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Enterococcus faecalis Heme-Dependent Catalase

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Enterococcus faecalis **cells cannot synthesize porphyrins and do not rely on heme for growth but can take up heme and use it to synthesize heme proteins. We recently described a cytochrome *bd* in *E. faecalis* strain V583 and here report the identification of a chromosomal gene, *katA*, encoding a heme-containing cytoplasmic catalase. The 54-kDa KatA polypeptide shows sequence similarity to members of the family of monofunctional catalases. A hexahistidyl-tagged version of the catalase was purified, and major characteristics of the enzyme were determined. It contains one protoheme IX group per KatA polypeptide. Catalase activity was detected only in *E. faecalis* cells grown in the presence of heme in the medium; about 2 and 10 μM hemin was required for half-maximal and maximal production of catalase, respectively. Our finding of a catalase whose synthesis is dependent on the acquisition of heme in the opportunistic pathogen *E. faecalis* might be of clinical importance. Studies of cellular heme transport and heme protein assembly and in vivo synthesis of metalloprotein analogs for biotechnological applications are impeded by the lack of experimental systems. We conclude that the *E. faecalis* cell potentially provides such a desired system.**

Enterococcus faecalis, formerly known as *Streptococcus faecalis*, is a gram-positive bacterium with a low G+C content in its genomic DNA. It is a common inhabitant of the intestines of animals, including humans, where it is part of the commensal flora. However, *E. faecalis* can cause disease in, e.g., immunodeficient persons, and it is frequently the causative agent of nosocomial infections. Lately, the emergence of multidrug-resistant strains has become a serious problem (10). Recognizing the medical importance of this bacterium, The Institute for Genomic Research has undertaken the genome sequencing of *E. faecalis* strain V583.

Heme is present as a prosthetic group in a variety of proteins, including, for example, catalases, cytochromes, and hemoglobins. *E. faecalis* cells do not synthesize heme, and genes for known porphyrin biosynthetic enzymes are not found in the genome sequence of strain V583. However, if *E. faecalis* cells are supplied with heme, synthesis of hemoproteins can take place (16, 19). We recently described a functional cytochrome *bd*-type quinol oxidase in *E. faecalis* V583 (27). The cytochrome *bd* is probably the terminal oxidase of a respiratory chain present in *E. faecalis* under certain conditions (1, 15). The presence of an aerobic respiratory chain is puzzling, however, in a bacterium generally considered to use a fermentative-energy metabolism. Aerobic respiration is more energy-efficient than fermentation, but it is also a source of reactive oxygen species. Cells have several protective mechanisms against these toxic compounds. Enzymatic detoxification of hydrogen peroxide in bacteria is mainly performed by catalases. Three classes of bacterial catalases have been described: monofunctional catalases, catalase-peroxidases, and manganese catalases (pseudocatalases) (30). The monofunctional catalases and the catalase-peroxidases contain heme as a prosthetic group.

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E. faecalis cells have generally been considered to be catalase negative (23) but may appear weakly positive for catalase under some conditions. The enzyme responsible for this catalase activity has not been demonstrated and has, for unclear reasons, often been attributed to a pseudocatalase, regardless of the fact that a heme-dependent *E. faecalis* catalase has been briefly described (17). We here report the identification of a gene, *katA*, encoding a heme-containing catalase and show that acquisition of heme is required for production of the active catalase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Bacillus subtilis* strains were grown at 37°C in Luria broth (LB) or minimal glucose medium or on tryptose blood agar base (Difco) plates. LB and Luria agar were used for growth of *Escherichia coli* strains. For maintenance and routine genetic work, *E. faecalis* was grown in Todd-Hewitt (TH) broth (Difco) or brain heart infusion broth (Difco) or on TH agar plates. For biochemical and physiological analysis, *E. faecalis* was grown in TSBG, which contains tryptone (15 g/liter; Lab M, Bury, England), soy peptone (5 g/liter; Lab M), NaCl (5 g/liter), 1% (wt/vol) glucose, 30 mM sodium 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.4), and 5 mM potassium phosphate buffer (pH 7.0). This medium contains less than 0.05 μM heme, since it was found to be unable to sustain the growth of *B. subtilis* strain 3G18Δ8 (deficient in the last step of heme synthesis [data not shown]).

When required, chloramphenicol was added to a final concentration of 5 μg/ml for *B. subtilis* and 25 μg/ml for *E. coli* and *E. faecalis*. Kanamycin was used at a concentration of 100 μg/ml for *B. subtilis* strains.

General DNA techniques. Bacterial chromosomal DNA was isolated essentially as described by Hoch (9). Electrocompetent *E. faecalis* cells were prepared as follows. Cells were grown in brain heart infusion broth in baffled flasks on a rotary shaker at 37°C for 16 h. Subsequently, the culture was diluted 50-fold into fresh medium and the incubation continued as before. After 4 h (optical density at 600 nm, 1.0 to 1.5), when the cells were in mid-exponential growth phase, they were collected by centrifugation at 5,000 × *g* at 4°C. The cells were washed four times in decreasing volumes of electroporation buffer (10% glycerol and 0.5 M sucrose). Finally, the cells were suspended in as small a volume of electroporation buffer as possible and stored at −80°C until use. Electroporation of 50 μl of the *E. faecalis* cell suspension was done by using 0.1 to 1 μg of plasmid DNA, cuvettes with a 2-mm gap length, and a Gene Pulser apparatus (Bio-Rad). A pulse length of 4.3 to 4.6 ms was obtained with the following settings: 2.5 kV, 25 μF, and 200 Ω. Immediately following the electric shock, the cells were suspended in 1 ml of TH broth at 37°C, supplemented with 0.5 M sucrose, and

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
Bacillus subtilis		
168A	*trpC2*	Laboratory stock
BLF03	*trpC2* Δ*katA*::*neo*	This work
PS2488	*trpC2* Δ*katA*::*cat*	2
3G18Δ8	*trpC2 ade met* Δ*hemH*::*ble*	8
Enterococcus faecalis V583		4
Escherichia coli SURE		Stratagene
Plasmids		
pHPSK	Cm^r Em^r	11
pLUF07	*katA* in pHPSK	This work
pLUF08	*katA*-His_6 in pHPSK	This work
pCat::Neo	*cat*::*neo* Km^r Ap^r	25
pAM401	Cm^r Tet^r	28
pLUF14	*katA* in pAM401, Cm^r	This work
pLUF15	*katA*-His_6 in pAM401, Cm^r	This work
pLUMB2	P_{aphA-3} in pBluescript KS	This work
pLUMB3	*katA*-His_6 in pLUMB2	This work
pLUMB5	P_{aphA-3}-*katA*-His_6 in pAM401, Cm^r	This work
pDG780	P_{aphA-3} Km^r	7
pBluescript KSII(−)	Ap^r	Stratagene

[a] Ap^r, Cm^r, Em^r, Tet^r, and Km^r indicate resistance to ampicillin, chloramphenicol, erythromycin, tetracycline, and kanamycin, respectively.

incubated without shaking at 37°C for 1 h. Finally, 150 μl of this suspension was plated on TH plates containing the appropriate antibiotic. For *E. coli*, preparation of electrocompetent cells and electroporation were done as described elsewhere (20). *B. subtilis* cells were transformed by using natural competence as described before (9).

Construction of *B. subtilis* BLF03. A catalase-negative derivative of *B. subtilis* strain 168A was obtained by transformation with chromosomal DNA isolated from strain PS2488 (*katA*::*cat*). One chloramphenicol-resistant transformant was subsequently transformed with the antibiotic cassette replacement plasmid pCat::Neo. A kanamycin-resistant clone was chosen and was designated BLF03. The successful deletion and replacement of *katA* in strain BLF03 was verified by catalase activity measurements on the cytoplasmic fraction.

Cloning of the *katA* gene and plasmid constructions. A DNA fragment containing the putative *E. faecalis* V583 *katA* gene was obtained by PCR with the Expand High Fidelity system (Roche Biochemicals) and primers C1 (5′-TGT GGA TCC TGG TGG TGT AAA CAG-3′) and C4 (5′-GAC GAA TTC TTA TGC TTG TTG CTT GAT-3′). The DNA fragment encoding the His-tagged KatA was obtained by using primers C1 and C5 (5′-GAC GAA TTC TTA GTG GTG GTG GTG GTG GTG TCC TGC TTG TTG CTT GAT-3′). The PCR products were cleaved with *Bam*HI and *Eco*RI and ligated into vector pHPSK. *B. subtilis* strain BLF03 (*katA*::*neo*) was transformed to chloramphenicol resistance with the two respective ligates. From each transformation, one clone was kept. Plasmids were isolated from the clones and designated pLUF07 (*katA*) and pLUF08 (*katA*-His_6), respectively.

To overproduce the native KatA and to express His_6-tagged KatA in *E. faecalis*, the genes encoding these proteins were cloned in the *E. coli-E. faecalis* shuttle plasmid pAM401. Plasmids pLUF07 and pLUF08 were cut with *Eco*RI and *Bam*HI, and the catalase-encoding fragments were inserted into pAM401, which was cut with the same enzymes. The resulting plasmids, pLUF14 (*katA*) and pLUF15 (*katA*-His_6), were first cloned in *B. subtilis* strain BLF03 and then introduced into *E. faecalis* strain V583 by electroporation.

For construction of a plasmid allowing constitutive expression of *katA*, the promoter of the kanamycin resistance gene (*aphA-3*) was amplified by PCR with *Taq* polymerase and primers MB3 (5′-TGCGGATCCATTTGAGGTGATAGG TAAGA-3′) and MB4 (5′-TTCAGATGCATTTATTATTTCCTTCCTC-3′) with plasmid pDG780 as the template. The use of these primers introduced *Bam*HI and *Nsi*I sites, respectively, in the PCR product. The PCR product was cloned into pBluescript KSII(−) cut with *Bam*HI and *Pst*I. The obtained ligate was used to transform *E. coli* SURE. One resulting plasmid with the desired structure was named pLUMB2. Primers MB5 (5′-GAGGGAATTCGTGAAAA ATCAACATTTAACTACGTC-3′) and MB6b (5′-ATTATGTCGACTTAGTG GTGGTGGTGGTGGTGTCC-3′) were used in PCR with *Pwo* polymerase (Roche Biochemicals) to amplify the gene encoding the His_6-tagged KatA with pLUF15 as the template. The primers included an *Eco*RI and a *Sal*I site, respectively. The PCR product was ligated into *Eco*RI- and *Sal*I-cut plasmid pLUMB2. *E. coli* SURE was transformed with this ligate. From the transformation, one clone was chosen and named pLUMB3. This plasmid was cut with *Bam*HI and *Sal*I, and the fragment, containing the *aphA-3* promoter and the gene encoding the His_6-tagged catalase, was inserted into pAM401 cut with the same enzymes. *E. coli* SURE was transformed with the ligate. A resulting plasmid designated pLUMB5 was isolated and introduced into *E. faecalis* strain V583 by electroporation.

Analysis of hydrogen peroxide resistance. Survival of *B. subtilis* strains after exposure to H_2O_2 was determined as follows. An overnight culture was diluted 100-fold in LB and incubated at 37°C on a rotary shaker for 3 h. The culture was then divided and put into two flasks. H_2O_2 was added to one flask to a final concentration of 8.8 mM, and the flasks were incubated as before for another 15 min. Samples were taken from both flasks and immediately diluted and plated on tryptose blood agar base plates. The degree of survival was determined by comparing the number of CFU in the hydrogen peroxide-treated culture to that in the untreated culture.

For *E. faecalis*, survival after hydrogen peroxide exposure was determined as for *B. subtilis*, except that the growth medium was TSBG and the culture was treated with different concentrations of H_2O_2.

Isolation of cytoplasmic fractions and KatA purification. To obtain cytoplasmic fractions from *B. subtilis* cells grown in LB, cultures were harvested when they were entering stationary phase, suspended in 50 mM potassium phosphate buffer, pH 8.0, and incubated with 1 mg of lysozyme (Sigma) per ml at 37°C for 40 min. The cytoplasmic cell fraction was obtained by centrifugation of the resulting lysate at 48,000 × *g* for 60 min at 4°C. Cytoplasmic fractions from *E. faecalis* were prepared from cells grown to early stationary phase unless otherwise stated. For full-scale preparations, cells from a 1-liter culture were washed and suspended in 10 ml of 20 mM MOPS (pH 7.4) and broken in a French pressure cell at 16,000 lb/in^2. For small-scale preparations, cells from a 40-ml culture were washed, suspended in 20 mM MOPS (pH 7.4), and lysed with a mini-BeadBeater machine (Biospec products, Bartlesville, Okla.). A 1.5-ml cell suspension was mixed with 1 ml of 0.1-mm-diameter zirconia-silica beads and shaken in the mini-BeadBeater at 5,000 rpm for two periods of 90 s separated by a 2-min pause on ice. The cytoplasmic fraction was separated from cell debris and membranes by centrifugation at 200,000 × *g* for 50 min (for cells broken in the French press) or at 48,000 × *g* for 1.5 h (for cells broken with beads). Protein

contents were determined by using the bicinchoninic acid assay (Pierce) with bovine serum albumin as the standard.

The His_6-tagged KatA was purified on a HiTrap chelating column (Pharmacia Biotech) loaded with Ni^{2+} ions in accordance with the manufacturer's instructions. Adsorption of the His_6-tagged catalase to the matrix was done in 50 mM potassium phosphate buffer, pH 8.0, supplemented with 300 mM NaCl and 1 mM imidazole. Elution of the catalase was achieved by raising the imidazole content of the buffer to 250 mM. The purified catalase was stored in the elution buffer at −80°C.

Biochemical analysis. Catalase activity was assayed by spectrophotometry and calculated by using the extinction coefficient for hydrogen peroxide (ε_{240} = 0.0436 μmol·cm·ml^{-1}). The decrease in absorbance at 240 nm was monitored upon addition of 1 to 10 μl of sample to a cuvette containing 3 ml of 0.06% H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0. The change of absorption during the first 10 s of the reaction was used to calculate activities. One unit is the amount of enzyme catalyzing the decomposition of 1 μmol of hydrogen peroxide per min under these test conditions.

The pyridine hemochromogen method was used to determine the heme content of the catalase (5). High-pressure liquid chromatography analysis was performed on heme extracted from purified catalase by using a Deltapak C_{18} column (Waters) and an acetonitrile gradient in 0.05% trifluoroacetic acid, as described by Sone and Fujiwara (24), with commercially available hemin (Sigma) used as the standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the Neville buffer system (13). MultiMarker (Novex) was used as a molecular mass reference. Gels were stained for protein with Coomassie brilliant blue R250.

Immunological techniques. His_6-tagged catalase purified from *B. subtilis* BLF03/pLUF08 was used for the production of rabbit polyclonal antibodies. For immunoblotting, the proteins were electrotransferred from an SDS-PAGE gel to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% (wt/vol) skim milk, reacted with a 2,000-fold dilution of rabbit anti-KatA antiserum, and developed by using horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) and the SuperSignal reagent (Pierce Chemical Co.).

Rocket immunoelectrophoresis was performed in 1% (wt/vol) low-electroendosmosis agarose (SeaKem) gel with a 12 mM sodium barbiturate buffer, pH 8.6. One percent rabbit KatA antiserum was added to the gel. Samples were loaded in a final volume of 10 μl, and the electrophoresis was run overnight at 75 V on a water-cooled (+4°C) bed. The relative content of antigen in the samples was determined from the areas of the immunoprecipitation rockets.

RESULTS

Identification of the *E. faecalis katA* gene. Structure and sequence data are available for several monofunctional catalases (6, 18, 22). Most of these enzymes are composed of four identical protein subunits of an approximate molecular mass of 60 kDa, each containing one heme group. The *B. subtilis* KatA sequence was used to search a prerelease of the *E. faecalis* V583 genome sequence made available by The Institute for Genomic Research. One gene, encoding a protein with 67% sequence identity to *B. subtilis* KatA, was found and designated *katA*. The coding region of *E. faecalis katA* is preceded by a putative promoter sequence and followed by an inverted repeat likely to function as a transcription terminator loop (Fig. 1A). The *katA* gene is flanked by an open reading frame (currently named EF1596) encoding a putative protein of unknown function and by *phrB*, which encodes deoxyribodipyrimidine photolyase. *E. faecalis* KatA contains both a domain that matches the Prosite entry for the catalase proximal active site (PS00438) and a domain that matches entry PS00437 for the proximal heme ligand.

Expression of *katA* in *B. subtilis*. The *E. faecalis katA* gene and a modified variant of the gene encoding a His_6-tagged version of KatA (*katA*-His_6) were obtained by PCR and cloned in plasmid pHPSK. The catalase genes in the two constructed

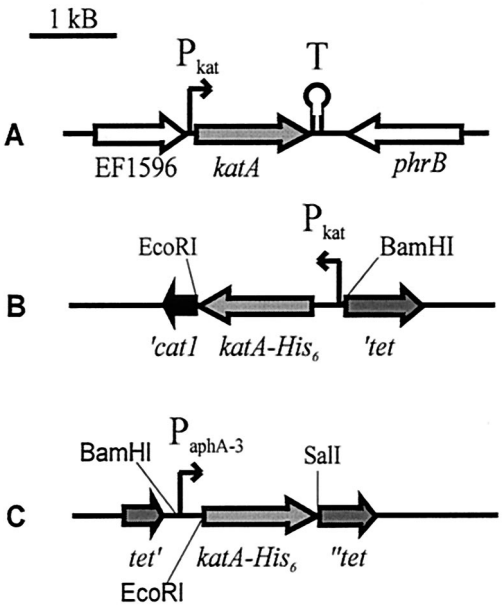

FIG. 1. Schematic presentation of the *katA* region in the *E. faecalis* V583 chromosome (A), pLUF15 (B), and pLUMB5 (C). P_{kat}, native promoter of *katA*; P_{aphA-3}, promoter of the kanamycin resistance gene (*aphA-3*); *katA*-His_6, recombinant gene encoding hexahistidyl-tagged KatA.

plasmids, pLUF07 and pLUF08, are under the control of the native *katA* promoter. These two plasmids were introduced into the *B. subtilis katA*-deficient strain BLF03. Liquid cultures of the parental strain 168A (*katA*$^+$) and BLF03 carrying pHPSK, pLUF07, or pLUF08 were exposed to 8.8 mM hydrogen peroxide for 15 min. This caused extensive killing of strain BLF03/pHPSK (<0.05% survival), whereas strains 168A, BLF03/pLUF07, and BLF03/pLUF08 had a survival level of 76 to 82% relative to that of untreated cells. This showed that the *E. faecalis katA* gene encodes an enzyme that can functionally complement a *B. subtilis* strain deficient in its vegetative catalase. Furthermore, the results showed that the presence of a His_6 tag at the C-terminal end of KatA does not interfere with the function of the protein.

Purification and characterization of KatA. The His_6-tagged KatA was purified from *B. subtilis* strain BLF03/pLUF08 by using metal affinity chromatography. The cytoplasmic cell fraction, from which the catalase was purified, showed an activity of 0.41 U/μg of protein whereas the activity of the purified catalase was 82 U/μg of protein, reflecting a 195-fold purification.

SDS-PAGE of the purified catalase preparation and staining for protein with Coomassie brilliant blue showed one polypeptide with an apparent subunit molecular mass close to the expected one of 54 kDa (Fig. 2, lane 2). The light absorption spectrum of the purified catalase was very similar to that of heme-containing catalases reported in the literature (spectrum not shown). High-pressure liquid chromatography analysis of heme extracted from the catalase showed the same retention time as that of protoheme IX (heme B). A stoichiometry of 1 ± 0.1 heme B group per 54-kDa polypeptide was found, based on the pyridine hemochromogen assay results. The combined

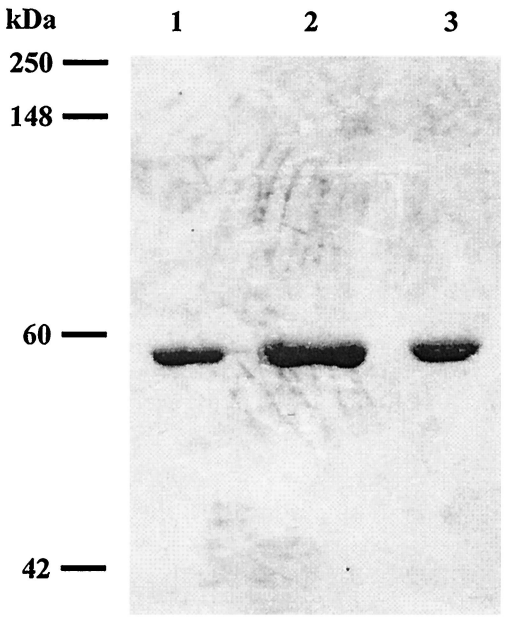

FIG. 2. SDS–10% PAGE of purified catalases. Lane 1, 1 μg of bovine liver catalase (Sigma Chemical Co.); lane 2, 2 μg of *E. faecalis* KatA-His_6 isolated from *B. subtilis* BLF03/pLUF08; lane 3, 1 μg of *E. faecalis* KatA-His_6 isolated from *E. faecalis* V583/pLUF15.

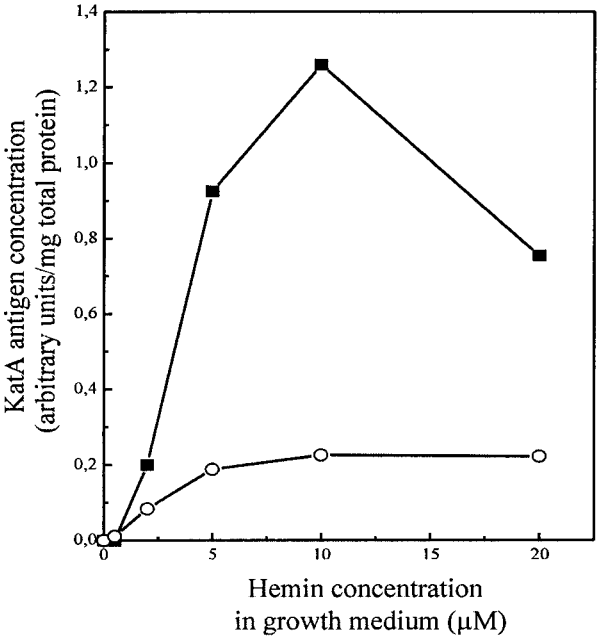

FIG. 3. Concentrations of KatA protein in the cytoplasmic cell fractions of *E. faecalis* strains V583/pLUMB5 (plasmid with the *katA*-His_6 gene under the control of the kanamycin promoter P_{aphA-3}) (○) and V583/pLUF15 (plasmid with the *katA*-His_6 gene under the control of the native promoter P_{kat}) (■) as a function of the concentration of hemin added to the growth medium (TSBG). KatA protein levels were determined by rocket immunoelectrophoresis. Three independent experiments were done; data from one of them are shown and are representative.

results show that the *E. faecalis katA* gene encodes a catalase belonging to the family of monofunctional catalases.

The isolated enzyme was used for production of rabbit polyclonal antibodies. The resulting antibodies recognize both wild-type *E. faecalis* catalase and the His_6-tagged variant of the enzyme. No cross-reaction could be seen with *B. subtilis* 168A catalase or with bovine liver catalase, as determined by rocket immunoelectrophoresis.

Active catalase is formed only when heme is present in the growth medium. TSBG medium contains less than 0.05 μM heme (see Materials and Methods). *E. faecalis* strains grown in TSBG lacked detectable catalase activity, but such activity was found in the cytoplasmic cell fraction from cells grown in the presence of 10 μM hemin (Table 2). The catalase activity found in strain V583 was relatively low. To increase the activity, and also to confirm that the heme-dependent production of catalase is due to KatA, the *katA*-His_6 gene was cloned in the multicopy plasmid pAM401, resulting in pLUF15. *E. faecalis* V583/pLUF15 contained high catalase activity when it was grown in the presence of hemin (Table 2).

TABLE 2. Catalase activity in cytoplasmic cell fractions isolated from different *E. faecalis* strains grown in TSBG in the absence and presence of 10 μM hemin

E. faecalis strain	Catalase activity (U/μg of protein)[a]	
	− hemin	+ hemin
V583/pAM401	<0.005	0.01 – 0.06
V583/pLUF15	<0.005	0.83 – 2.20
V583/pLUMB5	<0.005	0.13 – 0.45

[a] Numbers show spans of activity obtained in several independent experiments.

The His-tagged catalase produced in V583/pLUF15 grown in the presence of 10 μM hemin was purified. The isolated enzyme contained approximately 1 mol of heme B per mol of KatA polypeptide and was of the same apparent size as the corresponding protein isolated from *B. subtilis* BLF03/pLUF08 (Fig. 2, lane 3).

The transcription of genes encoding catalases is generally induced by oxidative-stress conditions. The expression of the *katA* gene in *E. faecalis* might therefore be induced by hemin in the growth medium. The purpose of this study was to analyze the requirement of heme for the assembly of active catalase and not the effects of heme on the transcription of the *katA* gene. Therefore, we constructed pLUMB5, a pAM401 derivative that contains the *E. faecalis katA*-His_6 gene under the control of the *aphA-3* promoter (Fig. 1C). The *aphA-3* promoter is unrelated to catalase and has been shown to be constitutive in *E. faecalis* (14). The production of KatA protein and catalase activity were also in *E. faecalis* V583/pLUMB5 dependent on hemin in the growth medium. Maximal production of catalase was obtained at a concentration of about 10 μM hemin in the medium (Fig. 3). Essentially the same hemin concentration-dependent production of the enzyme was obtained with V583/pLUF15, but the yield of catalase activity and KatA antigen obtained was approximately fivefold higher than that obtained with V583/pLUMB5 (Table 2 and Fig. 3). The production of catalase in the different strains was not dependent on the growth phase (data not shown).

By immunoblot analysis, we found a trace amount of KatA

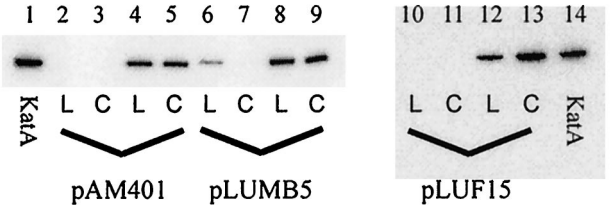

FIG. 4. Immunoblot of crude cell lysates (L) and cytoplasmic fractions (C) from *E. faecalis* strain V583/pAM401 (plasmid vector), V583/pLUMB5 (plasmid with the *katA*-His_6 gene under the control of the kanamycin promoter P_{aphA-3}), and V583/pLUF15 (plasmid with the *katA*-His_6 gene under the control of the native promoter region). The strains were grown in TSBG with (lanes 4, 5, 8, 9, 12, and 13) or without (lanes 2, 3, 6, 7, 10, and 11). 10 μM hemin added to the medium. Purified KatA-His_6 (0.25 ng) was added to lanes 1 and 14. Lanes 2 to 13 contained 2.5 μg of protein of cell lysate or the cytoplasmic cell fraction.

polypeptide but no catalase activity in fresh crude lysates of *E. faecalis* V583/pLUMB5 grown in TSBG without hemin added (Fig. 4, lane 6). KatA antigen was, however, not detected in the cytoplasmic fraction (Fig. 4, lane 7), i.e., in the supernatant obtained after high-speed centrifugation of the lysate, indicating that the KatA polypeptide is unstable in the absence of heme. Extracts of V583/pAM401 and V583/pLUF15 contained KatA antigen only when the cells had been grown in TSBG supplemented with hemin (Fig. 4).

Overproduction of catalase provides extensive protection against H_2O_2. *E. faecalis* V583 cells carrying pAM401 or pLUF15 and grown in TSBG with and without 10 μM hemin added were tested for resistance to hydrogen peroxide. In hemin-deficient medium, both strains were equally sensitive to hydrogen peroxide (Fig. 5). The presence of hemin in the growth medium augmented the survival of strain V583/pAM401 and abolished the extensive killing of strain V583/pLUF15, even at 100 mM hydrogen peroxide. Thus, overproduction of KatA mediates a heme-dependent protection of *E. faecalis* cells against very high concentrations of peroxide.

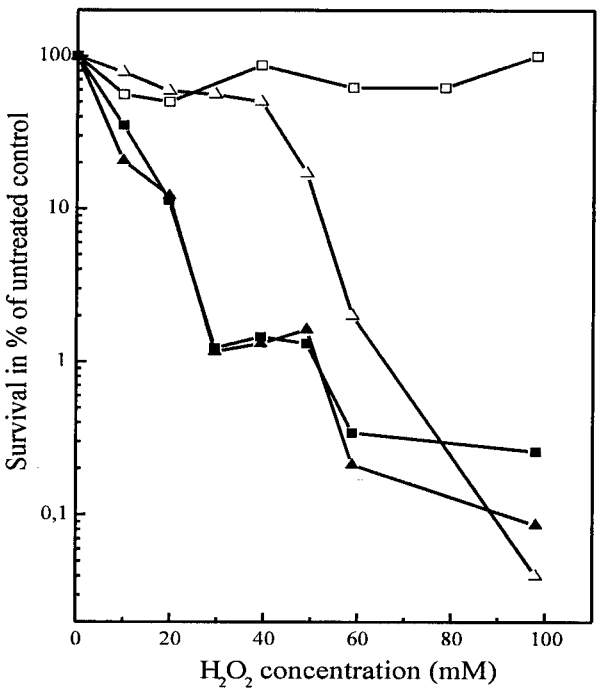

FIG. 5. Survival of *E. faecalis* V583/pAM401 (plasmid vector) (triangles) and V583/pLUF15 (plasmid with the *katA*-His_6 gene under the control of the native promoter region) (squares) following exposure to different concentrations of hydrogen peroxide for 15 min. The strains were grown in TSBG without (solid symbols) or with (open symbols) 10 μM hemin added.

DISCUSSION

Our results show that, if supplied with heme, *E. faecalis* cells can synthesize a water-soluble heme B-containing catalase. The enzyme is very similar to other monofunctional catalases. Three-dimensional crystal structures of similar bacterial catalases, e.g., *E. coli* HPII, show that the heme group is deeply buried within the protein subunits. In *E. faecalis* strain V583/pLUF15, which contains the *katA* gene under the control of the native promoter, no KatA protein could be detected in crude extracts or in cytoplasm from cells grown in the absence of heme. This indicates that no KatA or little KatA is produced under these conditions or that the protein is rapidly degraded. Crude extracts from strain V583/pLUMB5, which constitutively expresses *katA*, contained KatA also when it was grown in heme-deficient medium, but the protein, being apoprotein, appeared unstable, probably because the KatA polypeptide does not fold properly without its heme cofactor.

The physiological role of catalase is to remove the hydrogen peroxide that may form under oxic conditions and which is toxic to cells. The *E. faecalis katA* gene is expressed when the cells are grown in brain heart infusion broth under aerobic conditions (21). The normal habitat of *E. faecalis*, the gut, is mainly an anoxic environment. However, the bacterium encounters oxic environments, for example, during colonization of live tissues. Under these conditions, *E. faecalis* may produce large amounts of superoxide and hydrogen peroxide. Several enzymes present in *E. faecalis* are known to produce hydrogen peroxide, e.g., hydrogen peroxide-forming NADH oxidase, superoxide dismutase, and pyruvate oxidase. It is also possible that the *E. faecalis* respiratory chain, which is active in the presence of oxygen and heme, produces hydrogen peroxide. Production of hydrogen peroxide has been suggested to be an important factor of virulence and bacterial competition.

E. faecalis cells can also encounter oxidative stress mediated by exogenous superoxide and hydrogen peroxide produced by host defense cells, e.g., neutrophils. Conceivably, both bacterial hydrogen peroxide production and the expression of catalase are virulence factors. Catalase production, as well as the ability to use externally supplied heme, has been implicated in virulence in several pathogenic microorganisms, e.g., *Staphylococcus aureus* (3), *Helicobacter pylori* (12), and *Candida albicans* (29). Possibly, heme acquisition and catalase production by *E. faecalis* can be similarly incriminated as virulence determinants.

Heme is an essential molecule to most bacteria that use it as a prosthetic group in proteins. It is therefore difficult to study biosynthetic processes involving heme in these organisms. This problem can be circumvented by the use of *E. faecalis* as an

experimental system. The bacterium contains at least two hemoproteins, namely, cytochrome *bd*, in the cytoplasmic membrane, and the catalase in the cytoplasm. These hemoproteins can be exploited as reporter systems to monitor the uptake and intracellular transport of heme and the assembly of heme-containing proteins. Furthermore, *E. faecalis* cells offer a potentially powerful system for in vivo production of artificial hemoproteins of possible biotechnological importance. In contrast to most cells, *E. faecalis* and some other gram-positive bacteria are not sensitive to noniron metalloporphyrins (26). This property allows the growth of *E. faecalis* in the presence of heme analogs and allows synthesis, for example, of catalase containing such analogs (our unpublished data).

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REFERENCES

1. **Bryan-Jones, D. G., and R. Whittenbury.** 1969. Haematin-dependent oxidative phosphorylation in *Streptococcus faecalis*. J. Gen. Microbiol. **58:**247–260.
2. **Casillas-Martinez, L., and P. Setlow.** 1997. Alkyl hydroperoxide reductase, catalase, MrgA, and superoxide dismutase are not involved in resistance of *Bacillus subtilis* spores to heat or oxidizing agents. J. Bacteriol. **179:**7420–7425.
3. **Clements, M. O., and S. J. Foster.** 1999. Stress resistance in *Staphyllococcus aureus*. Trends Microbiol. **7:**458–462.
4. **Evers, S., D. F. Sahm, and P. Courvalin.** 1993. The vanB gene of vancomycin-resistant *Enterococcus faecalis* V583 is structurally related to genes encoding D-Ala:D-Ala ligases and glycopeptide-resistance proteins VanA and VanC. Gene **124:**143–144.
5. **Falk, J. E.** 1964. Porphyrins and metalloporphyrins, vol. 2, p. 181–241. Elsevier, Amsterdam, The Netherlands.
6. **Gouet, P., H. M. Jouve, and O. Dideberg.** 1995. Crystal structure of *Proteus mirabilis* PR catalase with and without bound NADPH. J. Mol. Biol. **249:**933–954.
7. **Guerout-Fleury, A.-M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
8. **Hansson, M., and L. Hederstedt.** 1994. Purification and characterisation of a water-soluble ferrochelatase from *Bacillus subtilis*. Eur. J. Biochem. **220:**201–208.
9. **Hoch, J. A.** 1991. Genetic analysis in *Bacillus subtilis*. Methods Enzymol. **204:**305–320.
10. **Huycke, M. M., D. F. Sahm, and M. S. Gilmore.** 1998. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. Emerg. Infect. Dis. **4:**239–249.
11. **Johansson, P., and L. Hederstedt.** 1999. Organization of genes for tetrapyrrole biosynthesis in gram-positive bacteria. Microbiology **145:**529–538.
12. **McGee, D. J., and H. L. Mobley.** 1999. Mechanisms of *Helicobacter pylori* infection: bacterial factors. Curr. Top. Microbiol. Immunol. **241:**155–180.
13. **Neville, D. M., Jr., and H. Glossmann.** 1971. Plasma membrane protein subunit composition. A comparative study by discontinuous electrophoresis in sodium dodecyl sulfate. J. Biol. Chem. **246:**6335–6338.
14. **Poyart, C., and P. Trieu-Cuot.** 1997. A broad-host-range mobilizable shuttle vector for the construction of transcriptional fusions to β-galactosidase in Gram-positive bacteria. FEMS Microbiol. Lett. **156:**193–198.
15. **Pritchard, G. G., and J. W. Wimpenny.** 1978. Cytochrome formation, oxygen-induced proton extrusion and respiratory activity in *Streptococcus faecalis* var. zymogenes grown in the presence of haematin. J. Gen. Microbiol. **104:**15–22.
16. **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.
17. **Pugh, S. Y., and C. J. Knowles.** 1983. Synthesis of catalase by "*Streptococcus faecalis* subsp. *zymogenes*." Arch. Microbiol. **136:**60–63.
18. **Putnam, C. D., A. S. Arvai, Y. Bourne, and J. A. Tainer.** 2000. Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism. J. Mol. Biol. **296:**295–309.
19. **Ritchey, T. W., and H. W. Seeley.** 1974. Cytochromes in *Streptococcus faecalis* var. *zymogenes* grown in a haematin-containing medium. J. Gen. Microbiol. **85:**220–228.
20. **Sambrook, J., and D. W. Russel.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Shepard, B. D., and M. S. Gilmore.** 1999. Identification of aerobically and anaerobically induced genes in *Enterococcus faecalis* by random arbitrarily primed PCR. Appl. Environ. Microbiol. **65:**1470–1476.
22. **Shima, S., A. Netrusov, M. Sordel, M. Wicke, G. C. Hartmann, and R. K. Thauer.** 1999. Purification, characterization, and primary structure of a monofunctional catalase from *Methanosarcina barkeri*. Arch. Microbiol. **171:**317–323.
23. **Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.).** 1986. Bergey's manual of systematic bacteriology, vol. 2. Williams and Wilkins, Baltimore, Md.
24. **Sone, N., and Y. Fujiwara.** 1991. Effects of aeration during growth of *Bacillus stearothermophilus* on proton pumping activity and change of terminal oxidases. J. Biochem. (Tokyo) **110:**1016–1021.
25. **Steinmetz, M., and R. Richter.** 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through in vivo recombination. Gene **142:**79–83.
26. **Stojiljkovic, I., V. Kumar, and N. Srinivasan.** 1999. Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. Mol. Microbiol. **31:**429–442.
27. **Winstedt, L., L. Frankenberg, L. Hederstedt, and C. von Wachenfeldt.** 2000. *Enterococcus faecalis* V583 contains a cytochrome *bd*-type respiratory oxidase. J. Bacteriol. **182:**3863–3866.
28. **Wirth, R., F. Y. An, and D. B. Clewell.** 1986. Highly efficient protoplast transformation system for *Streptococcus faecalis* and a new *Escherichia coli-S. faecalis* shuttle vector. J. Bacteriol. **165:**831–836.
29. **Wysong, D. R., L. Christin, A. M. Sugar, P. W. Robbins, and R. D. Diamond.** 1998. Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. Infect. Immun. **66:**1953–1961.
30. **Zamocky, M., and F. Koller.** 1999. Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis. Prog. Biophys. Mol. Biol. **72:**19–66.