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The *recA* operon: A novel stress response gene cluster in *Bacteroides fragilis*

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Abstract

Bacteroides fragilis, an opportunistic pathogen of humans, is a leading cause of bacteraemias and anaerobic abscesses which are often treated with metronidazole, a drug which damages DNA. This study investigated the responses of the *B. fragilis recA* three gene operon to the stress experienced during metronidazole treatment and exposure to reactive oxygen species simulating those generated by the host immune system during infection. A transcriptionally regulated response was observed using quantitative RT-PCR after metronidazole and hydrogen peroxide treatment, with all three genes being upregulated under stress conditions. In vivo and in vitro analysis of the functional role of the second gene of the operon was done using heterologous complementation and protein expression (in *Escherichia coli*), with subsequent biochemical assay. This gene encoded a functional bacterioferritin co-migratory protein (BCP) which was thiol-specific and had antioxidant properties, including protection of the glutamine synthetase III enzyme. This in vitro data supports the hypothesis that the genes of the operon may be involved in protection of the bacteria from the oxidative burst during tissue invasion and may play a significant role in bacterial survival and metronidazole resistance during treatment of *B. fragilis* infections.

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1. Introduction

The *Bacteroides* genus is one of the 5 predominant groups of bacteria in the intestinal microbiome, accounting for around 30% of gut microbes [1,2]. *Bacteroides fragilis*, a non-spore forming, Gram-negative, anaerobic rod represents only around 0.5% of the *Bacteroides* in the gut lumen where it grows as a commensal; it is, however, a virulent opportunistic pathogen [2]. It is isolated from the majority of clinical cases of bacterial septicaemia resulting from intestinal ruptures or surgeries and forms abscesses in the abdomen, pelvis, lungs and brain [3]. In order for *B. fragilis* to colonise the abdominal cavity, the cell has to survive high oxygen levels and the initial host immune onslaught. *B. fragilis* has been shown, in vitro, to have an extensive, complex and co-ordinated response to oxidative stress that involves at least 3 independent regulons, 28 proteins and alterations to its physiology at the metabolic level [3–5]. These genes may be transcriptionally responsive to oxygen, hydrogen peroxide or both [3]. In a second paper by this group, up to 45% of the transcriptome was shown to be alternatively regulated in response to oxidative stress [6]. Previous research by our group showed a link between the presence of the RecA protein and survival of *B. fragilis* cells in the presence of reactive nitrogen species (RNS) and reactive oxygen species (ROS) [7]. These observations suggest that the RecA protein from *B. fragilis* may also be important for enabling cell survival in the presence of the oxygen radicals associated with the innate immune response.

The *B. fragilis recA* gene was previously observed by RT-PCR to be transcribed, under normal growth conditions, on the same RNA transcript as two upstream open reading frames

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encoding a putative bacterioferritin co-migratory protein (BCP) and a putative saccharopine dehydrogenase (SDH) [7]. BCP proteins belong to the thiol-specific antioxidant (TSA) protein family [8]. These proteins are found in several bacteria where they catalyse the reduction of hydrogen peroxide and organic hydroperoxides [8,9], thereby preventing free radical formation and the resultant cellular oxidation damage. *B. fragilis* has KatA (catalase), AhpC (alkyl hydroperoxidase) and six other Tpx (thioredoxin peroxidase) proteins which can serve this protective function [10]. It is not known whether its *bcp* gene product may act in a similar way. The role of the *sdh* gene product is also not clearly understood in *B. fragilis*.

In this study, the functions of the *B. fragilis* BCP were investigated. The ability of the annotated *bcp* gene to complement an *Escherichia coli bcp*$^{-}$ strain (KD2301) functionally was evaluated, and the protein was also heterologously expressed and biochemically assayed for substrate preference, thiol peroxidase dependence and protective properties for the vital glutamine synthetase enzyme from *B. fragilis*. The transcriptional response of the *bcp* gene to exposure to metronidazole and hydrogen peroxide was measured along with the *recA* and *sdh* genes using quantitative RT-PCR methods (qPCR).

2. Methods and materials

2.1. Bacterial strains, plasmids and growth conditions

B. fragilis 638R was grown in supplemented brain heart infusion broth (BHISB) or on plates (BHISA) at 37 °C under anaerobic conditions [11]. All bacterial strains are described in Table 1. *E. coli* strains were grown in LB broth and plated on LB agar with appropriate antibiotic selection. *E. coli* KD2301 was grown with kanamycin (10 μg/ml) [12]. *E. coli* BL21DE3 was grown with no selection, while *E. coli* BL21DE3 and KD2301 strains expressing the pET22b1247pro plasmid were grown with ampicillin (100 μg/ml). All *E. coli* growth was under aerobic conditions at 30 °C.

2.2. Bioinformatic analysis

Protein and DNA sequences were obtained from the National Centre for Biotechnology Information (www.ncbi.nih.gov). BLAST 2.2.17 [13] was used to calculate the predicted percentage identity between protein sequences for the CDS from *B. fragilis* 638R (NC_016776.1) for the 3 ORFs BF638R1245, BF638R1246/7 and BF638R1248 that make up the three gene cluster. Conserved domains database (CDD) [14] searches were used to identify conserved domains in the protein sequences. KEGG analysis [15,16] was undertaken to establish whether the other enzymes in the metabolic pathways associated with the CDD protein domain searches were present in *B. fragilis*.

2.3. RNA isolation and northern blot analysis

RNA isolation and northern blot analysis were performed essentially as previously described [17]. The RNA was purified using the hot-phenol method except that chloramphenicol was not added to the culture prior to harvesting. For the northern blots, RNA (50 μg) was electrophoresed on 1% agarose gels containing, 1× MOPS (40 mM 3-[*N*-morpholino] propanesulphonic acid) and 2.2 M formaldehyde, and transferred to a nylon membrane. ^{32}P labelled probes were added to the membrane and allowed to hybridise overnight at 42 °C. Membranes were washed in decreasing concentrations of SSPE (5–0.1×) until low background radiation had been reached. DNA probes were labelled by random oligonucleotide priming with the incorporation of ^{32}P-dCTP. The *recA* and *bcp* probes were derived from PCR fragments that encompassed the central portion of the respective gene. At least 10^6 cpm of labelled probe/ml of hybridisation solution were added for all hybridisations.

2.4. Quantitative RT-PCR

2.4.1. Sample preparation and storage, and primer design

B. fragilis 638R was grown to mid-log phase ($OD_{600} = 0.6$) and then half of the culture was exposed to either 100 μM H_2O_2 or 1 μg/ml metronidazole. The other half of the culture was used as the uninduced control. Samples of 100 ml were taken for each treatment at time points 0, 15, 30 and 60 min. Three biological replicates were performed and each separated into three technical repeats for RNA extraction. RNA was extracted using the hot-phenol method of Aiba et al. [18] with the following modifications: after 16 h precipitation of the

Table 1
Strains and plasmids used in this study.

Strains/plasmids	Relevant characteristic/use[a]	Source/reference
B. fragilis		
638R	Clinical strain, $Rif^R Gent^R$	[39]
E. coli		
BL21DE3	*E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and $lacI^q$	[40]
BL21DE3(pET22b(+))	BL21DE3 with empty pET22b(+) vector	This study
BL21DE3(pET22b1247pro)	BL21DE3 derivative with pET vector expressing full-length *B. fragilis* BCP	This study
KD2301	BCP-deficient *E. coli* mutant derived from BL21DE3	[12]
KD2301(pET22b1247pro)	KD2301 derivative expressing full-length *B. fragilis* BCP	This study
Plasmids		
pET22b(+)	*pelB* coding sequence, His-tag coding C-terminal	Novagen
pET22b1247pro	pET22b(+) plasmid with full-length BCP protein under IPTG induction	This study

[a] Rif = rifampicin, Gent = gentamycin; R = resistant.

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