RESEARCH LETTER

Flavin mononucleotide (FMN)-based fluorescent protein (FbFP) as reporter for gene expression in the anaerobe *Bacteroides fragilis*

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Abstract

In this study, we show the expression of flavin mononucleotide-based fluorescent protein (FbFP) BS2 as a marker for gene expression in the opportunistic human anaerobic pathogen *Bacteroides fragilis*. *Bacteroides fragilis* 638R strain carrying *osu*::*bs2* constructs showed inducible fluorescence following addition of maltose anaerobically compared with nonfluorescent cells under glucose-repressed conditions. Bacteria carrying *ahpC*::*bs2* or *dps*::*bs2* constructs were fluorescent following induction by oxygen compared with nonfluorescent cells from the anaerobic control cultures. In addition, when these transcriptional fusion constructs were mobilized into *B. fragilis* IB263, a constitutive peroxide response strain, fluorescent BS2, was detected in both anaerobic and aerobic cultures, confirming the unique properties of the FbFP BS2 to yield fluorescent signal in *B. fragilis* in the presence and in the absence of oxygen. Moreover, intracellular expression of BS2 was also detected when cell culture monolayers of J774.1 macrophages were incubated with *B. fragilis ahpC*::*bs2* or *dps*::*bs2* strains within an anaerobic chamber. This suggests that *ahpC* and *dps* are induced following internalization by macrophages. Thus, we show that BS2 is a suitable tool for the detection of gene expression in obligate anaerobic bacteria in *in vivo* studies.

Introduction

The use of fluorescent proteins in biomedical research started over 10 years ago (Chalfie *et al.*, 1994). Since then, fluorescent proteins proved to be extremely useful as reporter tools in several cellular processes such as tracking protein movements in the cell, monitoring mitochondrial redox potential and transcriptional reporters (Wachter, 2006). In bacteria, green fluorescent proteins (GFPs) can be used to survey microorganisms in complex biological systems such as biofilms, soil and to visualize interactions of bacteria with plant or animal host tissues (Rosochacki & Matejczyk, 2002; Larrainzar *et al.*, 2005; Hoppe *et al.*, 2009; Chudakov *et al.*, 2010). Furthermore, GFPs can be transcriptionally and translationally fused to bacterial genes and expressed *in vivo* as an alternative to immunofluorescence. It can also be used to examine the function and localization of the gene products (Margolin, 2000). Currently, GFPs are a cornerstone tool used in *in vivo* imaging, fluorescence resonance energy transfer and quantitative transcriptional analysis. Several GFP-like derivatives have been engineered for better fluorescence and photostability (Heim *et al.*, 1995) as well as different color emissions (Shaner *et al.*, 2007). However, in the catalytic formation of the chromophore, GFP requires the presence of molecular oxygen (Heim *et al.*, 1994), thus rendering the protein colorless in anaerobic environments, making GFP unsuitable for use as a reporter gene in obligate anaerobic organisms.

Recent efforts to create a protein reporter for *in vivo* labeling and fluorescence either in the presence or in the absence of oxygen led to development of flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs) (Drepper *et al.*, 2007, 2010). Commercial FbFPs are derived from the blue-light photoreceptors YtvA from *Bacillus subtilis* and SB2 from *Pseudomonas putida* that contain the light oxygen voltage (LOV) domains. The LOV domains were first identified in plant phototrophins (Huala *et al.*, 1997) where they regulate several physiological processes such as phototropism, chloroplast relocation and stomatal opening (Briggs & Christie, 2002; Celaya & Liscum, 2005). LOV domain proteins were subsequently found in prokaryotes where they seem to participate in two-component systems

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for light sensing although the function of these light-sensing phenotypes is not yet fully understood for most species (Losi & Gärtner, 2008). LOV domains bind noncovalently to the oxidized FMN chromophore and when exposed to blue light (450 nm) undergo a reversible photocycle that leads to the formation of an FMN-cysteine C(4a) thiol adduct that exhibits weak autofluorescence (Salomon *et al.*, 2000). The photoactive cysteine residue in a truncated gene expressing only the LOV domain of YtvA protein (Cys53) from *B. subtilis* was substituted with an alanine by site-directed mutagenesis and adjusted for *Escherichia coli* codon usage bias (Drepper *et al.*, 2007). The modified protein, known as BS2, has a 25-fold increase in fluorescence intensity when compared with wild-type YtvA and exhibits a maximal light absorption at 449 nm and maximal emission at 495 nm (Drepper *et al.*, 2007).

An important characteristic of FbFP, including BS2, is that its fluorescence signal is not affected by the lack of oxygen (Drepper *et al.*, 2007). This property makes BS2 a useful tool to study gene expression in obligate anaerobes under different environmental conditions because of its ability to yield fluorescence under both anaerobic and aerobic conditions. In this study, we have used promoterless BS2 as a reporter gene to evaluate promoter activity in the anaerobe *Bacteroides fragilis* as a model organism. *Bacteroides fragilis* is an opportunistic human pathogen normally found as a component of microbial communities of the human lower intestinal tract (Smith *et al.*, 2006). One characteristic of this species is its high aerotolerance, which allows it to survive in aerobic environments for a long period of time (Rocha & Smith, 1999) and to survive host cellular immune defense in extraintestinal oxygenated tissues such as the intra-abdominal cavity (Rocha *et al.*, 2007; Sund *et al.*, 2008). Thus, in this study, we have analyzed the promoter activities of two characterized essential oxidative stress response genes under the control of the transcriptional regulator OxyR, the alkyl hydroperoxide reductase (*ahpCF*) and the nonspecific DNA-binding protein (*dps*) (Rocha *et al.*, 2000) transcriptionally fused to the promoterless BS2 fluorescent protein as a reporter gene. In addition, we also demonstrate the anaerobic expression of fluorescent BS2 under control of the maltose/starch inducible promoter *osu*. We show in this work that the fluorescent peptide BS2 is a useful tool to evaluate the expression *B. fragilis* genes under both anaerobic and aerobic conditions as well as in macrophage cell line assays.

Materials and methods

Strains and growth conditions

The *B. fragilis* strains 638R (Privitera *et al.*, 1979) and IB263 (Rocha & Smith, 1998) used in this study were routinely grown on BHIS (brain heart infusion supplemented with L-cysteine, hemin and $NaHCO_3$) at 37 °C under anaerobic conditions. Rifamycin (20 μg mL^{-1}), 100 μg mL^{-1} gentamycin and 10 μg mL^{-1} erythromycin were added to the media when required. The *E. coli* DH10B strain was grown routinely in Luria–Bertani medium (L-broth) containing 100 μg mL^{-1} ampicillin or 50 μg mL^{-1} spectinomycin when appropriate. For oxygen induction, overnight cultures in BHIS were diluted 1 : 25 in fresh media and grown to mid-log phase at an $OD_{550\,nm}$ of 0.5. The cultures were split and one half remained under anaerobic conditions. The other half was exposed to air in an orbital shaker incubator (250 r.p.m.) at 37 °C for 1 h. Chloramphenicol at 100 μg mL^{-1} was added immediately before harvesting bacterial cells by centrifugation. Maltose at 0.5% was added into anaerobic cultures when required.

Construction of transcriptional fusions to promoterless *bs2* gene

To clone the promoterless *bs2* gene into a *B. fragilis* shuttle expression vector, the *bs2* ORF (411 bp) from pGLOW-Bs2-stop (Evocatal GmbH, Dusseldorf, Germany) was PCR amplified using primers Bs2-BamHI-Forward (AAGAATGGATCCAAATAAGAAACAATT*ATGGCGTCGTTCCAGTCG*) and Bs2-SstI-Reverse (GCCGAGCTCGCATGCCTGC). The oligo primer Bs2-BamHI-Forward was designed to place the ribosome-binding site (RBS) of *B. fragilis ahpC* gene (Rocha & Smith, 1999) immediately upstream the *bs2* ATG codon (*bs2* nucleotides are shown in italics). This procedure was carried out to replace the *E. coli* RBS region and insert a native RBS chromosomal region to optimize translation of *bs2* gene in *B. fragilis*. The Bs2-SstI-Reverse primer was designed to contain the *bs2* stop codon and restriction cloning sites from the original pGLOW-Bs2-stop plasmid except that HindIII site was replace with an SstI site. The PCR product containing a 462-bp DNA fragment was A-tailed and cloned into pGEM-T (Promega, Madison, WI) according to the manufacturer's instructions to construct pER-151. pER-151 was digested with BamHI and SstI and the 447-bp DNA fragment containing the promoterless *bs2* gene was cloned into the BamHI and SstI sites of pFD1045, a shuttle vector containing the maltose/starch and oxygen inducible promoter of the *osu* operon (Spence *et al.*, 2006). This new construct, pER-153 (Fig. 1), was conjugated into *B. fragilis* 638R by triparental mating according to standard protocols (Rocha & Smith, 1999). Transconjugants were selected on BHIS plates containing rifamycin (20 μg mL^{-1}), 100 μg mL^{-1} gentamycin and 10 μg mL^{-1} erythromycin. The new strain, BER-85, was used for expression of BS2 under anaerobic conditions following addition of maltose. To construct the *ahpC*::*bs2* transcriptional fusion, the 447-bp BamHI/SstI DNA fragment containing promoterless *bs2* was

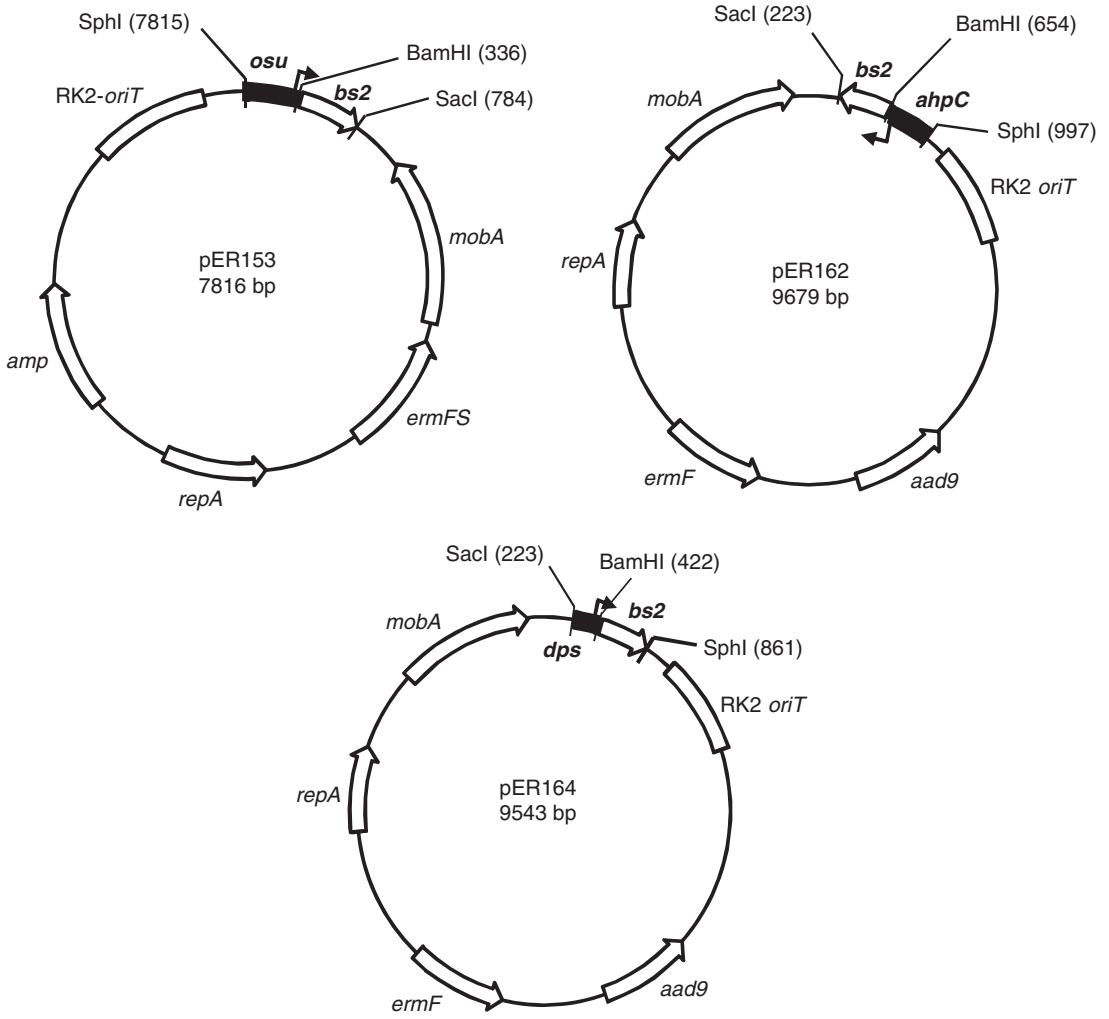

Fig. 1. Schematic representation of the shuttle vectors containing the transcription fusion constructs described in Materials and methods. A partial restriction map relevant for the cloning strategy is shown in each plasmid. aad9, spectinomycin resistance in *Escherichia coli*; *ermF* and *ermFS*, erythromycin resistance in *Bacteroides*. Broken arrows depict the transcription +1 nucleotide start site positioned at 54, 39 and 62 bp upstream of the transcription fusion junction for *osu*, *ahpC* and *dps* promoter regions, respectively.

cloned into the BamHI/SstI sites of pFD288 carrying a 330-bp DNA fragment of the *ahpC* promoter region in the SphI/BamHI sites (Rocha & Smith, 1999). The new construct, pER162 (Fig. 1), was conjugated into *B, fragilis* 638R and IB263 strains by triparental mating to construct BER-95 and BER-104, respectively. The *dps*::*bs2* construct was obtained by cloning the 441-bp BamH/SphI promoterless *bs2* gene into the BamHI/SphI sites of pUC19 containing 187 bp of the *dps* promoter region (Rocha *et al.*, 2000). The new construct, pER 163, carrying the *dps*::*bs2* transcriptional fusion was digested with SphI/SstI and cloned into the SphI/SstI sites of pFD288. The new construct, pER164 (Fig. 1), was conjugated into *B. fragilis* 638R and IB263 strains by triparental mating to construct BER-96 and BER-105, respectively.

Confocal laser microscopy

For the microscopy slides, 1 mL of bacterial cultures grown to mid-log phase in BHIS under conditions described above were centrifuged at 3000 ***g*** for 3 min and washed once in 1 mL of phosphate buffer saline (PBS) (4.3 mM dibasic sodium phosphate, 1.47 mM monobasic potassium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4). Bacteria were suspended in 1 mL PBS and a drop of this suspension was added to each slide and allowed to air-dry. The coverslips were mounted with glycerol and the slides were analyzed with a Confocal Microscope Zeiss LSM 510 using an excitation of 450 nm and an emission filter in the range of 475–525 nm. For dual channel

fluorescent color detection in slides stained with Alexafluor-546-phalloidin conjugate (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction, an excitation at 556 nm and emission at 573 nm were also used.

Macrophage assay

The J774.1 macrophage cell lines was grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The cells were grown over sterile coverslips placed inside six-well microplates at 37 °C in a 5% CO_2 humidified atmosphere. For all assays, six-well plates were seeded with approximately 2×10^5 macrophages mL^{-1} and incubated until confluence was reached. The bacterial cell numbers were determined spectrophotometrically at 600 nm. The assay was carried out by inoculating *B. fragilis* at an approximate multiplicity of infection of 100 into the six-well plates under anaerobic conditions. Infected monolayers were incubated for 1 h inside the anaerobic incubator to allow phagocytosis and internalization to occur. Then, the monolayers were washed three times with PBS without an antibiotic to remove unbound bacteria. The cells were then fixed with 3.7% formaldehyde for 10 min and washed three times with PBS. Macrophages were stained with Alexafluor-546-phalloidin conjugate. The coverslips were removed from the wells and placed on top of glass slides for laser confocal microscopy analysis as described previously.

Results

In this study, we show the use of the fluorescent protein BS2 as a reporter for *in vitro* and *in vivo* gene expression studies in the anaerobe *B. fragilis*. When the promoterless *bs2* gene was cloned in fusion with the starch/maltose and oxygen inducible promoter *osu* (Spence *et al.*, 2006), addition of maltose was able to induce expression of fluorescent BS2 under anaerobic conditions compared with uninduced culture controls. These results clearly demonstrate that expression of BS2 in cultures of *B. fragilis* BER-85 in the absence of oxygen yield an intense fluorescence characteristic of BS2. In addition, exposure of BER-85 strain to atmospheric air also yielded intense fluorescence, indicating that BS2 can be a useful fluorescent marker for gene expression in *B. fragilis* under both anaerobic and aerobic conditions (Fig. 2).

These findings prompted us to analyze the use of promoterless *bs2* as a reporter gene by constructing *bs2* transcriptional fusions to the oxygen and peroxide responsive promoters, *ahpC* and *dps*, which have been previously characterized in *B. fragilis* (Rocha *et al.*, 2000). Both *ahpC* and *dps* expression are under control of the peroxide transcriptional regulator OxyR (Rocha *et al.*, 2000). Thus, it seemed appropriate to investigate the expression of *ahpC*::*bs2* and *dps*::*bs2* constructs in response to oxygen and peroxide to further characterize expression of BS2 under both anaerobic and aerobic oxidative conditions. Figure 3 shows that *B. fragilis* 638R carrying the *ahpC*::*bs2* constructs (BER-95) were fluorescent compared with the anaerobic culture control (Fig. 3a and b). When the constitutive peroxide response strain, IB263, was transformed with the *ahpC*::*bs2* construct (BER-104), it produced fluorescence under both anaerobic and aerobic conditions (Fig. 3c and d). Similar findings were obtained when *B. fragilis* 638R carrying *dps*::*bs2* (BER-96) was exposed to oxygen; it also showed increased fluorescence compared with the anaerobic culture control (Fig. 4a and b). In addition, the IB263 *dps*::*bs2* strain (BER-105) also showed constitutive expression of BS2 independent of the presence or absence of oxygen, confirming that the protein BS2 is a useful tool as a fluorescent image marker for gene expression in the anaerobe *B. fragilis*.

The oxidative stress response has been demonstrated to play an important role in the ability of the opportunistic

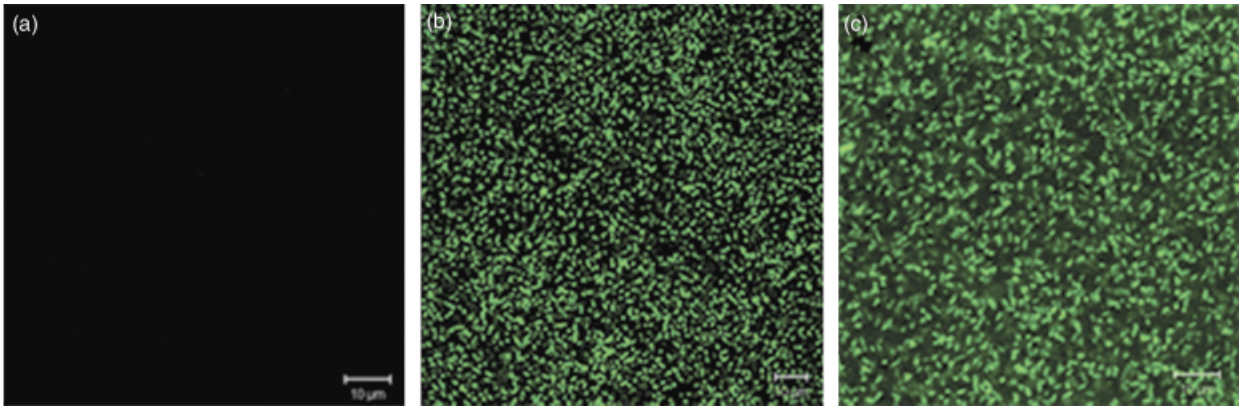

Fig. 2. Confocal laser microscopy images of *Bacteroides fragilis* BER-85 (638R *osu*::*bs2*). (a) Anaerobic culture control. (b) Anaerobic culture after addition of maltose for 1 h and (c) after exposure to oxygen for 1 h.

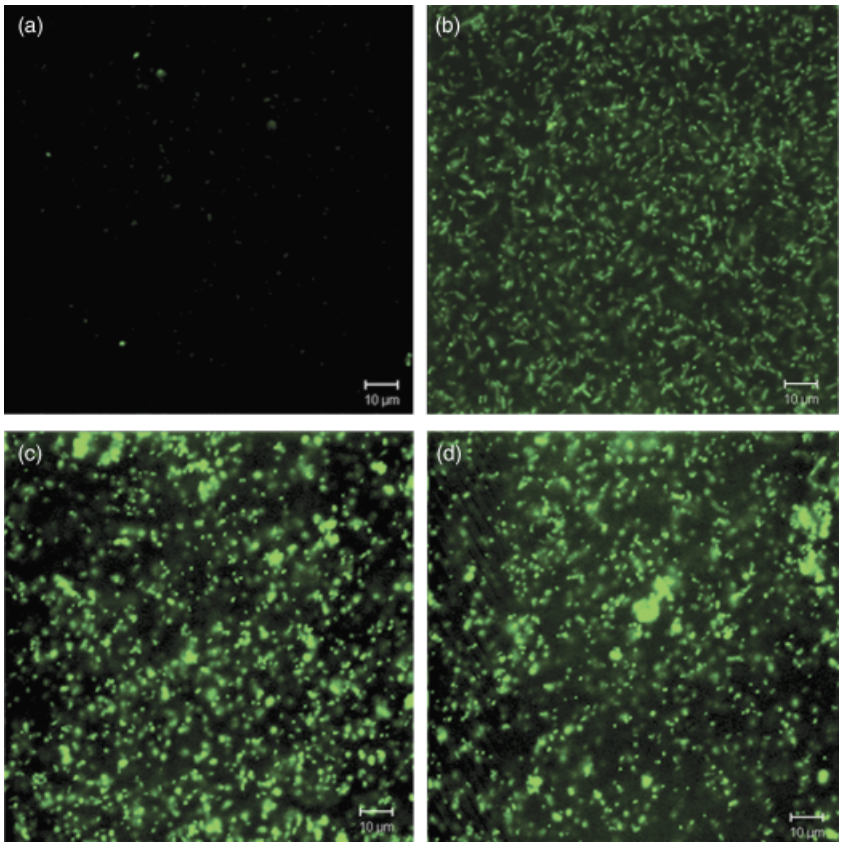

Fig. 3. Confocal laser microscopy images of *Bacteroides fragilis* strains expressing FbFP BS2 fluorescent protein. (a, b) *Bacteroides fragilis* BER-95 (638R *ahpC*::*bs2*): (a) anaerobic culture control; (b) after exposure to oxygen for 1 h. (c, d) *Bacteroides fragilis* BER-104 (IB263 *ahpC*::*bs2*): (c) anaerobic culture control; (d) after exposure to oxygen for 1 h.

intestinal colonizer *B. fragilis* to survive in intraperitoneal experimental infections (Rocha *et al.*, 2007; Sund *et al.*, 2008). However, expression of oxidative response genes *in vivo* has not been extensively investigated in *B. fragilis*. Thus, to investigate whether the *ahpC* and *dps* genes were induced following incubation with phagocytic cells, a J774.1 macrophage cell line assay *in vitro* was used to test whether *B. fragilis* BER-95 and BER-96 express the peroxide response genes following cellular internalization by macrophages. In this study, we showed that the expression of both *ahpC* (Fig. 5) and *dps* (Fig. 6) were visualized intracellularly as demonstrated by confocal laser microscopy, showing the expression of BS2 fluorescent protein in internalized *B. fragilis* strains carrying *ahpC*::*bs2* (BER-95) or *dps*::*bs2* (BER-96) transcriptional fusion constructs. Using the Z-STACK software function to analyze confocal laser microscopy image layers, we demonstrated that fluorescent *B. fragilis* cells were found to be in an intracellular compartment and not attached to the membrane surface of the macrophage cells. Thus, taken together, these findings show for the first time that BS2 can be used as a fluorescent marker to analyze gene expression in obligate anaerobic bacteria in both *in vitro* and *in vivo* systems.

Discussion

In addition to advances in fluorescent proteins derived from GFP, a new class of fluorescent proteins has recently been isolated and proven useful in environments deprived of oxygen. Drepper *et al.* (2007) demonstrated that FbFPs expressed in the facultative anaerobe *Rhodobacter capsulatum* in hypoxia was fully fluorescent. More recently, Drepper *et al.* (2010) have quantitatively monitored the fluorescent intensity of FpFP *in vivo* in *E. coli* under oxygen limitations by continuously measuring wavelength excitation at 460 nm and emission at 492 nm. A different study showed that FbFPs expressed in *Candida albicans* and *Saccharomyces cerevisiae* under anaerobic conditions render the cells fluorescent (Tielker *et al.*, 2009). In this work, we demonstrate that FbFPs expressed in the obligate anaerobe *B. fragilis* confer fluorescence to the cells when grown under anaerobic conditions. In the absence of oxygen, *B. fragilis* cells were remarkably fluorescent (Fig. 2), presenting an emission in the range of 475–505 nm when excited with light at 450 nm in agreement with previous works using FbFPs as the fluorescent marker (Drepper *et al.*, 2007; Tielker *et al.*, 2009).

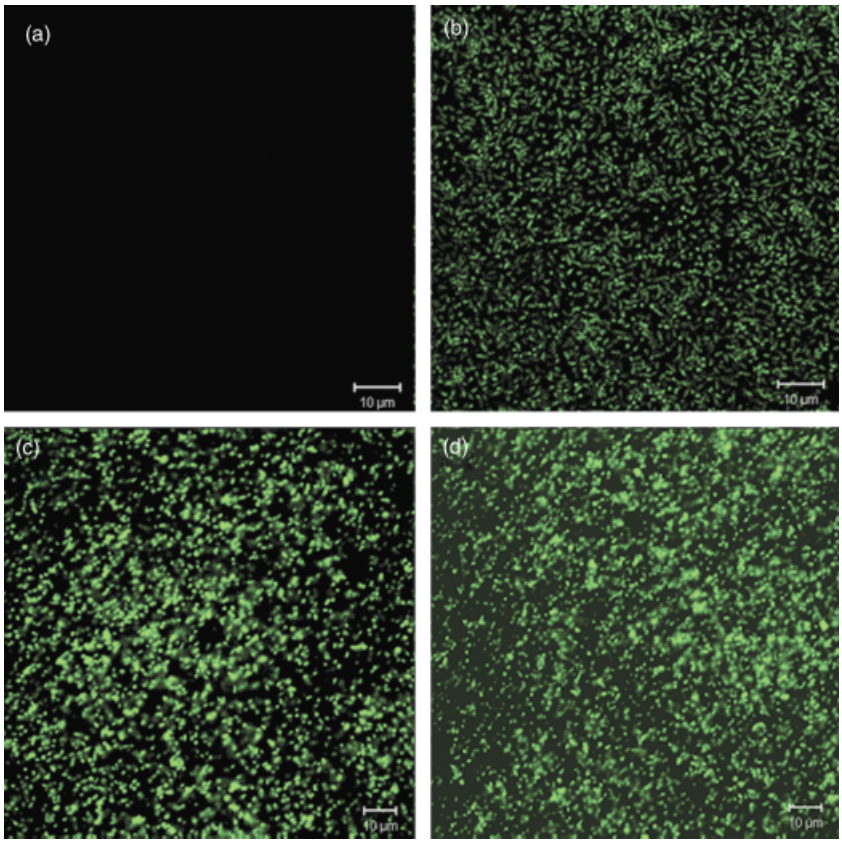

Fig. 4. Confocal laser microscopy images of *Bacteroides fragilis* strains expressing FbFP BS2 fluorescent protein. (a, b) *Bacteroides fragilis* BER-96 (638R *dps*::*bs2*): (a) anaerobic culture control; (b) after exposure to oxygen for 1 h. (c, d) BER-105 (IB263 *dps*::*bs2*): (c) anaerobic culture control; (d) after exposure to oxygen for 1 h.

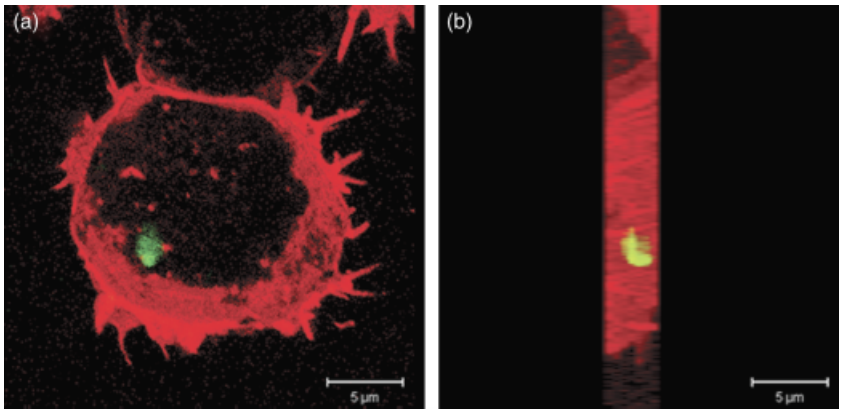

Fig. 5. Confocal laser microscopy of intracellular expression of BS2 in *Bacteroides fragilis* 638R *ahpC*::*bs2* (BER95) following internalization by the murine macrophage cell line J774.1. (a) Bacterial internalization was carried out for 1 h and visualized with laser confocal microscopy as described in Materials and methods. (b) The Z-Stacks of the image layers obtained in (a) were piled up and rotated to a 90° angle.

Although GFP protein derivatives have been engineered to increase photostability, intensity, broad pH range tolerance, faster maturation rates and different colors, which allow researchers to use multiple probes within the same image experimental set (Shaner *et al*., 2007), they are still dependent on molecular oxygen to display their fluorescence. This requirement for oxygen for proper post-translational modification of the protein fluorophore is a significant limitation to their use in anaerobic environments. Thus, our findings are important for the study of anaerobic bacteria as there is a lack of imaging tools to study molecular trafficking and gene expression in these organisms, which require anaerobic conditions during their growth and metabolism under both *in vitro* and *in vivo* conditions. This is particularly relevant with regard to *B. fragilis* because it will allow us to investigate this opportunistic anaerobic human pathogen under low or limited oxygen conditions similar to the ones that occur during anaerobic infection in human tissues.

In this regard, as a first step to understand gene expression in *B. fragilis* during infection, we demonstrate in this study that the *ahpC* and *dps* genes are expressed following

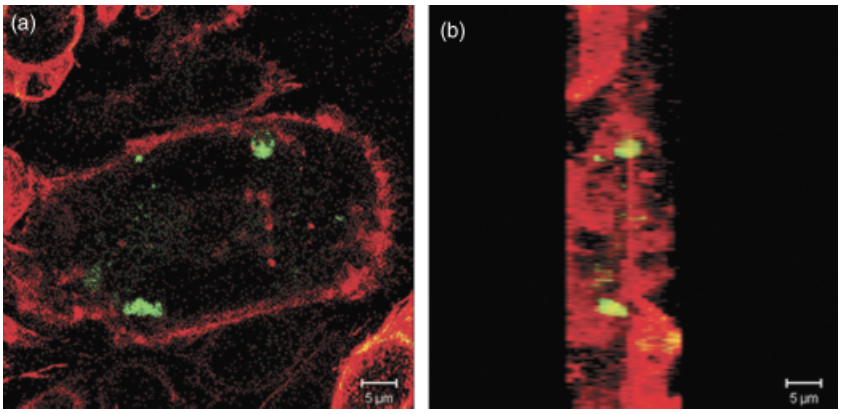

Fig. 6. Confocal laser microscopy of intracellular expression of BS2 in *Bacteroides fragilis* 638R *dps*::*bs2* (BER-96) following internalization by the murine macrophage cell line J774.1. (a) Bacterial internalization was carried out for 1 h and visualized with laser confocal microscopy as described in Materials and methods. (b) The Z-Stacks of the image layers obtained in (a) were piled up and rotated to a 90° angle.

incubation with a cell line macrophage. These findings indicate that *B. fragilis* cells were internalized by macrophages and that its intracellular environment induced *B. fragilis* oxidative stress response as demonstrated by the upregulation of the *ahpC*::*bs2* and *dps*::*bs2* transcription fusion. In animal models of intraperitoneal infection, the *B. fragilis* oxidative stress response is required for survival (Sund *et al.*, 2008). This is likely due to the fact that *oxyR* and *dps* mutant strains, which are deficient in the regulation of the peroxide stress response and protection against oxidative DNA damage, are unable to survive in the peritoneal cavity. Our findings correlate with the fact that intracellular upregulation of *ahpC* and *dps* expression may be an important defense for survival against phagocytic cells of the immune system. Although oxidative killing mechanisms of the phagocytic cells may contribute to *B. fragilis* oxidative stress response *in vivo*, the current study cannot rule out that other intracellular environmental conditions may also affect the expression of *ahpC* and *dps*. One such factor is the depletion of iron availability in the phagolysosome compartment. Induction of *ahpC* and *dps* expression is also affected by iron limitation *in vitro* (data not shown), which could account for additional regulation of *B. fragilis* genes following internalization by phagocytic cells.

In conclusion, these results indicate that the FbFPs are suitable to be used as transcriptional fusion reporters in obligate anaerobic bacteria. There seems to be a significant potential for FbFPs in the analysis of gene expression *in vivo* in anaerobic environments such as the human colon as well as in extraintestinal infections when used in combination with modern *in vivo* imaging techniques.

Acknowledgements

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