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Dichotomous Metabolism of *Enterococcus faecalis* Induced by Hematin Starvation Modulates Colonic Gene Expression

Toby D. Allen^{1,4}, Danny R. Moore^{1,4}, Xingmin Wang^{1,4}, Viviana Casu^{1,4}, Randal May⁴, Megan R. Lerner³, Courtney Houchen⁴, Daniel J. Brackett^{2,3}, and Mark M. Huycke^{1,4,*}

¹The Muchmore Laboratories for Infectious Disease Research, Department of Veterans Affairs Medical Center, Oklahoma City, Oklahoma 73104

²The Muchmore Laboratories for Research Service, Department of Veterans Affairs Medical Center, Oklahoma City, Oklahoma 73104

³Department of Surgery, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, 73104

⁴Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, 73104

Summary

Enterococcus faecalis is an intestinal commensal that cannot synthesize porphyrins and only expresses a functional respiratory chain when provided exogenous hematin. In the absence of hematin, *E. faecalis* reverts to fermentative metabolism and produces extracellular superoxide that can damage epithelial cell DNA. The acute response of the colonic mucosa to hematin-starved *E. faecalis* was identified by gene array. *E. faecalis* was inoculated into murine colons using a surgical ligation model that preserved tissue architecture and homeostasis. The mucosa was exposed to hematin-starved *E. faecalis* and compared to a control consisting of the same strain grown with hematin. At 1 hour post-inoculation six mucosal genes were differentially regulated and this increased to 42 genes at 6 hours. At 6 hours a highly significant biological interaction network was identified with functions that included NF- κ B signaling, apoptosis, and cell cycle regulation. Colon biopsies showed no histological abnormalities by hematoxylin and eosin staining. Immunohistochemical staining, however, detected NF- κ B activation in tissue macrophages using antibodies to the nuclear localization sequence for p65 and the F4/80 marker for murine macrophages. Similarly, hematin-starved *E. faecalis* strongly activated NF- κ B in murine macrophages *in vitro*. Furthermore, primary and transformed colonic epithelial cells activated the G2/M checkpoint *in vitro* following exposure to hematin-starved *E. faecalis*. Modulation of this cell cycle checkpoint was due to extracellular superoxide produced as a result of the respiratory block in hematin-starved *E. faecalis*. These results demonstrate that the uniquely dichotomous metabolism of *E. faecalis* can significantly modulate gene expression in the colonic mucosa for pathways associated with inflammation, apoptosis, and cell cycle regulation.

*Author for Correspondence: Mark M. Huycke, Medical Service (111), Department of Veterans Affairs Medical Center, 921 N.E. 13th Street, Oklahoma City, Oklahoma 73104, USA. Tel: 405-270-0501 ext. 3250, Fax: 405-297-5948, E-mail: mark-huycke@ouhsc.edu.

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Introduction

For several decades the colonic microbiota has been postulated to play a role in the etiology of sporadic colorectal cancer (CRC) (Augenlicht *et al.*, 2002; McGarr *et al.*, 2005; Rowland, 1995). This hypothesis is based on the observation that intestinal cancers occur almost exclusively in the colon where metabolically active bacteria are in direct proximity to mucosal surfaces at densities of 10^{11} colony forming units (CFU) per gram of fecal material. Several epidemiological studies have attempted to identify associations between at-risk populations for CRC and colonic bacteria (Benno *et al.*, 1986; Moore & Moore, 1995). This approach has been hampered, however, by the enormous complexity of the colonic microbiota and inadequate understanding of what constitutes exposure risks for commensals (Eckburg *et al.*, 2005). Despite these limitations, a credible rationale remains for studying the role of colonic commensals in CRC carcinogenesis.

Perhaps the best evidence supporting a role for colonic commensals in CRC derives from genetically engineered mice that develop intestinal tumors when conventionally colonized but have fewer tumors or no pathology when housed in pathogen-free or germ-free environments (Balish & Warner, 2002; Chu *et al.*, 2004; Dove *et al.*, 1997; Engle *et al.*, 2002; Kado *et al.*, 2001; Kim *et al.*, 2005; Maggio-Price *et al.*, 2006). The mechanisms by which commensals trigger inflammation or initiate genomic instability (a characteristic feature of sporadic CRC) remain uncertain. Commensals can modulate the intestinal mucosa through metabolism of fecal steroids, by producing short chain fatty acids, and inducing host genes (Augenlicht *et al.*, 2002; Bäckhed *et al.*, 2005; Debruyne *et al.*, 2001; McGarr *et al.*, 2005). None of these effects, however, are known to initiate or promote genomic or epigenetic changes in epithelial cells as antecedents to oncogenic transformation. Commensals that cause epithelial cell DNA damage, in contrast, are more likely to initiate chromosomal instability than bacteria that modulate epithelial cell metabolism but are otherwise not mutagenic.

Enterococcus faecalis is a Gram-positive minority constituent of the colonic microbiota that can directly damage epithelial cell DNA (Huycke *et al.*, 2002), promote chromosomal instability through a macrophage-induced bystander effect (Wang & Huycke, 2007), and trigger colitis and cancer in interleukin (IL)-10 knockout mice (Balish & Warner, 2002; Kim *et al.*, 2005). The bystander effect refers to oxidatively stressed or irradiated cells that produce chromosomal instability in neighboring cells through the production of diffusible mutagens (Lorimore & Wright, 2003). These effects are derived in part from the redox-active phenotype of *E. faecalis* that occurs when this microorganism is grown without heme. *E. faecalis* cannot synthesize porphyrins and requires this nutrient to form functional cytochrome *bd*, establish a proton gradient, and respire (Bryan-Jones & Whittenbury, 1969; Ritchey & Seeley, 1974). In contrast, growth of *E. faecalis* in the absence of heme leads to a block in respiration and the production of superoxide, hydrogen peroxide, and hydroxyl radical (Huycke *et al.*, 2001; Huycke *et al.*, 2002). These reactive oxygen species are known to damage DNA (Marnett, 2000), although it remains to be determined whether this redox-active phenotype promotes inflammation and colon cancer in IL-10 knockout mice that are monoassociated with *E. faecalis*.

To further explore the effect of *E. faecalis* on the colonic mucosa, we developed an *in vivo* colonic ligation model to examine host gene expression in wild-type mice. This model preserves tissue architecture and homeostasis and permits an analysis of gene expression in stromal and epithelial cell compartments. We compared *E. faecalis* grown with heme to *E. faecalis* grown without heme to determine the effect of altered bacterial metabolism on colonic mucosal gene expression. We believe this comparison reflects the naturally occurring potential dichotomous metabolism of *E. faecalis* in the intestinal environment. We found the induction and suppression of a small subset of genes involved in cell cycle regulation,

apoptosis, and NF- κ B signaling by hematin-starved *E. faecalis*. These findings indicate that the colonic mucosa rapidly responds to alternate metabolisms of *E. faecalis* and suggest novel mechanisms by which this commensal may promote inflammatory or potentially transforming events.

Methods

Bacteria, growth conditions, and superoxide assay

E. faecalis strain OG1RF is a human oral isolate that produces extracellular superoxide ($\sim 20 \mu\text{moles min}^{-1} 10^9 \text{ CFU}^{-1} \text{ in vitro}$), promotes chromosomal instability, and produces colonic inflammation and cancer when monoassociated with IL-10 knockout mice (Balish & Warner, 2002; Huycke & Moore, 2002; Kim *et al.*, 2005; Wang & Huycke, 2007). *E. faecalis* was grown overnight in closed tubes using brain-heart infusion broth (BD Diagnostics, Sparks, MD) with or without 10 μM hematin (Sigma, St. Louis, MO) and washed in sterile PBS before use in experiments. *E. faecalis* cannot synthesize porphyrins and is unable to form functional cytochrome *bd* unless supplied exogenous hematin (Huycke, 2002). Cytochrome *bd* is one of two terminal quinol oxidases expressed by *E. faecalis* that, when active, allows oxidative phosphorylation, promotes growth, and suppresses extracellular superoxide (Huycke *et al.*, 2001). Growth with hematin attenuates superoxide production by >10 -fold, an effect that persists for >6 hours *in vitro*. For all experiments, hematin-replete growth of *E. faecalis* was confirmed by measuring the attenuation of superoxide using the ferricytochrome *c* assay as previously described (Huycke *et al.*, 1996). Unless otherwise specified all chemicals were of analytical or molecular biology grade from Sigma.

Murine colonic ligation model

To assess the short-term effects of hematin-starved *E. faecalis* on the intact colonic mucosa we developed an intestinal ligation model. The technique is analogous to the ileal loop model used to investigate diarrheal toxins. Conventionally housed and fed 25-28 gram adult male BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized using 1-2% isoflurane on a carrier gas composed of 95% O_2 and 5% CO_2 . Through a 5 mm midline abdominal incision the proximal colon was identified at its juncture to the cecum. Two ligatures 0.5 cm apart were placed around the colon and a 2 mm incision made into the colon. A gavage needle was inserted into the colon and contents completely flushed per rectum using sterile PBS. The rectum was closed with a purse string ligature and the colon backfilled with 1.0 ml of PBS alone or PBS containing enterococci at $1 \times 10^8 \text{ CFU ml}^{-1}$. Immediately preceding instillation, D-glucose was added to PBS or the bacterial inocula to a final concentration of 5 mM. This sugar initiates extracellular superoxide production by *E. faecalis*, but not for bacteria grown with hematin (Huycke *et al.*, 2001). Following inoculation both colonic ligatures were tied to prevent backflow of enterococci and peritoneal contamination from proximal intestinal contents. Care was taken to preserve blood flow to the colon. The surgical area was washed with sterile PBS, the colon gently returned to the peritoneal cavity, the abdominal incision closed, and mice allowed to recover.

At 1 or 6 hours post-inoculation mice were anesthetized, abdomens re-opened, and colons surgically removed. The surgical manipulations were well tolerated with only one mouse not surviving to the end of the protocol. Contents were cultured for enterococci as previously described (Huycke *et al.*, 1992). Five mm colon biopsies were obtained for histopathology and immunohistochemistry. Biopsies were examined using hematoxylin and eosin, and modified Brown and Brenn, stains. Remaining colon segments were longitudinally opened and mucosal surfaces scraped with sterile razors for RNA extraction. Biopsies were fixed in formalin and scrapings snap frozen in liquid nitrogen. Mice ($n = 20$) were exposed to *E. faecalis* or PBS ($n = 6$) with independent experiments analysed by group (1 and 6 hours post-inoculation) for

mice exposed to hematin-starved *E. faecalis* ($n = 5$ per group), *E. faecalis* grown with hematin ($n = 5$ per group), or PBS ($n = 3$ per group). The animal protocol was approved by the Animal Studies Subcommittee of the VA Research and Development Committee.

Gene expression, network response, and transcriptional regulatory element analyses

Total RNA was isolated from colonic scrapings for mice exposed to hematin-replete or hematin-starved *E. faecalis* at 1 and 6 hours post-inoculation using the Atlas pure total RNA labeling system (BD Biosciences Clontech, Mountain View, CA). Probes were synthesized by reverse transcription using α - ^{33}P -dATP. Since the quantities of mucosal scrapings from individual colons were small, each sample was used in its entirety for probe synthesis. Extracted and labeled cDNA probes were hybridized overnight to 5K cDNA murine arrays (BD Biosciences Clontech). Separate arrays ($n = 5$ per group for a total of 20 arrays) were used for each probe prepared from colon scrapings. After high stringency washes, membranes were quantified (Storm 820 PhosphorImager, Amersham Biosciences, Piscataway, NJ) and expression of individual genes determined as optical density readings minus background (Array Vision software, Imaging Research, St. Catherine's, Ontario, Canada). Significantly up-regulated and down-regulated genes were analyzed using GeneSpring software (version 6.2, Silicon Genetics, Redwood City, CA, USA). After background subtraction, raw signals were normalized per spot, and by array, using an intensity dependent Lowess protocol. Signal intensities were normalized to the 50th percentile and comparisons between array results at 1 and 6 hour time points made using the Student's *t*-test with $P < 0.005$ considered significant. Fold-changes were calculated using the GeneSpring fold-change filter option. The Benjamini and Hochberg method was used to correct for multiple testing and minimize false discovery rates.

Biologically relevant response networks for significantly modulated genes were constructed using Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The Ingenuity Pathways Knowledge Base is the largest curated database on mammalian biology in the published literature. Findings on genes in human, mouse, and rat studies from peer-reviewed publications are encoded into an ontology by content and modelling experts. Manual extraction and curation identifies specific interactions that result in fewer false-positives than automated methods. Networks are algorithmically generated based on their connectivity and pathways of highly interconnected genes identified by statistical likelihood testing (Calvano *et al.*, 2005).

In silico analysis of differentially expressed genes was performed for transcription factor binding sites using the web-based Promotor Analysis and Interaction Network Tool software (Vadigepalli *et al.*, 2003). Comparisons were done using all genes on the murine array as the reference library.

Immunohistochemistry

Immunohistochemical analysis for the p65 component of NF- κ B was performed on serial sections of paraffin-embedded murine colon tissue. Antigen retrieval of deparaffinized sections was performed using the Decloaking Chamber (Biocare Medical, Concord, CA) with citrate buffer, or 0.1% Pronase (DAKO, Carpinteria, CA), and processed using the Sequenza™ staining methodology (Thermo Scientific, Waltham, MA). Endogenous peroxidase activity was quenched using Peroxidase-Blocking Reagent (Dako, Carpinteria, CA) followed by a blocking step with buffer containing 1% BSA (Jackson ImmunoResearch, West Grove, PA), 1% normal horse serum (Jackson ImmunoResearch), cold water fish gelatin, and Tween-20. Sections were stained using nuclear localization sequence (NLS)-specific anti-p65 antibody (1:300; Rockland Immunochemicals, Gilbertsville, PA) or anti-F4/80 monoclonal antibody (1:150; AbD Serotec, Raleigh, NC). The former antibody recognizes the NLS on p65 that is masked by I κ B. The F4/80 antigen is a surface marker expressed by mature murine

macrophages (Austyn & Gordon, 1981). After primary incubation with NLS-specific anti-p65 antibody, sections were incubated in HRP labeled EnVision+™ (Dako). Sections stained with anti-F4/80 antibody were developed using anti-rat secondary antibody (1:1000; Jackson ImmunoResearch) followed by incubation with ready-to-use SA-HRP solution (Dako). Following incubations, sections were developed with 3,3-diaminobenzidine substrate or Bajoran Purple (Biocare Medical) and counterstained with hematoxylin (Biocare Medical). The distribution of positive cells per field (100 ×) between groups was assessed in a randomized and blinded fashion and compared using ridit analysis with $P < 0.05$ considered significant (Fleiss, 1981). This method assumes that discrete measures represent intervals in an underlying continuous distribution without any assumptions about the distribution. Ridits range from 0 to 1 and the ridit for the control (or comparator) distribution is 0.50. A mean ridit is > 0.50 when more than half the time randomly selected measures from the experimental distribution have a value greater than randomly selected measures from the control distribution.

Colon sections were processed for netrin-1 immunohistochemistry using UltraVision LP Detection System HRP Polymer & AEC Chromogen (LabVision, Fremont, CA). Sections were blocked with H₂O₂ for 10 minutes to inhibit endogenous peroxidase activity followed by washes in Tris buffered saline with Tween 20 at pH 8.0. Following antigen retrieval, Ultra V Block (Dako) was applied for 5 minutes followed by a 1:20 dilution of rabbit anti-netrin-1 (Ab-1) primary antibody or control peptide per the manufacturer's instructions (Calbiochem, San Diego, CA). Sections were counter-stained with Immuno* Master Hematoxylin (American Master*Tech Scientific, Lodi, CA).

Cell lines

Chromosomally stable HCT116 colonic epithelial cells (American Type Culture Collection, Manassas, VA) were grown in 5% CO₂ at 37 °C using McCoy's 5A medium modified by L-glutamine and 25 mM HEPES (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS). RAW264.7 murine macrophages (ATCC) were grown under the same conditions using Dulbecco's Modified Eagle Medium modified with 4.5 g l⁻¹ glucose and L-glutamine (Invitrogen), and supplemented with 10% FBS. For experiments involving co-incubation with *E. faecalis*, bacteria were diluted to 1×10^9 CFU ml⁻¹ in fresh medium without FBS. YAMC cells are a non-transformed intestinal epithelial cell line derived from healthy tissue and were a gift from the Ludwig Institute for Cancer Research (Whitehead *et al.*, 1993). These cells were grown in 5% CO₂ at 33°C using RPMI-1620 (Invitrogen) supplemented with 5% FBS, 5 U ml⁻¹ recombinant murine interferon- γ (Peprotech Inc., Rocky Hill, NJ), and ITS Premix according to the manufacturer's instructions. After treatment, cells were washed with PBS and complete medium added containing gentamicin (10 μ g ml⁻¹) and penicillin (100 units ml⁻¹) to kill remaining extracellular bacteria. For H₂O₂-treated cells catalase (1200 units ml⁻¹) was also included in complete medium to eliminate residual H₂O₂.

Immunofluorescent assay

To visualize NF- κ B activation, *E. faecalis* treated RAW264.7 cells and HCT116 cells grown on chambered slides were fixed with paraformaldehyde and incubated with polyclonal anti-p65 IgG (1:100; Santa Cruz Biotechnology). A fluorescein isothiocyanate (FITC)-conjugated IgG (1:200; Santa Cruz Biotechnology) was used as the secondary antibody and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI) prior to laser scanning confocal microscopy (Zeiss model LSM-510 META, Jena, Germany).

NF- κ B-luciferase reporter assay

To verify NF- κ B activation, RAW264.7 cells were transfected with the pNF κ B-Luc reporter vector (Clontech) using Lipofectin Reagent (Invitrogen). Transfected cells were treated with *E. faecalis* for 1 hour and further incubated for 24 hrs. Lipopolysaccharide treatment (10 μ g

ml⁻¹) served as a control. Cell lysates were prepared using Reporter Lysis Buffer according to the manufacturer's instructions (Promega, Madison, WI). Luciferase activity was measured using the Luciferase Assay System (Promega) and a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA). Values were normalized to protein concentration (Bio-Rad, Hercules, CA).

Cell cycle and apoptosis assays

The ability of *E. faecalis* to activate colonic epithelial cell checkpoints or initiate apoptosis was assessed by flow cytometry. Following a 1 hour treatment with *E. faecalis*, HCT116 or YAMC cells were incubated in fresh medium containing FBS, gentamicin, and penicillin. Cells were fixed overnight with 70% ethanol and stained with propidium iodide (0.02 mg ml⁻¹) containing 0.1% Triton X-100 and 0.2 mg ml⁻¹ RNase A. Stained cells were analyzed using a FACSCalibur flow cytometer (B-D Immunocytometry Systems, San Jose, CA). Apoptotic cells were stained using the Annexin V FITC Apoptosis Detection Kit according to the manufacturer's instructions (Calbiochem, EMD Biosciences Inc., Darmstadt, Germany) and quantified by flow cytometry. Data were analyzed using CellQuest Pro software. Statistical analyses were performed using ModFit 2.2 software (Verity Software House, Topsham, ME). For each sample, >10,000 events were collected and groups compared using the Student's *t*-test with *P* < 0.05 considered significant.

qRT-PCR and gene silencing

Total mRNA was isolated from *E. faecalis*-treated cells using the NucleoSpin RNA II Kit (BD Biosciences) and 2 µg reverse-transcribed with an iScript cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed using a Mx3005P Q-PCR System following the manufacturer's instructions (Stratagene, La Jolla, CA). Primers used to assess expression of genes identified in the array experiment (Table 1) were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). *Netrin1* was silenced by RNA interference using siGENOME SMARTpool Reagent specific for human *NTN1* or with siCONTROL Non-Targeting siRNA Pool (Dharmacon, Lafayette, CO). Transient transfections were performed using DharmaFECT 4 Transfection Reagent according to the manufacturer's protocol and gene silencing confirmed by qRT-PCR. Groups were compared using the Student's *t*-test with *P* < 0.05 considered significant.

Western blotting

Total protein was extracted from cells and equal amounts analyzed by SDS/PAGE before transfer onto polyvinylidene difluoride membranes (Amersham Biosciences). Assays for netrin-1 were performed using goat polyclonal antihuman netrin-1 antibody and alkaline phosphatase conjugated rabbit anti-goat IgG as a secondary antibody (Santa Cruz Biotechnology Inc.), and detected using the ECFTM Western Blotting Detection System (Amersham Biosciences) according to the manufacturer's instructions.

Results and Discussion

E. faecalis activates NF-κB in colonic macrophages

To assess short-term effects of hematin-starved *E. faecalis* on colonic gene expression, we developed an *in vivo* ligation model that preserved mucosal architecture and homeostasis. The average concentrations of *E. faecalis* recovered from luminal contents at 1 and 6 hours post-inoculation were approximately 10-fold lower than initial inocula but not significantly different for mice administered hematin-starved *E. faecalis* compared to hematin-repleted *E. faecalis*. No histological abnormalities were noted for colon biopsies at either time point for any group.

In addition, epithelial or submucosal cocci were not visible by tissue Gram stain indicating that an acute mucosal infection had not occurred (data not shown).

Since hematin-starved *E. faecalis* induces COX-2 expression in macrophages *in vitro* (Wang & Huycke, 2007), and COX-2 is regulated *via* NF- κ B (Karin & Greten, 2005), we initially determined whether *E. faecalis* activated this redox-sensitive signaling pathway in the colon. NF- κ B was detected using an NLS-specific anti-p65 antibody that detects p65 only after it dissociates from I κ B. p65 is a member of the canonical NF- κ B pathway and activated by many stimuli including redox stress and exposure to bacteria (Karin & Greten, 2005). We found significantly increased numbers of cells with p65 nuclear staining in colons exposed to *E. faecalis* at 6 hours compared to PBS controls (Ridit = 0.57, P = 0.008). Although an increase in NF- κ B activation was found for colons exposed to hematin-starved compared to hematin-repleted *E. faecalis*, this difference was not statistically significant (Ridit = 0.52, P = 0.25). To identify mucosal cells with NF- κ B activation, we stained serial colon sections with the NLS-specific anti-p65 antibody and the anti-F4/80 monoclonal antibody. Nearly all cells positive for p65 also stained positive for F4/80 indicating that this redox-sensitive signaling pathway had been activated in tissue macrophages (Fig. 1a and b). In contrast, no staining for the NLS of p65 was noted in epithelial cells.

To determine if *E. faecalis* activated NF- κ B *in vitro*, we exposed macrophage and epithelial cell lines to hematin-starved bacteria. Strong nuclear staining, along with cytoplasmic staining, was noted in macrophages by laser scanning confocal microscopy (Fig. 1c). NF- κ B activation was noted as early as 3 hours post-inoculation and persisted for >48 hrs. In comparison, HCT116 cells exposed to *E. faecalis* did not lead to nuclear localization of p65 (data not shown). To verify NF- κ B activation in macrophages, we transfected RAW264.7 cells with the pNF κ B-Luc reporter plasmid. Compared to control, there was a >25-fold increase in NF- κ B activation at 24 hours following exposure to hematin-starved *E. faecalis* (Fig. 1d). Furthermore, manganese superoxide dismutase (MnSOD) significantly reduced NF- κ B activation (P = 0.005) indicating that extracellular superoxide from hematin-starved bacteria contributed to this effect. The addition of catalase did not further decrease NF- κ B activation by hematin-starved *E. faecalis*, although H₂O₂ alone activated NF- κ B in these cells.

NF- κ B regulates genes involved in cellular proliferation, immunity, and apoptosis (Karin & Greten, 2005). Activation of NF- κ B requires the phosphorylation of I κ B by I κ B kinase. This results in I κ B degradation and release of NF- κ B homo- and heterodimers to translocate into the nucleus. NF- κ B promotes tumorigenesis by inhibiting apoptosis, dysregulating tumor-specific immune responses, and producing reactive oxygen species that can damage genomic DNA. Our findings indicate that hematin-starved *E. faecalis* activates NF- κ B, in part, by producing extracellular superoxide. Although reactive oxygen species (including superoxide) can activate NF- κ B, this effect is unpredictable and typically cell-dependent (Gloire *et al.*, 2006). Many studies use H₂O₂ as an oxidative stress, although superoxide should also be considered as it may lead to dissimilar effects. For example, superoxide is required for IL-1 dependent NF- κ B activation in chondrocytes (Mendes *et al.*, 2003), enhances lipopolysaccharide-dependent NF- κ B activation in macrophages (Khadaroo *et al.*, 2003), and initiates NF- κ B activation in neutrophils (Mitra & Abraham, 2006). In this study, MnSOD significantly decreased *E. faecalis*-dependent induction of NF- κ B in RAW264.7 cells confirming that this anionic radical can potentiate NF- κ B activation beyond that seen with H₂O₂ alone.

Colonic mucosal gene response to hematin-starved *E. faecalis*

To determine how broadly the hematin-starved physiology of *E. faecalis* modulated gene expression in the colonic mucosa, we compared mRNA from mice for >5000 genes following exposure to hematin-starved *E. faecalis* to mRNA following exposure to hematin-repleted *E.*

faecalis. At 1 hour post-inoculation, 6 colonic mucosal genes were differentially regulated (*Sod2*, *Sod3*, *Agrt1*, *Vav1*, *Car4*, and *Nme6*; $P < 0.01$ for each). At 6 hrs, 25 genes were significantly down-regulated and 17 genes up-regulated (Table 2). Of the differentially regulated genes at the 1 hour time point, only *Sod3* and *Car4* were still differentially regulated at 6 hrs. For the 42 colonic mucosal genes differentially expressed by hematin-starved *E. faecalis*, 9 (21%) were related to inflammatory or stress responses and 10 (24%) involved pathways for cell cycle control, signaling, and apoptosis. In addition, several were expressed by immune effector cells, suggesting that acute colonic mucosal responses to hematin-starved *E. faecalis* involves innate and/or adaptive immunity.

To identify potential biological interaction networks for these differentially regulated genes, we subjected genes at the 6 hour time point to Ingenuity Pathways Analysis. Only one highly significant mucosal response network was identified (Fig. 2, $P < 0.0001$). Functions within this network included cell cycle regulation, inositol phosphate metabolism, NF- κ B signaling (RelA or p65), ERK/MAPK signaling, chemokines, T cell receptors, integrins, and fibroblast growth factor. Exploration of potential regulatory responses within the network was performed using *in silico* transcriptional regulatory element analysis. One hundred eleven transcriptional regulatory elements were associated with the 42 differentially regulated genes. When we compared the frequency of these elements to elements for all genes on the murine array, there were only 5 significantly over-represented elements: 3 for NF- κ B (including the sequence for p65 binding), HEN1, and GATA-1. In addition, 66 (59%) of the 111 transcriptional regulatory elements were significantly under-represented. In aggregate, these findings indicate that hematin-starved *E. faecalis* acutely activates NF- κ B signaling in the colonic mucosa.

These *in silico* analyses identified a single response network with 7 up-regulated mucosal genes. p65 was the major transcription factor in this network and in biopsies NF- κ B activity localized to tissue macrophages. Mechanism by which *E. faecalis* contacts tissue macrophages was not investigated, but may involve translocation of enterococci through follicle-associated M cells in the colon (Kraehenbuhl & Neutra, 2000). These specialized epithelial cells facilitate uptake of luminal bacteria and coordinates their interaction with innate and adaptive immune effector cells. Enterococci readily translocate the intact intestinal epithelium and, in murine models, are often recovered from the liver, spleen, and mesenteric lymph nodes (Wells *et al.*, 1990). This phenomenon may derive, in part, from ineffective killing of *E. faecalis* by macrophages (Gentry-Weeks *et al.*, 1999). In the colonic ligation model the concentration of luminal bacteria at 6 hours was 10-fold lower than the original inoculum. Epithelial translocation is one possible explanation for a decrease in colony counts.

Several genes within the mucosal response network were associated with NF- κ B signaling including *C3ar1*, *Cyr61*, and *Akap8l*. C3AR1 (complement component 3a receptor 1) induces NF- κ B activation when coupled to G α_{16} (Yang *et al.*, 2001). Similarly, Cyr61 (cysteine rich protein 61) is associated with NF- κ B signaling, inflammation, and angiogenesis (Klein *et al.*, 2002), as well as anti-apoptotic effects when over-expressed in breast cancer (Lin *et al.*, 2004). Although CYR61 has been implicated in the progression of breast cancer (Xie *et al.*, 2001), it can also act as a tumor suppressor (Chien *et al.*, 2004). Finally, AKAP8L (nuclear protein kinase A anchoring protein) can bind NF- κ B, although the significance of this interaction is unclear (Bouwmeester *et al.*, 2004). Several other genes in the mucosal response network have been implicated in cancer biology. For example, MCM2 (minichromosome maintenance 2 protein) binds to the nuclear scaffold created by AKAP8L and promotes apoptosis in cancer cells (Feng *et al.*, 2003). In contrast, TIMP2 (inhibitor of matrix metalloproteinase 2) inhibits apoptosis and allows tumor growth (Egeblad & Werb, 2002), although its over-expression is inhibitory (Gomez *et al.*, 1997). The net long-term effect of hematin-starved *E. faecalis* on the colonic mucosa cannot be discerned from this study, although the complexity the early response is apparent.

***E. faecalis* blocks G2/M transition in intestinal epithelial cells**

Since the mucosal response network indicated differential expression of genes involved in apoptosis, and because reactive oxygen species from *E. faecalis* can damage colonic epithelial cell DNA to initiate programmed cell death (Huycke *et al.*, 2002), we investigated the effect of superoxide on cell cycle checkpoints and apoptosis. Both HCT116 and YAMC cells that were exposed to hematin-starved *E. faecalis* for 1 hour developed marked arrest at the G2/M transition by 48 hours (Fig. 3a-d). This effect was partially reversed with MnSOD, and completely abolished when MnSOD and catalase were both added to bacteria-treated cells. The effect of catalase suggested H₂O₂, which spontaneously arises from the disproportionation of superoxide, also contributed to activation of the G2/M checkpoint. Treatment of HCT116 cells with H₂O₂ alone is known to cause this arrest (Chang *et al.*, 2003), but our findings showed a greater proportion of arrested cells following exposure to hematin-starved *E. faecalis* than H₂O₂ alone.

To determine whether G2/M checkpoint activation by hematin-starved *E. faecalis* was associated with an increase in apoptosis, we examined HCT116 cells for early apoptotic cells and noted no increase at 24 and 48 hours compared to no treatment controls (Fig. 3e). In contrast, HCT116 cells exposed to H₂O₂ showed significantly increased apoptosis at 24 hours. These findings did not involve NF- κ B since nuclear localization of p65 was not found by laser scanning confocal microscopy at 6, 24, 48, or 72 hours (data not shown). In contrast to transformed HCT116 cells, the primary non-transformed YAMC cells showed significantly increased apoptosis at 48 hours following exposure to hematin-starved *E. faecalis* or H₂O₂ treatment (Fig. 3f).

We previously demonstrated that *E. faecalis* can damage colonic epithelial cell DNA (Huycke *et al.*, 2002). In the current study we find that extracellular superoxide from *E. faecalis* activates the G2/M checkpoint in HCT116 and YAMC cells. This effect was partially reversed by MnSOD and completely abrogated when both MnSOD and catalase were used, suggesting that extracellular superoxide and H₂O₂ each contributed to cell cycle modulation. Arrest at the G2/M transition can be triggered by DNA double strand breaks to activate pathways that allow mitosis to proceed after DNA repair or alternately initiate apoptosis (Nougayrede *et al.*, 2006; Taieb *et al.*, 2006). Differing outcomes following exposure to hematin-starved *E. faecalis* was apparent for HCT116 and YAMC cells and demonstrates how cellular responses to DNA damage vary by cell type. Finally, we noted in a prior study that a minority of cells exposed to hematin-starved *E. faecalis* failed to repair DNA damage or initiate apoptosis and subsequently developed chromosomal instability (Wang & Huycke, 2007).

The DNA damaging effects of commensals on the colonic mucosa may not be limited to *E. faecalis*. Pathogenic and commensal strains of *E. coli* express hybrid peptide-polyketide and cytolethal distending toxins that produce DNA double strand breaks, arrest at the G2/M transition, and cell death (Nougayrede *et al.*, 2006; Taieb *et al.*, 2006). Other examples include commensal bacteria that utilize sulfate as an oxidant (in the assimilatory pathway) or terminal electron acceptor (in the dissimilatory pathway) to dispose of hydrogen reducing equivalents (Gibson *et al.*, 1988). The net result of this metabolism is hydrogen sulfide that, like superoxide, can be genotoxic to epithelial cells (Attene-Ramos *et al.*, 2006). DNA double strand breaks created by reactive oxygen species, hydrogen sulfide, or other clastogens should activate DNA damage repair responses and activate the G2/M checkpoint (Su, 2006). Ongoing DNA damage could lead the accumulation of mutations important to oncogenic transformation. Investigations are underway in our laboratory to explore these issues.

Regulation of netrin-1 by extracellular superoxide from *E. faecalis*

To further investigate the gene response network *in vitro*, we screened HCT116, YAMC, and RAW264.7 cells by qRT-PCR for differential regulation of selected up-regulated genes in the network known to be transcriptionally regulated by NF- κ B or involved in apoptosis (Table 1). Of these, only *NTN1* in HCT116 cells was significantly up-regulated by hematin-starved *E. faecalis* (Fig. 4). The inability to detect similar changes in gene expression *in vitro* using these cells compared to the *in vivo* model likely represents inherent differences between transformed cells and complex multicellular tissues in living animals (Waddell *et al.*, 2007). Indeed, the rationale for the *in vivo* model was to avoid oversimplification of host-commensal interactions found in homogenous *in vitro* culture systems.

Netrin-1 is an extracellular ligand secreted by intestinal epithelial cells that binds basolateral epithelial receptors such as DCC (Arakawa, 2004). When left unbound these receptors initiate signaling pathways that lead to apoptosis. The role of netrin-1 in tumorigenesis was established using *Apc*^{+1638N} mice where over-expression led to intestinal hyperplasia and high-grade tumors (Mazelin *et al.*, 2004). Addition of MnSOD reduced *E. faecalis*-induced *NTN1* expression by 2.5-fold ($P = 0.001$) indicating that extracellular superoxide was partially responsible (Fig. 4).

Although we detected abundant expression of netrin-1 in the murine colonic mucosa by immunohistochemistry, no significant differences in immunoreactivity was found for any group of mice. The lack of differential staining may have been due to insufficient time for tissue protein concentrations to change (*i.e.*, only 6 hrs). To determine whether increased netrin-1 expression in HCT116 cells explained the lack of increased apoptosis in HCT116 cells following exposure to *E. faecalis* (Fig. 3e), we used short interfering RNA to knock down *NTN1* expression. Gene silencing led to an 83-90% reduction in *NTN1* mRNA for cells exposed to hematin-starved *E. faecalis* compared to untransfected controls or cells transfected with scrambled short interfering RNA. The proportion of cells undergoing apoptosis in *NTN1* silenced cells, however, proved no different than for untransfected controls (data not shown). This result could have been due to a lack of functional receptors for netrin-1 on HCT116 cells or defects in the secretion of netrin-1. When Western blots were performed, netrin-1 was not detected (data not shown) implying that other mechanisms are responsible for inhibition of apoptosis in these cells following exposure to hematin-starved *E. faecalis*.

Several factors merit consideration when interpreting the results of this study. Because cDNA from mucosal scrapings were used, by necessity, in their entirety to synthesize probes, we could not check changes in gene expression for arrays by qRT-PCR. However, to overcome this limitation we performed 10 arrays per time point to increase the number of independent replicates. This resulted in a robust dataset for the identification of genes with differing expression. Furthermore, we defined altered gene expression using conservative rules so as to identify only those genes that were highly likely to have undergone induction or repression by hematin-starved *E. faecalis*. Additional genes may have been identified using less rigorous cutoffs. This, in turn, could have expanded the gene response network or identified other networks, but would not have changed the primary conclusion of this study that the metabolic activity of intestinal commensals can acutely alter colonic mucosal gene expression. The short-term nature of our colonic ligation model only permitted an examination of early response genes. Longer-term studies will require intestinal colonization or a gnotobiotic design. Finally, although superoxide from hematin-starved *E. faecalis* induced *NTN1*, knockdown of this gene had no effect on apoptosis since the secretion of this ligand appeared defective in these cells.

In summary, we found that the uniquely dichotomous metabolism of *E. faecalis*, a colonic commensal with a rudimentary respiratory chain that requires exogenous hematin for oxidative phosphorylation, can significantly modulate gene expression in the colonic mucosa. *In vivo*,

in silico, and *in vitro* analyses identified genes and signaling pathways that are associated with inflammation, apoptosis, and cell cycle regulation. In aggregate, these results suggest mechanisms by which *E. faecalis* might enhance epithelial cell susceptibility to DNA damage through the activation of tissue macrophages and by modulating apoptosis in epithelial cells.

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Abbreviations

CFU	Colony Forming Units
CRC	colorectal cancer
DAPI	4',6-diamidino-2-phenylindole
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
IL	interleukin
MnSOD	manganese superoxide dismutase
NF-κB	nuclear factor-kappa B
NLS	nuclear localization sequence
qRT-PCR	real-time reverse transcriptase PCR

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Fig. 1.

Hematin-starved *E. faecalis* activates NF-κB in macrophages. Serial sections of colon exposed to *E. faecalis* for 6 hours was stained with (A) NLS-specific anti-p65 antibody and (B) anti-F4/80 monoclonal antibody. NF-κB activity localized to F4/80-positive tissue macrophages (arrow, 40 × magnification). (C) RAW264.7 cells treated with *E. faecalis* (1 hr, 1×10^9 CFU ml⁻¹) exhibit cytoplasmic and nuclear localization of p65 (stained green with FITC at 63 × magnification) by laser scanning confocal microscopy at 24 hours post treatment, whereas untreated cells primarily exhibited cytoplasmic staining (nuclei stained blue with DAPI). (D) pNFκB-Luc-transfected macrophages showed increased NF-κB induction following treatment with *E. faecalis*; MnSOD significantly decreased *E. faecalis* dependent NF-κB induction whilst catalase caused no further reduction (see Materials and Methods for details; data are means ± SE for at least 6 experiments; compared to *E. faecalis*, *, $P = 0.005$; **, $P = 0.01$; ***, $P < 0.0002$).

Fig. 2. Colonic mucosal response network. Genes significantly up-regulated by hematin-starved *E. faecalis* at 6 hours post-inoculation are highlighted in bold and the network was identified by Ingenuity Pathways Analysis. Direct interaction refers to gene products with direct effects on a targets; indirect interaction refers to gene product effects on targets through intermediate effectors.

Fig. 3.

Hematin-starved *E. faecalis* alters the epithelial cell cycle and fails to induce apoptosis in colonic epithelial cells. Change in (A) HCT116 and (B) YAMC cell cycle at 24 and 48 hours following a 1 hour exposure to *E. faecalis*. Representative histograms from (C) HCT116 and (D) YAMC cells demonstrate a pattern of arrest at the G₂/M transition. Treatments; 1, H₂O₂ (200 μM); 2, *E. faecalis* (1×10^9 CFU ml⁻¹); 3, same as 2 with manganese superoxide dismutase (1200 U ml⁻¹); and 4, same as 3 with catalase (1200 U ml⁻¹); data are the mean $\pm$ SD for at least 3 experiments. Percentage of early apoptotic cells in (E) HCT116 and (F) YAMC cells at 24 (white bars) and 48 hours (black bars) following 1 hour exposure to *E. faecalis* or H₂O₂ (200 μM) compared to untreated cells (data are the mean $\pm$ SD for at least 5 experiments; *, $P = 0.03$; **, $P < 0.002$; ***, $P < 0.0001$ compared to control at timepoint).

Fig. 4.

Extracellular superoxide from hematin-starved *E. faecalis* induces *NTN1* expression in HCT116 cells. qRT-PCR of *NTN1*, after normalizing values to β -actin mRNA, shows significantly greater up-regulation at 24 hours ($P = 0.001$) following a 1 hour exposure to *E. faecalis* (1×10^9 CFU ml⁻¹) compared to cells exposed to *E. faecalis* with MnSOD (1200 U ml⁻¹; data are means $\pm$ SD for 6 experiments; compared to control, *, $P < 0.0001$).

Table 1

Primer pairs for qRT-PCR.

Target gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>NTN1</i> ; human	CCCTGCATAAAAGATCCCTGT	CAGTCTTCAGGCTCCTCCAC
<i>Ntn1</i> ; murine	TTGCATCAAGATTCCCTGTGG	GGCCTTGCAATAGGAGTCAC
<i>C3ar1</i> ; human, murine	TCCTCTGCTGCCCTCTCCTT	CAGGAAGACACTGGCAAACA
<i>Cyr61</i> ; human, murine	GAATCTACCAAAACGGGGAAA	GTTCTTGGGGACACAGAGGA
<i>Akap8l</i> ; human, murine	TGGGTATGGTATGGCCACTT	ATAAAACCGGAATCGGCACCTG
<i>Ac7B</i> ; human, murine	TGCCGACAGGATGCAGAAG	CTCAGGAGGAGCAATGATCTTGAT

Colonic mucosal genes with significantly altered expression following a 6 hour exposure to hematin-starved *E. faecalis* compared to *E. faecalis* grown with hematin.

Table 2

Gene symbol	Gene product	Fold change	P value
<u>Inflammation and stress response</u>			
<i>C3ar1</i> *	Complement component 3a receptor 1	+6.1	0.005
<i>Prdx3</i>	Peroxiredoxin 3	+6.1	0.001
<i>Tnfrsf10</i>	Tumor necrosis factor (ligand) superfamily, member 10	-83.3	3.7E-05
<i>Xrcc6</i>	X-ray repair complementing defective repair in Chinese Hamster cells 6	-66.7	1.5E-04
<i>Cxcl2</i>	Chemokine (C-X-C) ligand 2	-50	5.5E-04
<i>Tnfaip6</i>	Tumor necrosis factor induced protein 6	-50	7.6E-04
<i>Sod3</i> †	Superoxide dismutase 3, extracellular	-43.5	0.002
<i>F2rl1</i>	Coagulation factor II (thrombin) receptor-like 1	-38.5	8.9E-04
<i>Hspa4</i>	Heat shock protein 4	-29.4	0.005
<u>Cell cycle regulation, apoptosis, and cell signaling</u>			
<i>Mcm2</i> *	Minichromosome maintenance deficient 2 mitotin (<i>S. cerevisiae</i>)	+6.1	0.002
<i>Ntn1</i> *	Netrin 1	+5.3	0.004
<i>Gja10</i>	Gap junction membrane channel protein alpha 10	+4.9	1.2E-05
<i>Cyr61</i> *	Cysteine rich protein 61	+4.6	0.004
<i>Timp2</i> *	Tissue inhibitor of metalloproteinase 2	+3.7	9.6E-04
<i>Gpc3</i> *	Glypican 3	+3.7	0.005
<i>EfnA4</i>	Ephrin A4	-71.4	1.0E-04
<i>Cdc21l</i>	Cell division cycle 2-like 1	-52.6	0.001
<i>Ptp4a1</i>	Protein tyrosine phosphatase 4a1	-41.7	0.002
<i>Mapk6</i>	Mitogen-activated protein kinase 6	-34.5	0.002
<u>Transcriptional regulation and DNA binding</u>			
<i>Tal2</i>	T-cell acute lymphocytic leukemia 2	+4.6	5.5E-04
<i>Nr2c1</i>	Nuclear receptor superfamily 2, group C, member 1	-52.6	1.2E-04
<i>Arnt2</i>	Aryl hydrocarbon receptor nuclear translocator 2	-50	6.4E-04
<i>Hoxd8</i>	Homeo box D8	-47.6	0.004
<i>Gcl</i>	Germ cell-less homolog (<i>Drosophila</i>)	-45.5	0.005
<i>Mecox2</i>	Mesenchyme homeobox 2	-40	5.2E-04

Gene symbol	Gene product	Fold change	P value
<i>Six6</i>	Sine oculis-related homeobox 6 homolog (<i>Drosophila</i>)	-35.7	0.002
<i>Ddx3x</i>	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked	-34.5	0.001
<u>Signal transduction</u>			
<i>Gnaq</i>	Guanine nucleotide binding protein, alpha q polypeptide	-66.7	1.7E-04
<i>Arf4</i>	ADP-ribosylation factor-like 4	-58.8	3.3E-04
<i>Gpr34</i>	G protein-coupled receptor 34	-22.2	0.005
<u>Calcium ion binding and transport</u>			
<i>Fbln1</i> [*]	Fibulin 1	+6.2	0.001
<i>Nptx1</i>	Neuronal pentraxin 1	+5.8	0.004
<i>Cacnb3</i>	Calcium channel, voltage dependent, beta 3 subunit	+5.2	0.001
<u>Metabolism</u>			
<i>Yme1l1</i>	YME1-like 1 (<i>S. cerevisiae</i>)	+5.6	0.003
<i>Car4</i> [†]	Carbonic anhydrase 4	+2.4	7.3E-06
<i>Psmb2</i>	Proteasome (prosome, macropain) subunit, beta type 2	-11.6	5.1E-04
<i>Mlycd</i>	Malonyl-CoA decarboxylase	-9.8	0.004
<u>Structural and miscellaneous</u>			
<i>Tekt1</i>	Tektin 1	+9.2	0.002
<i>Akap8l</i> [*]	A-kinase (PRKA) anchor protein 8-like	+3.0	0.002
<i>Ppp3cc</i>	Protein phosphatase 3, catalytic subunit, gamma isoform	+2.2	1.8E-04
<i>Nte</i>	Neuropathy target esterase	-20.8	0.003
<i>Gdi1</i>	Guanosine diphosphatase (GDP) dissociation inhibitor 1	-4.2	0.007

^{*} Genes that are part of the mucosal response network induced by hematin starvation (see Fig. 2).

[†] Genes also differentially regulated at the 1 hour time point.