH maturation and that the link to biofilm physiology will be uncovered through mutagenesis of the other genes in the region and biochemical characterization of the SDMH.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the NSF (CBET 0753068) to W.T.S. and by NIH grant AI77782-01A2 to L.E.H.

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