A Novel *Enterococcus faecalis* Heme Transport Regulator (FhtR) Senses Host Heme To Control Its Intracellular Homeostasis

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**ABSTRACT** *Enterococcus faecalis* is a commensal Gram-positive pathogen found in the intestines of mammals and is also a leading cause of severe infections occurring mainly among antibiotic-treated dysbiotic hospitalized patients. Like most intestinal bacteria, *E. faecalis* does not synthesize heme (in this report, heme refers to iron protoporphyrin IX regardless of the iron redox state). Nevertheless, environmental heme can improve *E. faecalis* fitness by activating respiration metabolism and a catalase that limits hydrogen peroxide stress. Since free heme also generates toxicity, its intracellular levels need to be strictly controlled. Here, we describe a unique transcriptional regulator, FhtR (named FhtR for *faecalis* heme transport regulator), which manages heme homeostasis by controlling an HrtBA-like efflux pump (named HrtBAf for the HrtBA from *E. faecalis*). We show that FhtR, by managing intracellular heme concentration, regulates the functional expression of the heme-dependent catalase A (KatA), thus participating in heme detoxification. The biochemical features of FhtR binding to DNA, and its interaction with heme that induces efflux, are characterized. The FhtR-HrtBAf system is shown to be relevant in a mouse intestinal model. We further show that FhtR senses heme from blood and hemoglobin but also from cross-feeding by *Escherichia coli*. These findings bring to light the central role of heme sensing by FhtR in response to heme fluctuations within the gastrointestinal tract, which allow this pathogen to limit heme toxicity while ensuring expression of an oxidative defense system.

**IMPORTANCE** *Enterococcus faecalis*, a normal and harmless colonizer of the human intestinal flora can cause severe infectious diseases in immunocompromised patients, particularly those that have been heavily treated with antibiotics. Therefore, it is important to understand the factors that promote its resistance and its virulence. *E. faecalis*, which cannot synthesize heme, an essential but toxic metabolite, needs to scavenge this molecule from the host to respire and fight stress generated by oxidants. Here, we report a new mechanism used by *E. faecalis* to sense heme and trigger the synthesis of a heme efflux pump that balances the amount of heme inside the bacteria. We show in a mouse model that *E. faecalis* uses this mechanisms within the gastrointestinal tract.

**KEYWORDS** *Enterococcus faecalis*, heme homeostasis, heme transport, microbiota, stress adaptation, transcriptional regulation
as a leading cause of enterococcal infections, and it is the third most frequent source of hospital-acquired nosocomial infections (3). *E. faecalis* is thus considered a major public health threat due to its intrinsic resistance to antibiotics and the emergence of multidrug-resistant isolates (3). Selective outgrowth of enterococci following intestinal dysbiosis is frequent, regardless of whether it results from antibiotic treatment, intestinal inflammation, or infection (4). In addition to intrinsic and acquired antibiotic resistances, *E. faecalis* is resistant to other antimicrobial factors, such as bile, and tolerates a wide variety of stress factors such as temperature, pH, oxygen tension, or oxidation (1).

For most living organisms, heme (iron porphyrin) (in this report, heme refers to iron protoporphyrin IX regardless of the iron redox state, whereas hemin refers to ferric iron protoporphyrin IX) is an essential cofactor of enzymes such as cytochromes, catalases, or peroxidases (5). The importance of heme resides in the unique properties of its iron center, including the capacity to undergo electron transfer, to perform acid-base reactions and to interact with various coordinating ligands (6). Paradoxically, the high potential redox of heme iron catalyzes the production of reactive oxygen species (ROS). Oxidative stress generated by heme together with its accumulation in membranes explains its toxicity (7–9). Most bacteria carry the enzymatic machinery for endogenous heme synthesis. However, numerous bacteria lack some or all the enzymes needed for autosynthesis but still require this molecule for their metabolism (5). Interestingly, *E. faecalis*, like the majority of species constituting the gastrointestinal microbiota, cannot synthesize heme (10, 11). When heme is added to an aerated culture, *E. faecalis* activates a terminal cytochrome *bd* oxidase, causing a shift from fermentation to an energetically favorable respiratory metabolism (11, 12). *E. faecalis*, unlike other *Firmicutes* that cannot synthesize heme, also carries a gene that encodes a heme catalase (KatA; EC 1.11.1.6), limiting hydrogen peroxide stress when heme is available (10). Both activities contribute to the virulence of several Gram-positive pathogens (13, 14). Although the importance of heme as a cofactor for numerous cellular functions is established (5, 15), the mechanisms governing exogenous heme internalization and secretion that contribute to heme homeostasis vary among bacteria and are poorly understood. However, heme homeostasis must be strictly regulated in all bacteria to avoid toxicity (6, 8). Heme efflux is a documented defense mechanism against heme toxicity in some *Firmicutes*. (i) The Pef regulon comprises two multidrug resistance efflux pumps and a MarR-type heme-responsive regulator in *Streptococcus agalactiae* (16). (ii) The HatRT system involves a TetR family heme binding transcriptional regulator (HatR) and a major facilitator superfamily heme transporter (HatT) in *Clostridium difficile* (17). (iii) Heme homeostasis in several Gram-positive bacteria relies on HrtBA (heme-regulated transport) proteins, an ABC transporter, which promotes heme efflux (14, 18–20). Expression of hrtBA is controlled by hssRS genes, encoding a two-component heme sensor and response regulator in numerous Gram-positive pathogens, including *S. agalactiae*, *Staphylococcus aureus*, and *Bacillus anthracis* (14, 19–22). In contrast, the food bacterium *Lactococcus lactis* regulates HrtBA expression via the TetR family heme sensor HrtR (19). To date, the mechanisms involved in *E. faecalis* management of environmental heme are unknown.

In this work, we describe the mechanism by which a novel *E. faecalis* TetR regulator, called FhtR (for *faecalis* heme transport regulator), induces expression of HrtBA<sub>Ef</sub> (named HrtBA<sub>Ef</sub> for the HrtBA from *E. faecalis*), a conserved heme efflux transporter. We show that FhtR binds intracellular heme, resulting in derepression and increased transcription of hrtBA<sub>Ef</sub>. Heme iron coordination specifies FhtR as a heme sensor, and a critical role for the tyrosine 132 was defined. Our results also establish this system as a master mechanism of control of intracellular heme availability as shown by its requirement for the expression of the heme-dependent *E. faecalis* KatA. Finally, the relevance of the FhtR system to *E. faecalis* is shown in a mouse intestine model, suggesting the importance of FhtR for *E. faecalis* adaptation in the GIT. Our conclusions lead to a new picture of heme homeostasis in *E. faecalis*. 
RESULTS

The conserved heme efflux transporter HrtBA<sub>er</sub> is functional in <i>E. faecalis</i>. <i>E. faecalis</i> OG1RF genome encodes two adjacent open reading frames (ORFs), OG1RF_RS02770 and OG1RF_RS02775 sharing, respectively 24% and 45% amino acid (AA) sequence identity with HrtB and HrtA from <i>Staphylococcus aureus</i> (18) (see Fig. S1A and S1B in the supplemental material). We thus verified the role of these ORFs, referred to as HrtB<sub>er</sub> and HrtA<sub>er</sub> in heme efflux. Growth of an in-frame ΔhrtBA<sub>er</sub> deletion mutant was severely impaired at hemin concentrations ≥ 25 μM compared to the wild-type (WT) OG1RF strain (Fig. 1A). However, WT OG1RF could overcome up to 500 μM hemin, highlighting the involvement of HrtBA<sub>er</sub> in limiting heme toxicity in <i>E. faecalis</i>.

**FIG 1** HrtBA<sub>er</sub> controls and responds to heme intracellular concentration. (A) Deletion of hrtBA<sub>er</sub> increases sensitivity to hemin toxicity. Overnight cultures of WT and ΔhrtBA<sub>er</sub> strains were diluted to an OD<sub>600</sub> of 0.01 and grown with the indicated concentrations of hemin (in micromolar) for 10 h at 37°C in a microplate Spark spectrophotometer (Tecan). OD<sub>600</sub> was measured every 20 min. Values are the means ± standard deviations (error bars) from three biological replicates. (B) Heme accumulates in the ΔhrtBA<sub>er</sub> strain. WT and ΔhrtBA<sub>er</sub> strains were grown to an OD<sub>600</sub> of 0.5 prior to the addition of 5 μM hemin in the culture medium for an additional 1.5 h. Bacteria were pelleted by centrifugation, and heme content was determined by the pyridine hemochrome assay on cell lysates. Heme content was normalized to the protein concentration. Background from bacteria not incubated with hemin was subtracted. Results represent the means plus standard deviations (error bars) from three biological replicates. Statistical significance was determined by t test where **** = P < 0.0001. (C) Visualization of cellular heme accumulation in the ΔhrtBA<sub>er</sub> mutant. Cells, grown as described above for panel A, were incubated for 1.5 h with 5 μM hemin. The bacteria were photographed following centrifugation. The results are representative of three independent experiments. (D) HrtBA<sub>er</sub> reduces heme cytoplasmic concentration. WT and ΔhrtBA<sub>er</sub> strains carrying the intracellular sensor plasmid, pPhrt-hrtR-lac were grown after 1.5 h of incubation with 5 μM hemin. Results represent the means plus standard deviations from three biological replicates. Statistical significance was determined by t test where **** = P < 0.0001. (E) HrtBA<sub>er</sub> prevents hemin-induced oxidative stress. WT and ΔhrtBA<sub>er</sub> strains were grown as described above for panel B. β-Gal activity was quantified by luminescence in relative light units (RLU) after 1.5 h of incubation with 5 μM hemin. Results represent the means plus standard deviations from three biological replicates. Fluorescence background from bacteria not incubated with hemin was subtracted. Statistical significance was determined by t test where **** = P < 0.0001. (F) Induction of hrtBA<sub>er</sub> operon by hemin. The WT strain transformed with the reporter plasmid pPhrt-hrtR-lac was grown, and β-gal activity was determined as described above for panel D following incubation with the indicated concentrations of hemin. Results represent the means ± standard deviations from three biological replicates. Statistical significance was determined by one-way analysis of variance (ANOVA) with Dunnett’s multiple-comparison test comparing each concentration of hemin to no-hemin control with statistical significance indicated as follows: *, P = 0.0202; ****, P < 0.0001.
(Fig. S1C). The $\Delta frbA_{Ef}$ mutant grown in 5 $\mu$M heme-containing medium accumulated about twofold more intracellular heme than the WT strain, as evaluated by the pyridine hemochrome assay (23) (Fig. 1B). This result correlated with the intense red color of culture pellets from the $\Delta frbA_{Ef}$ mutant compared to the WT strain (Fig. 1C). Intracellular heme concentrations were also monitored using the intracytoplasmic heme sensor HrtR (19); $\beta$-galactosidase ($\beta$-gal) activity from the reporter plasmid $P_{hrtR}$-lac was about 4 times higher in $\Delta frbA_{Ef}$ compared to the WT exposed to 5 $\mu$M heme (Fig. 1D). Finally, accumulation of heme in the $\Delta frbA_{Ef}$ mutant correlated with a more than twofold increase of cellular ROS generated by heme as shown by the fluorescence of dihydrorhodamine 123 (24) (Fig. 1E). In conclusion, E. faecalis expresses a functional HrtBA$_{E}$ heme efflux transporter that modulates intracellular heme levels, thus reducing oxidative stress.

Transcriptional regulation of $fhtBA_{Ef}$ by heme was then investigated using a $fhtBA_{Ef}$ promoter reporter, $P_{fhtBA}$-lac. $\beta$-Gal expression in the WT strain was induced as a function of concentration between 0.1 and 2.5 $\mu$M hemin in the culture medium. Induction reached a maximum at concentrations below 5 $\mu$M (Fig. 1F). This concentration range is far below WT strain sensitivity to heme toxicity ($\geq 25$ $\mu$M) (Fig. 1A). We conclude that HrtBA$_{E}$ expression is induced at subtoxic heme concentrations.

**A new TetR regulator, FhtR, controls $fhtBA_{Ef}$ expression.** The above findings prompted us to investigate the mechanism of $fhtBA_{Ef}$ induction. Several Gram-positive pathogens regulate $fhtBA_{Ef}$ via an adjacent two-component system HssR and HssS (14, 20, 21). No hssR hssS genes were identified in or near the $fhtBA_{Ef}$ operon in E. faecalis OG1RF or other E. faecalis genomes. However, a monocistronic gene encoding a TetR family transcriptional regulator, OG1RF_RS02765, is adjacent to $fhtBA_{Ef}$ (Fig. 2A), sharing no significant AA identity with the hrtBA regulator, HrtR, in Lactococcus lactis (19). We hypothesized that OG1RF_RS02765 was the transcriptional regulator of $fhtBA_{Ef}$ and tentatively named it FhtR (for faecalis heme transport regulator) (Fig. 2A).

To investigate the role of FhtR in heme-dependent transcription of $fhtBA_{Ef}$, an fhtR in-frame deletion in strain OG1RF ($\Delta fhtR$) was constructed and transformed either with p$P_{fhtBA}$-lac or p$fhtr$ encompassing both ($P_{fhtBA}$-lac and $P_{fhtR}$-fhtR) expression cassettes (Fig. 2B). In contrast to the WT strain, $\beta$-gal was expressed in $\Delta fhtR$ mutant grown in 5 $\mu$M heme (Fig. 2B). Transformation of p$fhtr$ with $\Delta fhtR$ led to overcomplementation compared to the WT(p$P_{fhtBA}$-lac) strain (Fig. 2B). Moreover, on solid medium, $\Delta fhtR$ exhibited a complete absence of sensitivity to heme compared to the WT and complemented $\Delta fhtR$(p$fhtr$) strains (Fig. 2C). Similar results were obtained in liquid culture (Fig. S2). These results are in line with the observation that heme accumulation is reduced in the $\Delta fhtR$ mutant strain compared to the WT or $\Delta fhtR$(p$fhtr$) strain (Fig. 2D) and that HrtBA$_{Ef}$ may be constitutively expressed in the $\Delta fhtR$ mutant (Fig. 2B). Indeed, $P_{mut}$ was constitutively transcribed, with no effects of heme, nor of FhtR expression as shown using pmut-lac as the reporter (Fig. 2E), and by Western blotting (WB) using FhtR-hemagglutinin (HA) tagged fusion expressed from P$_{fhtR}$ (Fig. 2F). We conclude that E. faecalis uses a constitutively expressed, unique intracellular heme sensor, FhtR, to control $fhtBA_{Ef}$ expression.

**FhtR is a heme binding protein.** Members of the TetR family of transcriptional regulators act as chemical sensors (25, 26). Ligand binding alleviates TetR protein interactions with their respective operators, leading to promoter induction (25, 26). To verify that heme was the signal that relieves FhtR-mediated $fhtBA_{Ef}$ repression, recombinant FhtR was purified as a fusion to the maltose binding protein (MBP-FhtR) from Escherichia coli. MBP-FhtR appeared green (Fig. 3A, inset), and its UV-visible spectrum exhibited a strong Soret band, suggesting that FhtR scavenges endogenously produced heme (Fig. 3A). To purify an apoFhtR, MBP-FhtR was expressed from a heme synthesis-deficient E. coli strain (hemA::kan) (Fig. 3B, dashed line). The purified protein bound hemin $b$ in vitro (i.e., noncovalently) with a similar UV-visible spectrum as observed above for in vivo-bound heme: a Soret band at 407 nm and Q bands at 528 nm and 608 nm (Fig. 3B, holoFhtR). Size-exclusion chromatography profiles showed that both apo- and holo-MBP-FhtR eluted as a single peak corresponding...
FIG 2 FhtR controls hrtBAEf expression. (A) Schematic representation of the fhtR gene and hrtBAEf operon. The fhtR gene (OG1RF_RS02765) encodes a TetR family transcriptional regulator. The hrtBEf (OG1RF_RS02770) and hrtAEf (OG1RF_RS02775) genes encode a permease and ATPase, respectively. (B) FhtR controls hrtBAEf expression. WT and ΔfhtR strains carrying the reporter plasmid pPhrtBA-lac and a ΔfhtR strain carrying a plasmid, pfhtR, combining both P_{min}-lac and P_{ntr}-fhtR were grown to an OD_{600} of 0.5, and β-gal expression was quantified by luminescence as reported in the legend to Fig. 1 with the indicated concentrations of hemin. Results represent the means plus standard deviations (error bars) from three biological replicates. Statistical significance was determined by t test as follows: ns, not significant (P > 0.5); ****, P < 0.0001. (C) fhtR deletion abrogates heme toxicity. Stationary-phase cultures of WT, ΔfhtR, and ΔfhtR (p{fhtR}) strains were plated on solid medium. Hemin (10 μM, stock solution) was pipetted directly onto plates, which were incubated for 24 h. Inhibition zones appear as a circular clearing in the center of each panel. No inhibition zone was observed for the ΔfhtR strain. The results are representative of three independent experiments. (D) Visualization of the impact of FhtR on heme accumulation. WT, ΔfhtR, and ΔfhtR (pfhtR) strains were grown and incubated with 5 μM hemin as described above for panel A. The bacteria were pelleted by centrifugation and photographed. The results are representative of three independent experiments. (E) FhtR expression is constitutively induced. β-Gal expression upon hemin addition to the culture medium in WT and ΔfhtR strains transformed with the pP_{ntr}-lac reporter plasmid was determined by luminescence as described in the legend to Fig. 1. Results represent the means plus standard deviations from three biological replicates. (F) fhtR transcription is not mediated by hemin. The ΔfhtR strain transformed with pP_{ntr}-fhtR-HA or carrying an empty vector (control) was used to monitor FhtR expression by Western blotting (WB) using an antheremagglutinin (anti-HA) antibody (α-HA). Bacteria were grown to an OD_{600} of 0.5 and incubated with 2.5 μM hemin for 1.5 h. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on cell lysates (80 μg per lane). The results are representative of three independent experiments.
to the size of a dimer (132 kDa), in line with other TetR regulators (Fig. 3C) (25). The 608-nm charge transfer band and Soret at 407 nm are indicative of a ferric high-spin tyrosinate-ligated heme where heme is anchored through a proximal tyrosinate side chain (27, 28). Hemin pentacoordinate high-spin ligation to FhtR was further confirmed by electron paramagnetic resonance (EPR) spectroscopy (see below). The heme dissociation coefficient \(K_d\) was 310 nM as determined by MBP-FhtR fluorescence quenching over increasing concentrations of hemin (Fig. 3D). Heme titration by differential absorption spectroscopy at 407 nm showed that the saturation point corresponded to the binding of one molecule of hemin per MBP-FhtR monomer (Fig. 3D, inset). Altogether, these data demonstrate that FhtR is a heme binding protein, suggesting that heme interaction is the primary event leading to activation of hrtBAE transcription.

**Tyrosine 132 is a crucial heme axial ligand in FhtR.** According to UV-visible and EPR spectra, the likely candidate for axial ligand of oxidized heme is a tyrosine (Y) (see above). Several Y residues present in FhtR were substituted to phenylalanine (F) (Fig. S3A, in blue), F and Y both have phenyl ring structures, so that F substitution minimizes an impact on FhtR conformation. Although F lacks the hydroxyl group that...
coordinates heme, FhtR heme binding was not modified for several mutants tested individually (Fig. S3A). Only FhtRY132F was purified from E. coli (hemA::kan) strain as an apoprotein (dashed line) that was mixed with equimolar concentration of hemin (holoMBP-FhtR). Spectra were obtained as described for panel A with 20 μM complex and normalized to an OD280 of 1. (Inset) Magnification of the 500- to 700-nm region. Results are representative of three independent experiments. (C) EPR spectroscopy of MBP-FhtR and MBP-FhtRY132F in complex with hemin. EPR spectra of 60 μM bound hemin to WT (black line) and Y132 mutant (blue line) MBP-FhtR in 20 mM HEPES (pH 7) and 300 mM NaCl were recorded. (Inset) Magnification of the 900- to 1,500-gauss magnetic field range. The results are representative of three independent experiments. (D) Induction of the hrtBAEf operon by hemin. β-gal activity from the ΔfhtR strain transformed either with pPhrtBA-lac, pPhrt-fhtR or pPhrt-fhtRY132F was determined as described in the legend to Fig. 1C following incubation with the indicated concentrations of hemin. Results represent the means plus standard deviations from three biological replicates. Statistical significance was determined by one-way analysis of variance (ANOVA) with Dunnett’s multiple-comparison test comparing each concentration of hemin to pPhrtR (0 μM) control with statistical significance indicated as follows: ns, not significant (P > 0.5); ****, P < 0.0001.

We then compared FhtR and FhtRY132F activities in vivo. The ΔfhtR mutant was complemented either with pPhrt (pPhrBA-lac; PhrR-fhtR) or pPhrtY132F (pPhrBA-lac; PhrR-fhtR).
FhtR controls hrtBA<sub>Ef</sub> transcription by binding two distinct 14-nt palindromic repeat sequences. TetR family operators usually comprise a 10- to 30-nucleotide (nt) inverted repeat sequence with internal palindromic symmetry (25). Two such 14-nt-long palindromes were identified in the −10/−35 region of the hrtBA<sub>Ef</sub> promoter (called P1 and P2; Fig. 5A). An electrophoretic mobility shift assay (EMSA) was performed with apoMBP-FhtR, using a 325-bp DNA segment comprising the hrtBA<sub>Ef</sub> promoter (Fig. 5B) or a segment covering the internal hrb region as a control (Fig. 5A). FhtR-specific interaction with the P<sub>hrtBA</sub><sub>Ef</sub> DNA segment confirmed FhtR binding specificity. The shifted DNA migrated as two distinct bands (C1 and C2), in proportions that depended on the MBP-FhtR: DNA ratio (Fig. 5B), plausibly revealing that FhtR competed with P1 and P2 nucleotides (P1* and P2*) and analyzed DNA shifts by EMSA. Replacement of both distal and proximal operators (P<sub>hrtBA P1</sub>, P<sub>hrtBA P2</sub>) abolished the FhtR-induced DNA shift, confirming the role of palindromes in the interaction of FhtR with P<sub>hrtBA</sub><sub>Ef</sub> (Fig. 5C). Single replacement of P1 (P<sub>hrtBA P1</sub>) or P2 (P<sub>hrtBA P2</sub>) resulted in complete DNA shifts that migrated faster in the gel (C1) than seen with the native nucleotide sequence (Fig. 5C). We conclude that both P1 and P2 are FhtR binding sites.

We then tested the effects of heme on FhtR binding by EMSA. Addition of hemin to MBP-FhtR abolished the formation of the DNA-FhtR complex, as seen by the progressive disappearance of band shifts with increasing hemin concentrations (Fig. 5D). Complete release of FhtR from P<sub>hrtBA</sub><sub>Ef</sub> was obtained when hemin was in 10-fold molar excess over FhtR (Fig. 5D). Both C1 and C2 complexes were revealed when intermediate amounts of heme were added (0.1 and 1 μM; Fig. 5D). This suggests the release of MBP-FhtR from only one operator depending on the saturation level of FhtR with heme.

The role of the two operators in the control of hrtBA<sub>Ef</sub> was investigated in vivo, using P<sup>Δ</sup><sub>hrtBA</sub><sub>Ef</sub> or a P<sup>Δ</sup><sub>hrtBA P1</sub>, P<sub>hrtBA P2</sub> promoter variant to control lac gene (Fig. 5E). In contrast to P<sup>Δ</sup><sub>hrtBA</sub><sub>Ef</sub>-lac, which was strongly induced with 1 μM hemin, P<sup>Δ</sup><sub>hrtBA P1</sub>, P<sub>hrtBA P2</sub>-lac was constitutively expressed (Fig. 5E). Finally, the role of each operator was investigated (Fig. 5F). In the absence of heme, either P1 or P2 is sufficient for full P<sub>hrtBA</sub><sub>Ef</sub> repression by FhtR. Release of the promoter was facilitated in the presence of only P1 or P2 as shown with increased transcriptional activities of P<sup>Δ</sup><sub>hrtBA P1</sub> or P<sup>Δ</sup><sub>hrtBA P2</sub> in the presence of hemin compared to native P<sub>hrtBA</sub><sub>Ef</sub> (Fig. 5F). We propose that the presence of two operators provides strong repression of the hrtBA<sub>Ef</sub> promoter, thus preventing transcriptional leakage and allowing for fine tuning of HrtBA<sub>Ef</sub> expression. Taken together, these results demonstrate that FhtR is a heme sensor that directly controls heme homeostasis by regulating hrtBA<sub>Ef</sub> transcription.

FhtR controls HrtBA<sub>Ef</sub> the gatekeeper of intracellular heme availability. Our observation that FhtR regulates intracellular heme pools even at low heme concentrations led us to hypothesize that FhtR controls intracellular heme availability in <i>E. faecalis</i>. We tested this possible role of FhtR on the <i>E. faecalis</i> endogenous heme-dependent catalase (KatA). While katA transcription is not susceptible to heme induction, KatA protein stability relies on the presence of heme (10, 12). KatA-mediated H<sub>2</sub>O<sub>2</sub> catalysis was measured in WT, ΔfhtR, and ΔfhtR(pfhtR) strains (Fig. 6A). In the absence of heme, H<sub>2</sub>O<sub>2</sub> consumption was at a basal level (Fig. 5A), thus excluding major contributions of other enzymes in our conditions. In the presence of 1 μM hemin (Fig. 6A), the ΔfhtR mutant exhibited about 30% catalase activity compared to WT and complemented
ΔfhtR(pfhtR) strains, as evaluated by the percentage of catabolized H$_2$O$_2$ (Fig. 6A). This was further confirmed by comparing the amounts of KatA (holoKatA) by WB, using anti-KatA antibody (kindly provided by L. Hederstedt). In the absence of hemin, KatA was expressed at low levels in WT, ΔfhtR, and ΔfhtR(pfhtR) strains (Fig. 6B). Comparatively, addition of hemin strongly increased the amounts of KatA in WT and complemented ΔfhtR(pfhtR) strains, but not in the ΔfhtR mutant (Fig. 6B). Low KatA availability in the ΔfhtR mutant is readily explained by constitutive heme efflux (via HrtBA$_{pfht}$), and consequently depleted intracellular heme pools in this mutant. We then evaluated the survival capacity of *E. faecalis* OG1RF WT, ΔfhtR, and ΔfhtR(pfhtR) strains when challenged with 2.5 mM H$_2$O$_2$. In the absence of hemin, all strains grew equivalently without H$_2$O$_2$ (Fig. S5B). In contrast, while hemin addition rescued the survival of both the WT and ΔfhtR(pfhtR) strains, the ΔfhtR strain remained hypersensitive to H$_2$O$_2$. 

**FIG 5** FhtR controls *hrtBA$_{pfht}$* transcription via binding to two repeated 14-nt palindromic sequences. (A) Two 14-nt palindromic motifs are present upstream of *hrtBA$_{pfht}$*. The two palindromes are shown in blue (P1) and in red (P2); the ribosome binding sequence (RBS) is underlined, and the start codon is shown in green. (B) FhtR binds to the promoter region of *hrtBA$_{pfht}$*. EMSA shows binding of FhtR to P$_{hrtBA}$$_{pfht}$. The *hrtBA$_{pfht}$* promoter fragment (0.25 pmol) was incubated with increasing amounts of MBP-FhtR as indicated in molar ratios. DNA shift was visualized with GelRed (Biotium) following PAGE. The two shifted DNA-protein complexes (C1 and C2) and noncomplexed DNA (NC) are indicated. The results are representative of at least three independent experiments. (C) Roles of P1 and P2 on FhtR binding to the promoter region of *hrtBA$_{pfht}$*. EMSA was performed as described for panel B with either the native P$_{hrtBA}$ DNA fragment (WT) (as in panel A) or mutated fragments P$_{hrtBA}$P1*, P$_{hrtBA}$P2*, P$_{hrtBA}$P1P2*, and P$_{hrtBA}$P1P2*. MBP-FhtR: DNA (2.5 pmol: 0.25 pmol). The results are representative of at least three independent experiments. (D) Effect of hemin on the binding of FhtR to the *hrtBA$_{pfht}$* promoter. The *hrtBA$_{pfht}$* promoter DNA (0.25 pmol) was incubated with 2.5 pmol of MBP-FhtR together with increasing amounts of hemin as indicated (molar ratios) and analyzed by EMSA as described in the legend to panel B. The results are representative of at least three independent experiments. (E) Substitution of the two palindromic nucleotide sequences, P1 and P2, in P$_{hrtBA}$ abrogates FhtR-mediated control of *hrtBA$_{pfht}$* transcription. The WT strain was transformed either with the reporter plasmid pP$_{hrtBA}$-lac or pP$_{hrtBA}$P1*,P2*-lac. β-Gal activity was determined as described in the legend to Fig. 1 following incubation with 2.5 μM hemin. Results represent the means plus standard deviations from three biological replicates. Statistical significance was determined by *t* test with statistical significance indicated as follows: ns, not significant (*P* > 0.5); ****, *P* < 0.0001. (F) Substitution of either P1 or P2 nucleotide sequences in P$_{hrtBA}$ enhances its transcriptional activation by hemin. The WT strain was transformed either with the reporter plasmid pP$_{hrtBA}$-lac, pP$_{hrtBA}$P1*-lac, or pP$_{hrtBA}$P2*-lac. β-Gal activity was determined as described in the legend to Fig. 1 following incubation with hemin. Results represent the means plus standard deviations from three biological replicates. Statistical significance was determined by one-way ANOVA with Tukey’s multiple-comparison test with significance indicated as follows: ns, not significant (*P* > 0.5); *, *P* = 0.0140; ****, *P* < 0.0001.
Deletion of \( hrtBAEf \) in the \( DfhtR \) strain (\( DfhtRDhrtBAEf \)) restored the survival capacity in the presence of hemin (Fig. S5C). Thus, poor survival of \( DfhtR \) reflects the lack of heme needed to stabilize KatA (Fig. 6D). Finally, the OG1RF mutant (\( katA::tetR \)) was hypersensitive to hemin toxicity, showing that KatA was required for controlling oxidative stress generated by heme (Fig. 1E and Fig. 4E). Taken together, these results identify FhtR as the direct and indirect regulator of HrtBA\( _{Ef} \)-mediated heme efflux and KatA activity, respectively, with both mechanisms lowering heme stress in \( E.\ faecalis \). FhtR is thus a key mediator of heme homeostasis, and consequently, of oxidative stress response in \( E.\ faecalis \) generated by \( H_2O_2 \).

Heme sensing in the gastrointestinal tract. \( E.\ faecalis \) is a normal resident of the GIT of vertebrates, an ecosystem where heme is available (29–32). We therefore investigated whether hrtBA\( _{Ef} \)-mediated heme management is required by \( E.\ faecalis \) in the GIT in a murine gastrointestinal model. We generated \( E.\ faecalis \) OG1RF strains expressing the \( luxABCDE \) (\( lux \)) operon from \( Photorhabdus\ luminescens \) driven by the following: (i) \( P_{hrtBA} \ (pPhrtBA_{lux}) \), which emits light specifically in the presence of hemin (Fig. 7A); (ii) a constitutive promoter P23 (\( plux \)), constitutively emitting light for bacterial tracking (14); or (iii) a control promoterless vector, \( pP_{1/lux} \). Cultures of these strains were

\( \text{FIG 6} \) FhtR controls intracellular utilization of heme. (A) \( fhtR \) deletion limits heme-dependent KatA activity. KatA enzymatic activity in \( E.\ faecalis \) was assessed on WT, \( \Delta fhtR \), and \( \Delta fhtR(pfhtR) \) grown overnight (ON) with 1 \( \mu M \) hemin. Catalase activity was determined on an equivalent number of bacteria incubated with 100 \( \mu M \) \( H_2O_2 \) for 1 h with the spectrophotometric FOX1 method based on ferrous oxidation in xylenol orange. Results are expressed as the percentage of \( H_2O_2 \) metabolized in respective strains grown without hemin. Results represent the means plus standard deviations from three biological replicates. Statistical significance was determined by one-way analysis of variance (ANOVA) with Dunnett’s multiple-comparison test comparing each strain to the WT strain control with significance indicated as follows: ns, \( P > 0.05; **, P = 0.008 \). (B) Expression of KatA is impaired in the \( \Delta fhtR \) mutant. Equivalent amounts of protein (20 \( \mu g \)) from lysates of WT, \( \Delta fhtR \), and \( \Delta fhtR(pfhtR) \) strains as described for panel A were separated by SDS-PAGE, and immunoblots were probed with an anti-KatA polyclonal antibody. The presented results are representative of three independent experiments. (C and D) The \( \Delta fhtR \) mutant is hypersensitive to \( H_2O_2 \). ON cultures of WT, \( \Delta fhtR \), and \( \Delta fhtR(pfhtR) \) strains were diluted to an \( OD_{600} \) of 0.01 and grown to an \( OD_{600} \) of 0.5 in the absence of hemin (C) or in the presence of 1 \( \mu M \) hemin (D). Cultures were distributed in wells on a 96-well plate and supplemented with 2.5 \( \text{mM} \) \( H_2O_2 \) or not supplemented with \( H_2O_2 \). \( OD_{600} \) was monitored every 20 min in a microplate spectrophotometer (Spark; Tecan). \( OD_{600} \) at time zero was normalized to 0. Results represent the means ± standard deviations from three biological replicates. (E) KatA limits heme toxicity. ON cultures of WT and \( katA::tetR \) strains were diluted to an \( OD_{600} \) of 0.01 and grown in 96-well plate in the presence of the indicated concentration of hemin as described for panel D. Results represent the means ± standard deviations from three biological replicates.
orally inoculated in the digestive tracts of mice, and light emission from whole live animals was measured in an in vivo imaging system IVIS200, 6 h postinoculation (Fig. 7B). This time delay corresponded to the maximum light emission from the tracking strain OG1RF(plux) (Fig. S6A). Luminescence signaling from the ingested *E. faecalis* pPhrtBA-lux sensor strain also localized in the abdomen, similar to the tracking strain (Fig. 7B). Examination of dissected organs revealed that the heme sensor-associated luminescence was mainly detected in the cecum (Fig. 7C), correlating with the high bacterial load of this organ [WT(plux); Fig. 7C]. A significant signal was also detected in the feces from inoculated animals, further highlighting that *E. faecalis* was able to scavenge and internalize heme within the digestive tract to induce hrtBA<sub>Ef</sub> expression (Fig. 7D). Finally, mice and human fecal samples (as well as fecal waters [Fig. S6B and S6C]) from healthy individuals were able to induce luminescence from WT(pPhrtBA-lux) in vitro, excluding the possibility that induction of PhrtBA <i>in vivo</i> could result from the inoculation procedure (Fig. 7E). Therefore,
FhtR heme sensor activity is active and relevant to _E. faecalis_ heme management in the lumen of the GIT.

**Heme sources for _E. faecalis_ in the GIT.** The results described above imply that _E. faecalis_ internalizes heme in the intestinal environment to activate FhtR. Thus, an interesting question remains as to the identities of heme sources that are accessible to _E. faecalis_ in the GIT. Normal bleeding (occult blood), exacerbated in intestinal pathologies, as well as food (as meat) are considered main sources of heme within the GIT (the second being excluded in mice) (29–32). We thus visualized the ability of hemoglobin (Hb) or blood deposited on plates as schematized (Fig. 8A) to induce PhrtBA from the heme sensor strain WT(pPhrtBA-lux) as shown with hemin (Fig. 8B). Similarly, luminescence was induced in proximity of Hb and blood deposits as heme sources (Fig. 8C and D). This result suggests that heme from physiologically available sources is internalized by _E. faecalis_. Crossfeeding of metabolites, including heme between bacteria, has been reported (29, 33). The possibility that _E. faecalis_ could scavenge heme from intestinal resident heme-synthesizing bacteria, such as _E. coli_—a phylum that becomes prevalent together with _E. faecalis_ throughout dysbiosis—was evaluated. The WT (pPhrtBA-lux) heme sensor strain was grown in contact with _E. coli_ (as the heme source) as illustrated in Fig. 8A. Strikingly, induction of luminescence was localized to areas of
overlap between the two bacteria (Fig. 8E) and required heme synthesis by *E. coli*, as no sensing could be detected with a heme-defective *hemA::kan* mutant (Fig. 8F). This result suggests that heme synthesized by *E. coli* is internalized by *E. faecalis*. Thus, heme crossfeeding between bacterial symbionts in the gut might provide a heme source for *E. faecalis*. We conclude that the *E. faecalis* heme sensor is activated by the heme sources available in the GIT.

**DISCUSSION**

*E. faecalis* is a core member of the microbiome, and it is also the cause of a variety of severe infections (34). The central role of heme in reprogramming *E. faecalis* metabolism and fitness led us to investigate how heme homeostasis is controlled. A novel heme sensor, FhtR, is shown here to regulate heme intracellular homeostasis in *E. faecalis*. FhtR-heme complexes derepress the *hrtBA* operon, leading to HrtBA-mediated management of intracellular heme pools. While expression of HrtBA is a conserved strategy in multiple Gram-positive organisms, *E. faecalis* appears to be the first example of an opportunistic pathogen where HrtBA is not controlled by the two-component system HssRS. BLAST analysis of FhtR homologs in several Gram-positive bacteria showed that the regulator is present only in enterococci, vagococci, and carnobacteria (see Fig. S7A and S7B in the supplemental material for FhtR alignments and phylogenetic tree). FhtR shares no homology with HrtR, a TetR regulator of *hrtRBA* in *Lactococcus lactis* (19). In contrast to HrtR which autoregulates its own expression, *fhtR* is monocistronic and expressed constitutively, implying that only HrtBA expression is controlled by heme (19).

We characterized FhtR as a heme binding protein through pentacoordinated ligation of the heme ion, implying a tyrosine. This state of coordination is mostly found in heme receptors that transiently bind heme, such as IsdA, IsdC, and IsdH in *S. aureus* or HmA in *Escherichia coli* (35). FhtR blocks *hrtBA* transcription by binding to two distinct 14-nt inverted repeats sequences in its promoter region. Alleviation of repression occurs when the heme-FhtR complex loses its affinity for its DNA binding sites. Conformational changes upon ligand binding is a shared mechanism among TetR regulators, leading to uncoupling from DNA (26). We thus hypothesize that these events, which we verified *in vitro*, explain FhtR regulation of the *hrtBA* efflux pump in *E. faecalis*. The unique features of FhtR in *E. faecalis* compared to other regulators of *hrtBA* genes encoding efflux pumps support the idea that control of HrtBA-mediated heme homeostasis may vary among bacteria as a function of their lifestyle. It is thus tempting to speculate that differences in host niches, and in heme utilization and metabolism, might explain disparities in bacterial heme sensing mechanisms.

Heme efflux by HrtBA is reported as a bacterial detoxification mechanism that prevents intracellular heme overload (8, 14, 16, 17, 19). We showed here that HrtBA induction is required for *E. faecalis* survival when heme concentrations reached toxic levels (>25 μM). Yet, *hrtBA* was induced at heme concentrations as low as 0.1 μM, suggesting that heme efflux is also needed at nontoxic levels. Interestingly, *E. faecalis* carries a gene that encodes the heme-dependent catalase whose activity relies on the amount of heme in the cytoplasm that is indirectly regulated by FhtR. This enzyme not only binds heme and thus lowers free heme levels, but it also actively lowers oxidative stress generated by heme. It will be of interest to determine the hierarchy of heme binding between FhtR and catalase *in vivo*.

To date, no heme import function has been identified in *E. faecalis* or in other tested Gram-positive bacteria that cannot synthesize heme (13, 18). BLAST analysis of these bacteria failed to identify genes of the *isd* heme import system described in *Staphylococcus aureus* (36, 37). In *S. aureus*, heme receptors and transporters are induced in iron-depleted growth media, and imported heme is used as an iron source (36). Thus, our findings led us to question the need for a dedicated transport system to internalize exogenous heme in *E. faecalis* and to propose an alternative hypothesis. We noted that HrtBA is a member of the MacB family of efflux pumps that is distinct from other structurally characterized ABC transporters (38). A model based on MacB
transport of antibiotics and antimicrobial peptides in *Streptococcus pneumoniae* proposed that transmembrane conformational changes promote lateral entry of substrates in the membrane before they reach the cytoplasm (39). On the basis of the previous and present data (23), we propose that HrtB, has the integral role as the heme “gatekeeper” in the cell. Like MacB and antimicrobial substrates (40), membrane-bound heme could either enter passively into the intracellular compartment and be effluxed by HrtB before this step. Altogether, our results place HrtBA at the forefront of heme homeostasis in *E. faecalis* that is dependent on the key role of FhtR to adapt to the dichotomy between toxicity and benefits of heme which may be crucial in the host.

In vivo bioluminescence imaging of *E. faecalis* using an FhtR-based sensor identified the GIT as an environment where HrtBA is expressed. The gut lumen of healthy individuals contains heme, independently of the nature of ingested food or of the microbiota (29–32). Heme in the GIT is reported to mainly originate from Hb from normal bleeding (occult blood) (41). Accordingly, *E. faecalis* was able to internalize heme from blood and Hb in vitro. In addition, a common microbiota constituent, *Escherichia coli*, is shown to be a heme donor, suggesting a novel basis for intestinal bacterial interactions. As several phyla composing the core microbiota are heme auxotrophs with vital heme requirements, it is tempting to hypothesize that normal or disease-associated phyla composing the core microbiota are heme auxotrophs with vital heme requirements. Interestingly, *E. faecalis* causes a variety of severe infections, most often among antibiotic-treated hospitalized patients with intestinal dysbiosis favoring high *E. coli* and enterobacterial populations (42). It will be interesting to evaluate the impact of HrtBA on heme homeostasis in *E. faecalis* fitness and virulence in in vivo models. Taken together, our results suggest that the FhtR sensor and the HrtBA heme gatekeeper allow *E. faecalis* to optimize its adaptation to variable heme pools in the host.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Bacterial strains and plasmids used in this work are listed in Table S1 in the supplemental material. *E. coli* NEB10 (New England Biolabs) was grown in LB medium, and *E. coli* C600 hemA-kan was grown in M17 medium supplemented with 0.5% glucose (M17G). Experiments with *E. faecalis* were all performed using strain OG1RF and derivatives (Table S1). *E. faecalis* was grown in static conditions at 37°C in M17G. When needed, antibiotics were used for *E. coli* at 50 μg·ml⁻¹ kanamycin and 100 μg·ml⁻¹ ampicillin; for *E. faecalis*, 30 μg·ml⁻¹ erythromycin was used. Oligonucleotides used for plasmid constructions are listed in Table S2. Hemin (Fe-PPIX) (Frontier Scientific) was prepared from a stock solution of 10 mM hemin chloride in 50 mM NaOH. In this report, heme refers to iron protoporphyrin IX regardless of the iron redox state, whereas hemin refers to ferric iron protoporphyrin IX. For growth homogeneity, WT and mutant strains were transformed with the promoterless pTCV-lac plasmid control to complemented strains. Plasmid construction and *E. faecalis* gene deletion are described in Text S1 in the supplemental material.

**β-Galactosidase assays.** Stationary-phase cultures were diluted at an optical density at 600 nm (OD₆₀₀) of 0.01 in M17G and grown to an OD₆₀₀ of 0.5. Hemin was added to cultures, which were further grown for 1.5 h. β-Galactosidase activity was quantified by luminescence in a Spark microplate luminometer (Tecan) using the β-gal substrate (Promega) as described (19).

**Cellular ROS quantification.** Stationary-phase cultures were diluted at an OD₆₀₀ of 0.01 in M17G and grown to an OD₆₀₀ of 0.5. Hemin was added to cultures, which were further grown for 1.5 h. Bacteria were washed twice with phosphate-buffered saline (PBS) plus 0.5% glucose by centrifugation at 4°C to remove extracellular heme. Cell pellets were resuspended in PBS plus 0.5% glucose supplemented with 25 μM dithiodr-hodamide 123, a cell-permeant fluorescent ROS indicator (Invitrogen). Cell suspensions were distributed into the wells of a 96-well plate. After 15-min incubation, optical density at 600 nm and fluorescence (excitation 500 nm; emission, 536 nm) were measured in a Spark microplate spectrophurometer (Tecan).

**Bacterial lysate preparation.** Bacteria were pelleted at 3,500 × g for 10 min, resuspended in 20 mM HEPES (pH 7.5) and 300 mM NaCl and disrupted with glass beads (Fastprep; MP Biomedicals). Cell debris was removed by centrifugation at 18,000 × g at 4°C for 15 min from the bacterial lysate supernatant. Proteins were quantified by the Lowry method (Bio-Rad).

**Heme concentration determination in bacterial lysates.** Equivalent amounts of proteins (in a volume of 250 μl) were mixed with 250 μl of 0.2 M NaOH, 40% (vol/vol) pyridine, and 500 μM potassium ferricyanide or 5 μl of 0.5 M sodium dithionite (diluted in 0.5 M NaOH), and 500- to 600-nm absorption spectra were recorded in a UV-visible spectrophotometer Libra S22 (Biochrom). Dithionite-reduced minus ferricyanide-oxidized spectra of pyridine hemochromes were used to determine the amount of heme b by monitoring the value of the difference between absorbance at 557 nm and 540 nm using a difference extinction coefficient of 23.98 mM⁻¹·cm⁻¹ (43).

**Recombinant MBP-FhtR purification.** MBP-FhtR and MBP-FhtR variants were purified by affinity chromatography on amylose resin as reported previously (19). Briefly, *E. coli* NEB10 or C600 ΔhemA strains
were grown to an OD₆₀₀ of 0.6 or 0.3, respectively, and expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) overnight (ON) at room temperature (RT). Cells were pelleted at 3,500 × g for 10 min, resuspended in 20 mM HEPES (pH 7.5) and 300 mM NaCl containing 1 mM EDTA (binding buffer), and disrupted with glass beads (Fastprep; MP Biomedicals). Cell debris was removed by centrifugation at 18,000 × g for 15 min at 4°C. MBP-tagged proteins were purified by amylose affinity chromatography (New England Biolabs) following the manufacturer’s recommendations: the soluble fraction was mixed with amylose resin and incubated on a spinning wheel at 4°C for 1 h. The resin was then centrifuged and washed three times with binding buffer. Purified proteins were eluted in binding buffer containing 10 mM maltose and dialyzed against 20 mM HEPES (pH 7.5) and 300 mM NaCl.

Heme-dependent catalase expression and activity. KataA expression was monitored on immunoblots with a polyclonal anti-Kata antibody (10). Catalase activity was determined on whole bacteria incubated with 100 μM H₂O₂, with the spectrophotometric FOX1 method based on ferrous oxidation in xylene-ol orange as described previously (44, 45). Absorption was measured at 560 nm.

Electrophoretic mobility shift assay. A 325-bp DNA fragment containing the hrtBA promoter (P₉₉₉₉) was amplified by PCR from E. faecalis OG1RF genomic DNA with primer pair (O21-O22) (Table S2). In P₉₉₉₉, the two 14-nt palindromic sequences P1 (5′-TTATCAATCGTAA-3′) and P2 (5′-TTATCGATGATAA-3′) were randomly altered to P1* (5′-ACTCTGGATCATATA-3′) and P2* (5′-ATACCGTATAAAG-3′) to generate three DNA variants P₉₉₉₉ P1* P2*, P₉₉₉₉ P1* P2*, and P₉₉₉₉ P1* P2*. These fragments were cloned into pUC plasmid (pUC-V5, pUC-V52, and pUC-V53; Table S1) (Proteogenix, France) that were used as templates to PCR amplify the promoter region DNA variants with the primer pair (O21-O22) (Table S2). All PCR reactions were cloned into pT7V (Table S1). EMSA (electrophoretic mobility shift assay) was performed in 20 mM Tris-HCl (pH 8), 50 mM KCl, 0.2 mM MgCl₂, 1 mM EDTA, 0.2 mM dithiothreitol (DTT), and 5% glycerol as reported previously (19). Binding was analyzed by gel electrophoresis on a 7% polyacrylamide gel in Tris-borate-EDTA (TBE) buffer stained with GelRed (Biotium) following electrophoresis.

Ethics statement. Animal experiments were conducted in strict accordance with the recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioural Research with Animals of the EEC council (Directive 2010/63/EU). The protocols were approved by the Animal Care and Use Committee at the Research center of Jouy-en-Josas (COMETHEA; protocol number 15–61) and by the Ministry of Education and Research (APAFIS 2277-2015081917023093 V4). All efforts were undertaken to minimize animal suffering. All experimental procedures were performed in biosafety level 2 facilities.

In vivo heme sensing assay in the mouse GIT. For inoculation in the digestive tract, E. faecalis strains were prepared as follows. E. faecalis OG1RF precultures were diluted and grown in M17G to an OD₆₀₀ of 0.5 that was determined to correspond to 6 × 10⁸ CFU/ml. Bacteria were then centrifuged at 6,000 rpm at 4°C for 15 min, and pellets were resuspended in PBS to a final concentration of 2 × 10⁹ cells/ml. Bacterial stocks were aliquoted and frozen in liquid nitrogen. Aliquots were kept at −80°C until use. Bacterial counts were confirmed by plating serial dilutions of cultures. Six-week-old female BALB/c mice (Janvier, France) were orally administered by gavage of 10⁸ CFU using a feeding tube. Image acquisition of in vivo luminescence imaging procedure is described in Text S1 in the supplemental material.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

TEXT S1, DOCX file, 0.03 MB.
FIG S1, TIF file, 2.4 MB.
FIG S2, TIF file, 2.2 MB.
FIG S3, TIF file, 2.3 MB.
FIG S4, TIF file, 1.7 MB.
FIG S5, TIF file, 2.9 MB.
FIG S6, TIF file, 2.7 MB.
FIG S7, TIF file, 2.8 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.01 MB.

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