Dichotomous metabolism of *Enterococcus faecalis* induced by haematin starvation modulates colonic gene expression

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*Enterococcus faecalis* is an intestinal commensal that cannot synthesize porphyrins and only expresses a functional respiratory chain when provided with exogenous haematin. In the absence of haematin, *E. faecalis* reverts to fermentative metabolism and produces extracellular superoxide that can damage epithelial-cell DNA. The acute response of the colonic mucosa to haematin-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 haematin-starved *E. faecalis* and compared with a control consisting of the same strain grown with haematin. At 1 h post-inoculation, 6 mucosal genes were differentially regulated and this increased to 42 genes at 6 h. At 6 h, a highly significant biological interaction network was identified with functions that included nuclear factor-κB (NF-κB) signalling, apoptosis and cell-cycle regulation. Colon biopsies showed no histological abnormalities by haematoxylin and eosin staining. Immunohistochemical staining, however, detected NF-κB activation in tissue macrophages using antibodies to the nuclear localization sequence for p65 and the F4/80 marker for murine macrophages. Similarly, haematin-starved *E. faecalis* strongly activated NF-κB in murine macrophages *in vitro*. Furthermore, primary and transformed colonic epithelial cells activated the G2/M checkpoint *in vitro* following exposure to haematin-starved *E. faecalis*. Modulation of this cell-cycle checkpoint was due to extracellular superoxide produced as a result of the respiratory block in haematin-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.

**INTRODUCTION**

For several decades, the colonic microbiota has been postulated to play a role in the aetiology 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} c.f.u. (g faecal material)^{-1}. 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 tumours when conventionally colonized but have fewer tumours 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 the metabolism of faecal steroids, by producing short-chain fatty acids and by inducing host genes (Augenlicht et al., 2002; Bäckhed et al., 2005; Debruyne et al., 2001; McGarr et al., 2005). None of these effects, however, is 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; 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) with or without 10 μM haematin (Sigma), and washed in sterile PBS before use in experiments. E. faecalis cannot synthesize porphyrins and is unable to form functional cytochrome bd unless supplied with exogenous haematin (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 haematin attenuates superoxide production by >10-fold, an effect that persists for >6 h in vitro. For all experiments, haematin-replete growth of E. faecalis was confirmed by measuring the attenuation of superoxide using a ferricytochrome c assay as described previously (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 haematin-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 diarrhoeal toxins. Conventionally housed and fed 25–28 g adult male BALB/c mice (Jackson Laboratory) were anaesthetized using 1–2 % isoflurane in a carrier gas composed of 95 % O₂ and 5 % CO₂. Through a 5 mm midline abdominal incision, the proximal colon was identified at its juncture with the caecum. Two ligatures 0.5 cm apart were placed around the colon and a 2 mm incision was made into the colon. A gavage needle was inserted into the colon and the contents were completely flushed through the rectum using sterile PBS. The rectum was closed with a purse-string ligature and the colon backfilled with 1.0 ml PBS alone or PBS containing enterococci at a concentration of 1 × 10⁷ c.f.u. ml⁻¹. Immediately preceding instillation, 1-glucose was added to the PBS or the bacterial inoculum to a final concentration of 5 mM. This sugar initiates extracellular superoxide production by E. faecalis, but not for bacteria grown with haematin (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 was gently returned to the peritoneal cavity, the abdominal incision was closed and mice were allowed to recover.

At 1 or 6 h post-inoculation (p.i.), mice were anaesthetized, the abdomens reopened and the 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 described previously (Huycke et al., 1992). Colon biopsies of 5 mm were obtained for histopathology and immunohistochemistry.
Biopsies were examined using haematoxylin and eosin staining, and a modified Brown and Brenn stain. The remaining colon segments were opened longitudinally and the mucosal surfaces scraped with sterile razors for RNA extraction. Biopsies were fixed in formalin and scrapings were 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 h p.i.) for mice exposed to haematin-starved *E. faecalis* (n=5 per group), *E. faecalis* grown with haematin (n=5 per group) or PBS (n=3 per group). The animal protocol was approved by the Animal Studies Subcommittee of the Veterans Affairs Research and Development Committee.

**Gene expression, network response and transcriptional regulatory element analyses.** Total RNA was isolated from colonic scrapings for mice exposed to haematin-replete or haematin-starved *E. faecalis* at 1 and 6 h p.i. using an Atlas pure total RNA labelling system (BD Biosciences Clontech). Probes were synthesized by reverse transcription using [γ-32P]dATP. As the quantities of mucosal scrapings from individual colons were small, each sample was used in its entirety for probe synthesis. Extracted and labelled cDNA probes were hybridized overnight to 5000 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) and expression of individual genes was determined as absorbance readings minus background (ArrayVision software; Imaging Research). Significantly upregulated and downregulated genes were analysed using GeneSpring software version 6.2 (Silicon Genetics). 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 the 1 and 6 h time points were made using Student’s t-test with P<0.005 considered significant. Fold changes were calculated using the GeneSpring fold change filter option. The Benjamini–Hochberg method was used to correct for multiple testing and to 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 are 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 Promoter Analysis and Interaction Network Tool software (Vadigepalli et al., 2003). Comparisons were carried out using all genes on the murine array as the reference library.

**Immunohistochemistry.** Immunohistochemical analysis of the p65 component of NF-κB was performed on serial sections of paraffin-embedded murine colon tissue. Antigen retrieval of deparaffinized sections was performed using a decloaking chamber (Biocare Medical) with citrate buffer or 0.1% pronase (Dako) and processed using the Sequenza staining method (Thermo Scientific). Endogenous peroxidase activity was quenched using peroxidase-blocking reagent (Dako) followed by a blocking step with buffer containing 1% BSA (Jackson ImmunoResearch), 1% normal horse serum (Jackson ImmunoResearch), coldwater fish gelatin and Tween 20. Sections were stained using nuclear localization sequence (NLS)-specific anti-p65 antibody (diluted 1:300; Rockland Immunochemicals) or anti-F4/80 mAb (diluted 1:150; AbD Serotec). The former antibody recognizes the NLS on p65 that is masked by IκB. 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 horseradish peroxidase (HRP)-labelled EnVision+ (Dako). Sections stained with anti-F4/80 antibody were developed using anti-rat secondary antibody (diluted 1:1000; Jackson ImmunoResearch), followed by incubation with ready-to-use streptavidin–HRP solution (Dako). Following incubation, sections were developed with 3,3-diaminobenzidine substrate or Bajoran Purple (Biocare Medical) and counterstained with haematoxylin (Biocare Medical). The distribution of positive cells per field (magnification 100×) between groups was assessed in a randomized and blind 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. Ridges 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 of 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 and AEC chromogen (LabVision). Sections were blocked with H2O2 for 10 min 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 min followed by a 1:20 dilution of rabbit anti-netrin-1 (Ab-1) primary antibody or control peptide following the manufacturer’s instructions (Calbiochem). Sections were counterstained with Immuno* master haematoxylin (American Master*Tech Scientific).

**Cell lines.** Chromosomally stable HCT116 colonic epithelial cells (AmericanTypeCultureCollection) were grown in 5% CO2 at 37 °C using McCoy’s 5A medium modified by l-glutamine and 25 mM HEPES (Invitrogen) and supplemented with 10% fetal bovine serum (FBS). RAW264.7 murine macrophages (American Type Culture Collection) were grown under the same conditions using Dulbecco’s modified Eagle’s medium modified with 4.5 g glucose 1l−1 and l-glutamine (Invitrogen) and supplemented with 10% FBS. For experiments involving co-incubation with *E. faecalis,* bacteria were diluted to 1×10⁸ c.f.u. 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 recombinant murine gamma interferon (PeproTech) ml−1 and ITS Premix (BD) according to the manufacturer’s instructions. After treatment, cells were washed with PBS and complete medium was added containing gentamicin (10 μg ml−1) and penicillin (100 U ml−1) to kill any remaining extracellular bacteria. For H2O2-treated cells, catalase (1200 U ml−1) was also included in the complete medium to eliminate residual H2O2.

**Immunofluorescent assay.** To visualize NF-κB 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 (diluted 1:100; Santa Cruz Biotechnology). A fluorescein isothiocyanate (FITC)-conjugated IgG (diluted 1:200; Santa Cruz Biotechnology) was used as the secondary antibody and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) prior to laser-scanning confocal microscopy (LSM-510 META; Zeiss).
NF-κB–luciferase reporter assay. To verify NF-κB activation, RAW264.7 cells were transfected with the pNFκB-Luc reporter vector (Clontech) using Lipofectin reagent (Invitrogen). Transfected cells were treated with E. faecalis for 1 h and further incubated for 24 h. LPS treatment (10 μg ml⁻¹) served as a control. Cell lysates were prepared using reporter lysis buffer according to the manufacturer’s instructions (Promega). Luciferase activity was measured using a luciferase assay system (Promega) and a TD-20/20 luminometer (Turner Designs). Values were normalized to protein concentration.

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 h 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 RNase A ml⁻¹. Stained cells were analysed using a FACSAir and flow cytometer (BD Immunocytometry Systems). Apoptotic cells were stained using the Annexin V FITC apoptosis detection kit according to the manufacturer’s instructions (Calbiochem, EMD Biosciences) and quantified by flow cytometry. Data were analysed using CellQuest Pro software. Statistical analyses were performed using ModFit version 2.2 software (Verity Software House). For each sample, >10000 events were collected and groups were compared using Student’s t-test with P<0.05 considered significant.

Quantitative real-time RT-PCR (qRT-PCR) and gene silencing. Total mRNA was isolated from E. faecalis-treated cells using a NucleoSpin RNA II kit (BD Biosciences) and 2 μg was reverse-transcribed with an iScript cDNA synthesis kit according to the manufacturer’s instructions (Bio-Rad Laboratories). qRT-PCR was performed using an Mx3005P Q-PCR System following the manufacturer’s instructions (Stratagene). Primers used to assess expression of genes identified in the array experiment (Table 1) were purchased from Integrated DNA Technologies. The gene for netrin-1 was reverse-transcribed 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 colon exposed to E. faecalis at 6 h compared with PBS controls (P=0.008). Although an increase in NF-κB activation was found for colon exposed to haematin-starved compared with haematin-replete E. faecalis, this difference was not statistically significant (P=0.52, P=0.25). To identify mucosal cells with NF-κB activation, we stained serial colon sections with the NLS-specific anti-p65 antibody and an anti-F4/80 mAb. Nearly all cells positive for p65 also stained positive for F4/80, indicating that this redox-sensitive signalling pathway in the colon. NF-κB was detected using an NLS-specific anti-p65 antibody that detects p65 only after it dissociates from IκB. p65 is a member of the canonical NF-κB pathway and is 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 colon exposed to E. faecalis at 6 h compared with PBS controls (ridit=0.57, P<0.008). Although an increase in NF-κB activation was found for colon exposed to haematin-starved compared with haematin-replete E. faecalis, this difference was not statistically significant (ridit=0.52, P=0.25). To identify mucosal cells with NF-κB activation, we stained serial colon sections with the NLS-specific anti-p65 antibody and an anti-F4/80 mAb. Nearly all cells positive for p65 also stained positive for F4/80, indicating that this redox-sensitive signalling pathway had been activated in tissue macrophages (Fig. 1a, b). In contrast, no staining for the NLS of p65 was noted in epithelial cells.

To determine whether E. faecalis activated NF-κB in vitro, we exposed macrophage and epithelial cell lines to haematin-starved bacteria. Strong nuclear staining, along

### RESULTS AND DISCUSSION

#### E. faecalis activates NF-κB in colonic macrophages

To assess the short-term effects of haematin-starved E. faecalis on colonic gene expression, we developed an *in vivo* infection model that preserved mucosal architecture and homeostasis. The mean concentrations of E. faecalis recovered from luminal contents at 1 and 6 h p.i. were approximately 10-fold lower than the initial inocula but were not significantly different for mice administered haematin-starved E. faecalis compared with haematin-replete 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 a tissue Gram stain, indicating that an acute mucosal infection had not occurred (data not shown).

As haematin-starved E. faecalis induces COX-2 expression in macrophages *in vitro* (Wang & Huycke, 2007) and COX-2 is regulated via NF-κB (Karin & Greten, 2005), we initially determined whether E. faecalis activated this redox-sensitive signalling pathway in the colon. NF-κB was detected using an NLS-specific anti-p65 antibody that detects p65 only after it dissociates from IκB. p65 is a member of the canonical NF-κB pathway and is 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 colon exposed to E. faecalis at 6 h compared with PBS controls (ridit=0.57, P=0.008). Although an increase in NF-κB activation was found for colon exposed to haematin-starved compared with haematin-replete E. faecalis, this difference was not statistically significant (ridit=0.52, P=0.25). To identify mucosal cells with NF-κB activation, we stained serial colon sections with the NLS-specific anti-p65 antibody and an anti-F4/80 mAb. Nearly all cells positive for p65 also stained positive for F4/80, indicating that this redox-sensitive signalling pathway had been activated in tissue macrophages (Fig. 1a, b). In contrast, no staining for the NLS of p65 was noted in epithelial cells.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTN1 (human)</td>
<td>CCGTCGATCAGGAGATTCTGG</td>
<td>CAGTCGCTTCAGCCTGAC</td>
</tr>
<tr>
<td>Ntnt1 (murine)</td>
<td>TGGCCTCGGTTGCTGTT</td>
<td>GGGCTGGAAGAACGAGTC</td>
</tr>
<tr>
<td>C3ar1 (human, murine)</td>
<td>TCGCTGCGCTGCTGCTT</td>
<td>GAGCTCCGACGTGACAC</td>
</tr>
<tr>
<td>Cyr61 (human, murine)</td>
<td>TGGGTATGATGATGACG</td>
<td>GTGTGGTGACAGAGAG</td>
</tr>
<tr>
<td>Akap8l (human, murine)</td>
<td>TGGGCAGGAGTGAGAG</td>
<td>ATAAAACGGAATCAGGAG</td>
</tr>
<tr>
<td>ActB (human, murine)</td>
<td>TGGGCAGGAGTGAGAG</td>
<td>ATAAAACGGAATCAGGAG</td>
</tr>
</tbody>
</table>
with cytoplasmic staining, was noted in macrophages by laser-scanning confocal microscopy (Fig. 1c). NF-κB activation was noted as early as 3 h p.i. and persisted for 48 h. In comparison, HCT116 cells exposed to E. faecalis did not lead to nuclear localization of p65 (data not shown). To verify NF-κB activation in macrophages, we transfected RAW264.7 cells with the pNFκB-Luc reporter plasmid. Compared with the control, there was a 25-fold increase in NF-κB activation at 24 h following exposure to haematin-starved E. faecalis (Fig. 1d). Furthermore, manganese superoxide dismutase (MnSOD) significantly reduced NF-κB activation (P<0.005), indicating that extracellular superoxide from haematin-starved bacteria contributed to this effect. The addition of catalase did not further decrease NF-κB activation by haematin-starved E. faecalis, although H₂O₂ alone activated NF-κB in these cells.

NF-κB regulates genes involved in cellular proliferation, immunity and apoptosis (Karin & Greten, 2005). Activation of NF-κB requires the phosphorylation of IκB by IκB kinase. This results in IκB degradation and release of NF-κB homo- and heterodimers to translocate into the nucleus. NF-κB promotes tumorigenesis by inhibiting apoptosis, dysregulating tumour-specific immune responses and producing reactive oxygen species that can damage genomic DNA. Our findings indicate that haematin-starved E. faecalis activates NF-κB, in part, by producing extracellular superoxide. Although reactive oxygen species (including superoxide) can activate NF-κB, 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-κB activation in chondrocytes (Mendes et al., 2003), enhances LPS-dependent NF-κB activation in macrophages (Khadaroo et al., 2003) and initiates NF-κB activation in neutrophils (Mitra & Abraham, 2006). In this study, MnSOD significantly

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**Fig. 1.** Haematin-starved E. faecalis activates NF-κB in macrophages. Serial sections of colon exposed to E. faecalis for 6 h were stained with NLS-specific anti-p65 antibody (a) and anti-F4/80 mAb (b). NF-κB activity was localized to F4/80-positive tissue macrophages (indicated by arrows, magnification 40×). (c) RAW264.7 cells treated with E. faecalis (1 h, 1×10⁶ c.f.u. ml⁻¹) exhibited cytoplasmic and nuclear localization of p65 (stained green with FITC; magnification 63×) by laser-scanning confocal microscopy at 24 h 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 Methods for details). Data are means ± SEM for at least six experiments. *P<0.005; **P=0.01; ***P<0.0002 compared with E. faecalis.
decreased *E. faecalis*-dependent induction of NF-κB in RAW264.7 cells, confirming that this anionic radical can potentiate NF-κB activation beyond that seen with H₂O₂ alone.

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

To determine how broadly the haematin-starved physiology of *E. faecalis* modulated gene expression in the colonic mucosa, we compared mRNA from mice for >5000 genes following exposure to haematin-starved *E. faecalis* with mRNA following exposure to haematin-replete *E. faecalis*. At 1 h p.i., six colonic mucosal genes were differentially regulated (*Sod2*, *Sod3*, *Agtr1*, *Vav1*, *Car4* and *Nmet6*; *P*<0.01 for each). At 6 h, 25 genes were significantly downregulated and 17 genes upregulated (Table 2). Of the differentially regulated genes at the 1 h time point, only *Sod3* and *Car4* were still differentially regulated at 6 h. For the 42 colonic mucosal genes differentially expressed by haematin-starved *E. faecalis*, nine (21%) were related to inflammatory or stress responses and ten (24%) involved pathways for cell-cycle control, signalling and apoptosis. In addition, several were expressed by immune effector cells, suggesting that acute colonic mucosal responses to haematin-starved *E. faecalis* involve innate and/or adaptive immunity.

To identify potential biological interaction networks for these differentially regulated genes, we subjected genes at the 6 h 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-κB signalling (Rea or p65), ERK/MAPK signalling, 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 and eleven transcriptional regulatory elements were associated with the forty-two differentially regulated genes. When we compared the frequency of these elements with elements for all genes on the murine array, there were only five significantly over-represented elements: three for NF-κB (including the sequence for p65 binding), HEN1 and GATA-1. In addition, 66 (59%) of the 111 transcriptional regulatory elements were significantly under-represented. Overall, these findings indicate that haematin-starved *E. faecalis* acutely activates NF-κB signalling in the colonic mucosa.

These in silico analyses identified a single response network with seven upregulated mucosal genes. p65 was the major transcription factor in this network, and in biopsies NF-κB activity localized to tissue macrophages. The 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 coordinate their interaction with innate and adaptive immune effector cells. Enterococci readily translocate across 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 h 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-κB signalling including *C3ar1*, *Cyr61* and *Akap8l*. *C3ar1* (complement component 3a receptor 1) induces NF-κB activation when coupled to GZ16 (Yang et al., 2001). Similarly, *Cyr61* (cysteine-rich protein 61) is associated with NF-κB signalling, inflammation and angiogenesis (Klein et al., 2002), as well as anti-apoptotic effects when overexpressed 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 tumour suppressor (Chien et al., 2004). Finally, AKAP8L (nuclear protein kinase A anchoring protein) can bind NF-κB, 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 metalloprotease 2) inhibits apoptosis and allows tumour growth (Egebäld & Werb, 2002), although its overexpression is inhibitory (Gomez et al., 1997). The net long-term effect of haematin-starved *E. faecalis* on the colonic mucosa cannot be discerned from this study, although the complexity of the early response is apparent.

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

As 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 exposed to haematin-starved *E. faecalis* for 1 h developed marked arrest at the G₂/M transition by 48 h (Fig. 3a, b, c, 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 that 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.
Table 2. Colonic mucosal genes with significantly altered expression following a 6 h exposure to haematin-starved *E. faecalis* compared with *E. faecalis* grown with haematin

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene product</th>
<th>Fold change</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td><strong>Inflammation and stress response</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3ar1*</td>
<td>Complement component 3a receptor 1</td>
<td>+6.1</td>
<td>0.005</td>
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<td>Prdx3</td>
<td>Peroxiredoxin 3</td>
<td>+6.1</td>
<td>0.001</td>
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<tr>
<td>Tnfsf10</td>
<td>Tumour necrosis factor (ligand) superfamily, member 10</td>
<td>−83.3</td>
<td>3.7 × 10⁻⁵</td>
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<td>Xrc6</td>
<td>X-ray repair complementing defective repair in Chinese hamster cells 6</td>
<td>−66.7</td>
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<td>Hspa4</td>
<td>Heat-shock protein 4</td>
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<tr>
<td><strong>Cell-cycle regulation, apoptosis and cell signalling</strong></td>
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<td>Mcm2*</td>
<td>Minichromosome maintenance deficient 2 mitotin (Saccharomyces cerevisiae)</td>
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<td>0.002</td>
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<td>Netrin 1</td>
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<td>Gap junction membrane channel protein z10</td>
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<td>Cysteine-rich protein 6</td>
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<td>0.004</td>
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<td>Timp2*</td>
<td>Tissue inhibitor of metalloproteinase 2</td>
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<td>Gpc3*</td>
<td>Glypican 3</td>
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<td>Ephrin A4</td>
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<td>Cdc25l1</td>
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<td>Mitogen-activated protein kinase 6</td>
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<td>Tal2</td>
<td>T-cell acute lymphocytic leukaemia 2</td>
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<td>Hoxd8</td>
<td>Homeobox D8</td>
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<td>Ddx3x</td>
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<td>Neuronal pentraxin 1</td>
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<tr>
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<td>0.004</td>
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<td>Tekt1</td>
<td>Tektin 1</td>
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<td>A-kinase (PRKA) anchor protein 8-like</td>
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<td>Ppp3cc</td>
<td>Protein phosphatase 3, catalytic subunit, γ isoform</td>
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<td>1.8 × 10⁻⁴</td>
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<td>Neuropathy target esterase</td>
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<td>Guanosine diphosphatase dissociation inhibitor 1</td>
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</table>

*Genes that are part of the mucosal response network induced by haematin starvation (see Fig. 2).
†Genes also differentially regulated at the 1 h time point.
following exposure to haematin-starved *E. faecalis* than H$_2$O$_2$ alone.

To determine whether G$_2$/M checkpoint activation by haematin-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 h compared with no-treatment controls (Fig. 3e). In contrast, HCT116 cells exposed to H$_2$O$_2$ showed significantly increased apoptosis at 24 h. These findings did not involve NF-$\kappa$B, as nuclear localization of p65 was not found by laser-scanning confocal microscopy at 6, 24, 48 or 72 h (data not shown).

In contrast to transformed HCT116 cells, the primary non-transformed YAMC cells showed significantly increased apoptosis at 48 h following exposure to haematin-starved *E. faecalis* or H$_2$O$_2$ treatment (Fig. 3f).

We demonstrated previously that *E. faecalis* can damage colonic epithelial-cell DNA (Huycke *et al.*, 2002). In the current study, we found that extracellular superoxide from *E. faecalis* activates the G$_2$/M checkpoint in HCT116 and YAMC cells. This effect was partially reversed by MnSOD and completely eliminated when both MnSOD and catalase were used, suggesting that extracellular superoxide and H$_2$O$_2$ each contributed to cell-cycle modulation. Arrest at the G$_2$/M transition can be triggered by DNA double-strand breaks to activate pathways that allow mitosis to proceed after DNA repair or, alternatively, to initiate apoptosis (Nougayrede *et al.*, 2006; Taieb *et al.*, 2006). Differing outcomes following exposure to haematin-starved *E. faecalis* were apparent for HCT116 and YAMC cells, and demonstrated how cellular responses to DNA damage vary by cell type. Finally, we noted in a prior study that a minority of cells exposed to haematin-starved *E. faecalis* failed to repair DNA damage or to 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 peptidopolyketide and cytolethal distending toxins that produce DNA double-strand breaks, arrest at the G$_2$/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 G$_2$/M checkpoint (Su, 2006). Ongoing DNA damage could lead to the accumulation of...
Regulation of netrin-1 by extracellular superoxide from *E. faecalis*

To investigate further the gene response network *in vitro*, we screened HCT116, YAMC and RAW264.7 cells by qRT-PCR for differential regulation of selected upregulated genes in the network known to be transcriptionally regulated by NF-κB or involved in apoptosis (Table 1). Of these, only *NTN1* in HCT116 cells was significantly upregulated by haematin-starved *E. faecalis* (Fig. 4). The inability to detect similar changes in gene expression *in vitro* using these cells compared with the *in vivo* model probably 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 homogeneous *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 mutations important to oncogenic transformation. Investigations are underway in our laboratory to explore these issues.

**Fig. 3.** Haematin-starved *E. faecalis* alters the epithelial cell cycle and fails to induce apoptosis in colonic epithelial cells. (a, b) Changes in the HCT116 (a) and YAMC (b) cell cycle at 24 and 48 h following a 1 h exposure to *E. faecalis*, and (c, d) representative histograms from HCT116 (c) and YAMC (d) cells demonstrating a pattern of arrest at the G2/M transition. Treatments: 1, H2O2 (200 μM); 2, *E. faecalis* (1×10⁶ c.f.u. ml⁻¹); 3, as in treatment 2 plus MnSOD (1200 U ml⁻¹); 4, as in treatment 3 plus catalase (1200 U ml⁻¹). Data are the means ± so of at least three experiments. (e, d) The percentage of early apoptotic cells in HCT116 (e) and YAMC (f) cells at 24 h (white bars) and 48 h (black bars) following 1 h exposure to *E. faecalis* or H2O2 (200 μM) compared with untreated cells. Data are the means ± so of at least five experiments. *, P<0.03; **, P<0.002; ***, P<0.0001 compared with the control at each time point.
receptors initiate signalling pathways that lead to apoptosis. The role of netrin-1 in tumorigenesis was established using Apc<sup>+/+1638N</sup> mice where overexpression led to intestinal hyperplasia and high-grade tumours (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 were 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 h). To determine whether increased netrin-1 expression 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 compared with cells exposed to E. faecalis with MnSOD (1200 U ml<sup>-1</sup>). Data are the means ± SD for six experiments. *P<0.0001 compared with control.

In summary, we found that the uniquely dichotomous metabolism of E. faecalis, a colonic commensal with a rudimentary respiratory chain that requires exogenous haematin for oxidative phosphorylation, can significantly modulate gene expression in the colonic mucosa. In vivo, in silico and in vitro analyses identified genes and signalling pathways that are associated with inflammation, apoptosis and cell-cycle regulation. Overall, 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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Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. Nature 431, 80–84.


