Oxidative stress-related responses of Bifidobacterium longum subsp. longum BBMN68 at the proteomic level after exposure to oxygen

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Bifidobacterium longum subsp. longum BBMN68, an anaerobic probiotic isolated from healthy centenarian faeces, shows low oxygen (3 %, v/v) tolerance. To understand the effects of oxidative stress and the mechanisms protecting against it in this strain, a proteomic approach was taken to analyse changes in the cellular protein profiles of BBMN68 under the following oxygen-stress conditions. Mid-exponential phase BBMN68 cells grown in MRS broth at 37 °C were exposed to 3 % O2 for 1 h (I) or 9 h (II), and stationary phase cells were subjected to 3 % O2 for 1 h (III). Respective controls were grown under identical conditions but were not exposed to O2. A total of 51 spots with significant changes after exposure to oxygen were identified, including the oxidative stress-protective proteins alkyl hydroperoxide reductase C22 (AhpC) and pyridine nucleotide-disulfide reductase (PNDR), and the DNA oxidative damage-protective proteins DNA-binding ferritin-like protein (Dps), ribonucleotide reductase (NrdA) and nucleotide triphosphate (NTP) pyrophosphohydrolases (MutT1). Changes in polynucleotide phosphorylase (PNPase) plus enolase, which may play important roles in scavenging oxidatively damaged RNA, were also found. Following validation at the transcriptional level of differentially expressed proteins, the physiological and biochemical functions of BBMN68 Dps were further proven by in vitro and in vivo tests under oxidative stress. Our results reveal the key oxidative stress-protective proteins and DNA oxidative damage-protective proteins involved in the defence strategy of BBMN68 against oxygen, and provide the first proteomic information toward understanding the responses of Bifidobacterium and other anaerobes to oxygen stress.

INTRODUCTION

Bifidobacteria are anaerobes in the Actinomycetales branch of the high-G+C Gram-positive micro-organisms. Many species of Bifidobacterium have been isolated from the mammalian gastrointestinal tract (Schell et al., 2002). They are considered probiotics due to their health-promoting properties in humans (Blandino et al., 2008; Masco et al., 2006; Trebichavsky et al., 2009), and are used live in numerous food preparations (Masco et al., 2005; Shah, 2000).

Abbreviations: CBB, Coomassie Brilliant Blue; 2-DE, 2D electrophoresis; DOC, dissolved oxygen concentration; PNDR, pyridine nucleotide-disulfide reductase; PNPase, polynucleotide phosphorylase; ROS, reactive oxygen species; SOD, superoxide dismutase; sqRT-PCR, semiquantitative RT-PCR.

Although bifidobacteria are considered strict anaerobes, their sensitivity to oxygen differs among species and strains (Biavati & Mattarelli, 2001). Bifidobacterium longum subsp. longum, B. longum subsp. infantis and Bifidobacterium breve can grow in L-shaped tubes with occasional shaking (10 s every 30 min) (Shimamura et al., 1992). Bifidobacterium bifidum JCM 1255T and B. longum subsp. longum JCM 1217T can grow under 5 % oxygen, and Bifidobacterium boum JCM 1211T and Bifidobacterium thermophilum JCM 1207T under 20 % oxygen in the culture-bottle headspace with vigorous shaking (150 r.p.m.) at 37 °C (Kawasaki et al., 2006). Bifidobacterium animalis subsp. lactis 1941, Bifidobacterium pseudolongum subsp. pseudolongum 1944 and B. longum subsp. longum 55815 are able to grow well in static culture in flasks placed in a hypoxic glove box under 21 % oxygen with agitation by magnetic stirrer (Talwalkar & Kailasapathy, 2003).
Bifidobacterium has neither a respiratory chain nor catalase (Biavati & Mattarelli, 2001). Exposure to oxygen causes the accumulation of reactive oxygen species (ROS), mainly H₂O₂, leading to cell death (Kawasaki et al., 2006, 2009a; Talwalkar & Kailasapathy, 2004). Bifidobacterium responses to oxygen stress have been studied at the individual enzyme level (Kawasaki et al., 2006; Shimamura et al., 1992; Talwalkar & Kailasapathy, 2003, 2004). The activities of NADH oxidase, NADH peroxidase and superoxide dismutase (SOD), enzymes which can minimize oxygen toxicity, have been compared under aerobic and anaerobic conditions in Bifidobacterium species (Shimamura et al., 1992). NADH oxidase and NADH peroxidase are induced in Bifidobacterium when strains are exposed to oxygen (Talwalkar & Kailasapathy, 2003), whereas the SOD level is independent of oxygen sensitivity, although it is considered important in detoxifying molecular oxygen (Talwalkar & Kailasapathy, 2004). Genome sequence analysis has indicated that B. longum subsp. longum does not have NADH peroxidase or SOD, although it does have an NADH oxidase homologue, together with three other predicted proteins that decrease oxidative damage, namely thiol peroxidase, alkyl hydroperoxide reductase (AhpC) and peptide methionine sulfoxide reductase (Schell et al., 2002). Although NADH oxidase and NADH peroxidase have been proven to play a role in the Bifidobacterium response to oxidative stress, overall changes in the protein network and detailed mechanisms governing this response are unknown.

B. longum subsp. longum BBMN68 (CGMCC 2265) is a human gastrointestinal tract strain isolated from the faeces of a healthy centenarian from Bama Yao Autonomous County, a location in China that is famous for the longevity of its population and its unique ecological characteristics (Hao et al., 2011). The full-length genome sequence of this strain has been completed (GenBank accession no. NC_014656) and subjected to KEGG annotation (http://www.genome.jp/kegg/). BBMN68, a potential probiotic (Yang et al., 2009a, b) used in the food industry, is sensitive to oxygen, and in fact shows lower oxygen tolerance than other reported strains (Talwalkar & Kailasapathy, 2003). Oxygen exposure of Bifidobacterium is inevitable during its handling and processing in the food industry. To understand the response of B. longum subsp. longum to oxygen stress in this ecological niche, we applied a proteomic approach together with semiquantitative RT-PCR (sqRT-PCR) and cell physiology assays to characterize and compare protein profiles, gene expression and growth changes in this strain under oxygen stress versus anaerobic conditions. AhpC C22 and pyridine nucleotide-disulfide reductase (PNDR), both related to protein protection, were found to be upregulated following O₂ stress. Differential expression was also found for the nucleotide triphosphate (NTP) pyrophosphohydrolases (MutT1), DNA-binding ferritin-like protein (Dps), ribonucleotide reductase (NrdA), polynucleotide phosphorylase (PNPase) and enolase, all potentially involved in DNA and RNA protection under oxygen stress in BBMN68. The physiological and biochemical functions of recombinant BBMN68 Dps expressed in Escherichia coli were confirmed by in vitro DNA protection and cell survival in vivo under oxidative stress. To the best of our knowledge, this is the first proteomic analysis of the mechanisms underlying the response of B. longum subsp. longum to oxidative stress.

METHODS

Strain, medium and culture conditions. B. longum subsp. longum strain BBMN68 (CGMCC 2265) was isolated from healthy centenarians in Bama longevity villages in China (Yang et al., 2009a, b). MRS broth (Oxoid) was used for BBMN68 culture at 37°C in infusion vials and Hungate tubes under modulated atmospheres. The head-space of the injection vials (300 ml capacity) and Hungate tubes were initially sparged with a gas mixture of 80% N₂/10% H₂/10% CO₂ to dissipate the residual oxygen in the MRS medium.

Overnight BBMN68 culture was inoculated (1 %, v/v) by syringe into injection vials containing 100 ml pre-warmed MRS medium, and the culture was grown at 37°C under anaerobic conditions (80% N₂/10% H₂/10% CO₂). When growth reached the mid-exponential phase (OD₆₀₀ 0.6), an oxygen concentration of 1–5% (v/v) was established in the injection vial headspace by published methods (Lobo et al., 2007; Mukhopadhyay et al., 2007): the ratio of the volumes of the headspace and liquid medium was kept at 2:1 (v/v) in the injection vials, and the required oxygen content in the headspace gas was achieved by injecting air with a syringe (based on an O₂ concentration of 21% by volume in air). After the headspace gas component had been modulated, injection vials were incubated at 37°C with gentle horizontal shaking (100 r.p.m.). Samples were collected at different time points.

Bacterial growth and determination of oxygen concentration in the medium. For the growth curve assay, strain BBMN68 was grown at 37°C in MRS broth in injection vials as above with different headspace O₂ contents. At each sampling time, 1 ml culture was withdrawn via syringe and centrifuged for 5 min at 8000 g, 4°C, and the cell-containing pellet was resuspended in 1 ml 50 mM Tris/HCl buffer, pH 6.8, for OD₆₀₀ determination. Viable cell counts were determined in duplicate at each interval by standard colony counting. Briefly, after decimal dilutions, a 1 ml aliquot of the culture was plated on MRS agar (Oxoid) containing 0.05 g cystine hydrochloride I⁻, and colonies were counted after incubation at 37°C for 48 h in an anaerobic jar filled with mixed gas (80% N₂/10% H₂/10% CO₂).

For dissolved oxygen concentration (DOC) assays, 80 ml MSR culture was collected from the injection vial and quickly measured under anaerobic conditions by a JPB-607 Dissolved Oxygen Analyser (Shanghai Precision & Scientific Instrument Co.).

Preparation of whole-cell proteins. BBMN68 cells in 100 ml cultures were harvested under the following conditions. (I) Cultures in the mid-exponential phase were treated with 3% (v/v) O₂ for 1 h. Non-O₂-treated cultures grown under otherwise identical conditions served as the control. (II) Cultures in the mid-exponential phase were treated with 3% O₂ for 9 h, and non-treated cultures grown under the same conditions served as the corresponding control. (III) Cultures in the stationary phase were treated with 3% O₂ for 1 h and corresponding controls were provided. The cells were harvested by centrifugation at 8000 g for 5 min at 4°C and quickly washed twice with 20 ml 50 mM Tris/HCl buffer, pH 6.8. The cell pellets were immediately frozen in liquid N₂ and stored at −80°C for later use. All cultures were grown in triplicate (biological replicates). Equal
Oxygen stress in *Bifidobacterium longum* BBMN68

For whole-cell protein extraction, the cell-containing pellet (about 0.20 g) was resuspended in 3 ml lysis buffer containing 7 M urea, 2 M thiourea, 4 % (v/v) CHAPS, 50 mM DTT and complete protease inhibitor cocktail (Roche). The cell suspension was sonicated for 25 min on ice using an ultrasonic processor (Ningbo Scientz Biotechnology Co., Scientz-IID) at 240 W with 2 s pulses at 6 s intervals. Nuclease mix (10 μl ml⁻¹, GE Healthcare) was added to the cell lysate and the mixture was incubated for 1 h at room temperature, then centrifuged for 30 min at 18 000 g at 4 °C. The supernatant was collected and the protein concentration was quantified using the 2-D Quant kit (GE Healthcare). Three independent gels (IPG strips; technical replicates) were run for each sample in the 2D-PAGE below.

### 2D gel electrophoresis (2-DE) and image analysis

For electrophoresis in the first dimension, total whole-cell protein (400 μg) was loaded onto the IPG strips (18 cm, pH 4–7; GE Healthcare) which had been rehydrated overnight with 350 μl rehydration solution (7 M urea, 4 % CHAPS, 50 mM DTT, 1 %, v/v, IPG buffer, pH 4–7). IEF was performed in an IGPorphor system (GE Healthcare), applying the following voltage gradient: 100 V for 3 h, from 100 to 500 V for 2 h, 500 V for 2 h, from 500 to 1000 V for 1 h, 1000 V for 1 h, from 1000 to 8000 V for 4 h, 8000 V for 9 h, for a total of 91 kVh. The strips were equilibrated twice for 15 min each in 10 ml equilibration buffer (50 mM Tris/HCl, pH 8.8, 6 M urea, 30 %, v/v, glycerol, 2 %, w/v, SDS, and 0.002 %, w/v, bromophenol blue) containing 2 % (w/v) DTT in the first equilibration and 2.5 % (w/v) iodoacetic acid in the second. For electrophoresis in the second dimension, SDS-PAGE (12.5 %) was performed using an Etan Dalt twelve (GE Healthcare) at 0.5 W per gel for 0.5 h, then at 6 W per gel until the dye front reached the gel bottom. Protein was stained using a sensitive colloidal Coomassie Brilliant Blue (CBB) G250 procedure (Candido et al., 2004). The gels were scanned at 400 d.p.i. (Perfection 4900 scanner, Epson). Image analysis was carried out with ImageMaster 2D Platinum version 5.0 software (GE Healthcare). The gels corresponding to the samples were compared and matched so as to attribute a common identified spot to the same spot on different images. The volume of each spot on the three replicate gels was normalized to the total spot volume from the reference gel, quantified, and then subjected to one-way analysis of variance (ANOVA).

### In-gel digestion and protein identification

Protein spots of interest were manually excised from all replicate gels and transferred to a 0.6 ml tube, then washed twice with MilliQ water for 10 min, and destained twice with 25 mM ammonium bicarbonate in 50 % (v/v) acetonitrile. After dehydrogenation in 100 % acetonitrile for 20 min, the gel pieces were vacuum-dried and rehydrated for 1 h at 4 °C with 0.015 mg trypsin ml⁻¹ in 25 mM ammonium bicarbonate and 1 mM CaCl₂, then incubated for 12 h at 37 °C. The resulting tryptic fragments were extracted three times in 10 μl aliquots of 0.1 % (v/v) trifluoroacetic acid (TFA) in 50 % acetonitrile for 1 h at 37 °C, and concentrated to between 5 and 6 μl by vacuum centrifugation. Peptides from in-gel-digested proteins were mixed with matrix solution [α-cyano-4-hydroxycinnamic acid (CHCA) in 0.1 % TFA and 50 % acetonitrile] and spotted (1 μl) on a 384-MPT AnchorChip plate (Bruker) twice, then left to air-dry at room temperature. The analyses were performed using an Autoflex II TOF-TOF instrument (Bruker Daltonics). The mass spectrometer was operated under 19 kV accelerating voltage, and a mass range between 600 and 4000 Da was obtained in the reflection mode. Proteins were identified by peptide mass fingerprinting (PMF) using Mascot (v2.1, Matrix Science) to search the National Center for Biotechnology Information (NCBI) nr protein database. Parameters for searches were as follows:

- **m/z tolerance**: 0.10 Da
- **specificity**: Peptide tolerance
- **database**: NCBI nonredundant protein database
- **search options**: Mowse score cut-off, 50 %
- **other options**: Max missed cleavages: 2, Monoisotopic mass
- **variable modifications**: Carbamidomethylation of cysteine, oxidation of methionine
- **fixed modifications**: Scoring: 50 %

Protein sequences were identified by MALDI-TOF MS using the Mass Spectrometry Facility at the Department of Biochemistry. All identifications were confirmed using the Mascot search tool (www.matrixscience.com). Parameters for searches were as follows:

- **m/z tolerance**: 10 ppm
- **specificity**: Peptide tolerance
- **database**: NCBI nonredundant protein database
- **search options**: Mowse score cut-off, 50 %
- **other options**: Max missed cleavages: 2, Monoisotopic mass
- **variable modifications**: Carbamidomethylation of cysteine, oxidation of methionine
- **fixed modifications**: Scoring: 50 %

### Gene-expression analysis by sqRT-PCR

Total RNA was extracted from BBMN68 cultures using TRIzol Reagent (Sigma) according to the manufacturer’s instructions, and treated with RNase-free DNase. Then, 1–2 μg total RNA was used to synthesize the first-strand cDNA using a SuperScript RT-PCR kit (Invitrogen). Both RNA and cDNA concentrations were determined spectrophotometrically (GeneDrop ND-1000, Gene Company Limited). For all analyses, sqRT-PCR assays were performed in a 25 μl system containing PCR Master Mix (MBI), 30 ng first-strand cDNA template and 0.4 μM primers (Table 1). The PCR conditions were as follows: 94 °C for 5 min, 27–34 cycles of 94 °C for 30 s, annealing for 30 s, 72 °C for 30 s, and a last run at 72 °C for 10 min. Gene-expression levels were quantified as band fluorescence intensity on a 2 % agarose gel after electrophoresis using 1D Image Analysis software (Scientific Imaging Systems). Band intensities were normalized to the 16S rDNA transcript band for relative quantification (Diaz et al., 2006; Le Fourn et al., 2008).

### Cloning, recombinant expression, and protein purification of Dps from BBMN68

PCR primers were designed to amplify the *dps* gene from BBMN68 (GI: 312133610). The forward primer (5’-CCATAGACACTTGCAATTGTGGC-3’) included an Ndel restriction endonuclease site (bold type) directly upstream of the start codon (underlined). The reverse primer (5’-TTCTCGAGTTGAGCGC-3’) included a Xhol site (bold type), and the stop codon was removed to allow attachment of a His-tag to the C-terminus of the recombinant protein. The resulting PCR product was digested with Ndel and Xhol and ligated into Ndel- and Xhol-digested pET-31b (+) (Novagen). The final pET-31b (+) -dps was transformed into *E. coli* BL21(DE3) (Transgen) for protein expression (Supplementary Fig. S1).

The overexpressed recombinant Dps was affinity-purified with nickel nitritolriacetate (Ni-NTA) agarose (Qiagen) according to the manufacturer’s instructions. The purity of Dps was checked by CBB R-250 staining after 12.5 % SDS-PAGE (Supplementary Fig. S2). The presence of purified protein was validated by MALDI-TOF MS (Supplementary Fig. S3). The purified protein was dialysed against 50 mM acetate buffer (pH 5.0) overnight. Protein concentration was quantified using the 2-D Quant kit (GE Healthcare).

### Assays for DNA protection in vitro and oxidative stress protection in vivo

The recombinant BBMN68 Dps was subjected to a DNA protection assay following a published method (Grove & Wilkinson, 2005), with minor modifications. Briefly, Dps was cross-linked in 50 mM acetate buffer (pH 5.0) with 0.1 % (v/v) glutaraldehyde at 37 °C for 2 h. Then, Dps and linearized pUC19 DNA (0.1 μg) were incubated for 1 h at 37 °C in a 20 μl reaction volume. Fe(NH₄)₂(SO₄)₂ (final concentration 100 μM) and H₂O₂ (final concentration 5 mM) were added. After incubation for 5 min at room temperature, reaction mixtures were loaded onto a 1 % agarose gel containing Gold View, and DNA was resolved by agarose gel electrophoresis in Tris-acetate EDTA buffer at 115 V for 15 min, and then photographed under UV illumination.

Survival of *E. coli* cells overexpressing Dps was tested by a H₂O₂ disk-diffusion assay (López-Gomollón et al., 2009). Transformed *E. coli* strain BL21(DE3) was grown in Luria–Bertani (LB) broth at 37 °C with 180 r.p.m. shaking. When OD₆₀₀ reached 0.5, IPTG was added.
to each culture (0.5 mM final concentration). After 2 h of IPTG induction at 30 °C, 400 μl culture was spread on LB agar plates (12 cm Petri dishes) containing 0.5 mM IPTG. Aliquots of 2 μl 30% H2O2 and twofold serial dilutions of H2O2 were spotted onto the Whatman paper disks (diameter 0.6 cm) on top of an LB agar plate coated with E. coli. The growth inhibition areas were measured following incubation of the cells for 16 h at 37 °C.

Iron incorporation by Dps and ferroxidase activity. Dps iron-binding and ferroxidase activity assays were carried out according to published methods (Gupta & Chatterji, 2003; Huang et al., 2010; Leong et al., 1992), with some modifications. Briefly, Dps that had been dialysed against 50 mM acetate buffer (pH 5.0) overnight was incubated with 1 mM ferrous ammonium sulfate for 1 h at room temperature (25 °C). The reaction mixture was separated by 10% native PAGE. The gel was stained with 100 mM K3[Fe(CN)6] for 20 min in the dark, then transferred to a solution containing 10% (v/v) methanol and 10% (v/v) TCA. After 30 min, the gel was destained with a solution containing 5% (v/v) sulfosalicylic acid and 10% (v/v) TCA. The gel was scanned and then further stained with CBB G250. BSA and horse spleen ferritin served as negative and positive controls, respectively.

For the ferroxidase activity assay, 2 μg Dps μl⁻¹ and 100 μM ferrous ammonium sulfate in 50 mM acetate buffer (pH 5.0) were incubated at room temperature. At different time points, 250 μl aliquots of the reaction were taken and A305 was determined, then 25 μl 30 mM ferrozine solution dissolved in 1 M ammonium acetate was added to terminate the reaction, and A570 was measured. The absorbance of controls without Dps addition in the same reaction systems was assayed in parallel at 305 and 570 nm.

RESULTS

Growth of B. longum subsp. longum BBMN68 under different oxygen exposures

Following the oxygen stress treatments (1, 3, 4 and 5% O2 in the headspace) begun at the mid-exponential phase (OD600 0.6), 1% O2 was found to have little effect on strain growth, whereas 3% O2 reduced both the growth rate and maximum growth of BBMN68, and exposure to 4 and 5% O2 resulted in complete growth inhibition (Fig. 1a).

The viability of BBMN68 grown under 3% O2 in the headspace was measured (Fig. 1b): the log(c.f.u. ml⁻¹) reached 8.90 ± 0.19 in the absence of O2 and 8.50 ± 0.06 in the presence of 3% O2 after 2 h. The data illustrated that 3% O2 causes an obvious reduction in growth rate and viability but is not lethal. These results were in accordance with the lower oxygen tolerance of this strain.

Changes in DOC in the MRS broth with BBMN68 cultures and blank medium following O2 treatment are shown in Fig. 2. In the mid-exponential and stationary phases, 3% O2 in the headspace generated DOC values of 2.7±0.07 and 3.2±0.07 mg l⁻¹ in the broths, respectively. For the cultures with mid-exponential phase cells exposed to 3% O2, the DOC decreased dramatically from 2.7±0.07 to 2.0±0.05 mg l⁻¹ (Fig. 2): 26% of the dissolved oxygen was consumed in the first 2 h, and there was no obvious change in DOC for up to 8 h afterward. For cultures with stationary phase cells exposed to 3% O2, no change in DOC was observed (Fig. 2). DOC generation is complex, and correlated with O2 dissolution and consumption. Fig. 2 indicates that the dissolved oxygen in the broth subjected the cells to oxygen stress.

Differentially expressed proteins under oxidative stress conditions

Differentially expressed proteins were first identified by ANOVA: protein spots showing a significant difference at P<0.05 were selected for further screening based on the fold change in relative volume. The fold change in the relative amounts of most proteins between oxygen stress and control conditions was found to range from 1.0 to 2.5, and a threshold of biological significance of 1.7-fold was selected for analysis of the data. This relatively low fold-change value was chosen because, in general, the change in protein expression following environmental stress in B. longum subsp. longum appears to be low: earlier studies with this organism have set thresholds of 1.5- and 1.9-fold to indicate a significant change in protein level (Sánchez et al., 2005, 2007). The proteins of BBMN68 differentially expressed under oxygen stress are shown in Fig. 3, and all of the identified proteins are annotated and listed in Table 2.

Changes in the BBMN68 protein profile following its response to O2 under condition I are shown in Fig. 3(a, b). A total of 27 proteins were differentially expressed between the stressed and control samples: 17 were upregulated and 10 were downregulated. Twenty-three of these spots were successfully identified: spots 1 and 2 were identified as alkyl hydroperoxide reductase C22 (AhpC); these two distinct spots were increased and decreased in the gel by O2 treatment, respectively. Based on an earlier report (Wolf et al., 2008), AhpC expression did not show any

### Table 1. Primers used in sqRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16s rDNA</td>
<td>BL1626</td>
<td>CGATGCAACGGCAAGAACCC</td>
<td>AATCCGCGTGGGCAACAGG</td>
</tr>
<tr>
<td></td>
<td>BL0615</td>
<td>TCGAGGAGCTGACATTTGAGG</td>
<td>GACGGTGGAAACATGATGGC</td>
</tr>
<tr>
<td>ahpC</td>
<td>BL0670</td>
<td>AAGATCGAGATTCGGAGGAGG</td>
<td>CTGGGCTGATGTGGTTCCTC</td>
</tr>
<tr>
<td>nrdA</td>
<td>BLD_1592</td>
<td>ACATGTCTACCTCGGTTCAC</td>
<td>CGGGAAAGAACGGGTCAGC</td>
</tr>
<tr>
<td>mutT1</td>
<td>BLD_1410</td>
<td>AACGTGACCGCCGCGGAACCT</td>
<td>TTGGCGATACGGGGTTGTCCT</td>
</tr>
<tr>
<td>dps</td>
<td></td>
<td></td>
<td>CGATCGAGGCGCTTGAATGCTC</td>
</tr>
</tbody>
</table>
measurable quantitative change, but rather a shift from its reductive (spot 2) to oxidative (spot 1) isoform. Spots 8 and 9 were sigma 54 modulation protein/ribosomal protein S30EA (also called ribosome-associated protein Y; pY). This protein has been reported to bind to dsRNA and the small ribosomal subunit to stabilize the ribosome against dissociation when bacteria are under environmental stress (Ye et al., 2002); it also arrests translation by interfering with aminoacyl-tRNA binding to the ribosomal A site (Agafonov et al., 2001), which may lead to a reduction in the protein biosynthesis rate. This allows cells to adjust to the oxidative stress by improving translation fidelity. DNA-binding ferritin-like protein (Dps), ribonucleotide reductase (NrdA), PNDR and NTP pyrophosphohydrolases (MutT1) were all upregulated under condition I.

Under condition II, a total of 17 protein spots were found to be differentially expressed between the stressed and control samples: six were upregulated and 11 were downregulated (Fig. 3c, d). Fifteen of these protein spots were successfully identified. It is interesting to note that one AhpC spot showed a strong increase upon oxidative stress while the other did not. The expression of AhpC (spot 28 in Fig. 3d) was increased at both the protein and transcriptional levels following the O2 treatment (Figs 3d and 4). This is in agreement with earlier reports on the obligate anaerobes Bacteroides fragilis and Staphylococcus sp. under oxidative stress (Rocha & Smith, 1999; Wolf et al., 2008). NrdA was also upregulated.

Under condition III, oxidative stress induced differential expression of only eight protein spots, four upregulated and four downregulated (Fig. 3e, f). Among them, seven spots were successfully annotated. Spot 45 (CTP synthase), spots 47 and 48 (sigma 54 modulation protein/ribosomal protein S30EA) and spot 46 (glycyl-tRNA synthetase) were upregulated, and these proteins are related to protein synthesis, while the three downregulated spots were identified as metabolic enzymes (Table 2). No oxidative stress-response proteins were found under condition III.

Under all three O2 treatments, the oxygen-sensitive enzymes glyceraldehyde-3-phosphate dehydrogenase C
cloned the BBMN68 Dps (BBMN68_1349) and over-
DNA protection presented 2-DE results (data not shown).
this analysis generally showed good agreement with the
and replicates of the samples from condition I subjected to
(2-D DIGE) has recently been established in our laboratory,
method of 2-D fluorescence-difference gel electrophoresis
members of a gene family (Zhang
different regulation of the rates of mRNA and protein
encoded proteins did not. Such inconsistencies between
upregulation under condition III, whereas the levels of their
nrdA mRNA under condition II was in agreement with their
AhpC, BL1626, NrdA, MutT1 and Dps were upregulated
shown in Fig. 4. The mRNA expression profiles of these genes are
carried out. The mRNA expression profiles of these genes are
proteins were selected (Table 1) and sqRT-PCR analysis was
stress, genes encoding five of the differentially expressed
To monitor the mRNA levels of genes induced by oxidative
physiological activity of Dps was also assayed by
monitoring the resistance of the strains to H2O2.
(Fig. 3. 2-DE maps of all proteins of B. longum subsp. longum strain BBMN68 under different oxidative-stress conditions. (a) Non-oxidative stress control for condition I, (b) treatment for condition I (3 % O2 stress for 1 h at the mid-exponential phase); (c) non-oxidative stress control for condition II, (d) treatment for condition II (3 % O2 stress for 9 h from the mid-exponential phase); (e) non-oxidative stress control for condition III, (f) treatment for condition III (3 % O2 stress for 1 h in the stationary phase). The protein spots with significantly changed expression are indicated by arrows and numbered. The identified proteins are listed in Table 2; unidentified spots are circled. MM, molecular mass.

Analysis of selected genes by sqRT-PCR
To monitor the mRNA levels of genes induced by oxidative
stress, genes encoding five of the differentially expressed
proteins were selected (Table 1) and sqRT-PCR analysis was
carried out. The mRNA expression profiles of these genes are
shown in Fig. 4. The mRNA level and protein abundance of
AhpC, BL1626, NrdA, MutT1 and Dps were upregulated
under condition I. The upregulated expression of ahpC and
nrdA mRNA under condition II was in agreement with their
protein levels. However, nrdA, ahpC and dps showed
upregulation under condition III, whereas the levels of their
encoded proteins did not. Such inconsistencies between
proteomic and transcriptional levels of a specific protein are
sometimes found, possibly due to an error in the methods,
different regulation of the rates of mRNA and protein
turnover, or coverage of several protein isoforms or several
members of a gene family (Zhang et al., 2008, 2009). The
method of 2-D fluorescence-difference gel electrophoresis
(2-D DIGE) has recently been established in our laboratory,
and replicates of the samples from condition I subjected to
this analysis generally showed good agreement with the
presented 2-DE results (data not shown).

DNA protection in vitro and cell survival in vivo
Dps protein exhibited one of the largest expression changes
when BBMN68 was under oxygen stress (Fig. 4). We
cloned the recombinant Dps (BBMN68_1349) and over-
expressed the recombinant protein in E. coli (Supplementary
Figs S1–S3). Fig. 5 shows non-specific DNA
binding of Dps and the protection effect revealed by
electromobility shift assays. Dps actively bound to the DNA
and retarded its mobility in a quantity-dependent fashion
(Fig. 5a). Under oxidative conditions, naked DNA without
Dps binding and protection was rapidly degraded and the
band disappeared (lane 1, Fig. 5b), while DNA with Dps
binding showed a normal DNA–Dps electromobility shift
(lane 3, Fig. 5b). The results illustrated that DNA
protection is successfully achieved by the formation of
DNA–Dps complexes under oxidative stress.

The in vivo physiological activity of Dps was also assayed by
monitoring the resistance of the strains to H2O2. E. coli
cells harbouring pET-31b(+)–dps showed higher survival
than controls under exposure to a wide range of H2O2
concentrations (Fig. 6).

The biochemical function of recombinant BBMN68 Dps
was assayed by monitoring iron incorporation and
ferroxidase activity. Dps protein can bind two Fe2+
at its ferroxidase centres, and convert H2O2 to H2O via the
oxidation of Fe2+ to Fe3+, decreasing the production of
ROS through the Fenton reaction. Fig. 7 shows the
recombinant Dps-sequestered Fe2+ as visualized by
K₃[Fe(CN)₆] staining, giving a royal blue-stained band
(Leong et al., 1992; Gupta & Chatterji, 2003). This
illustrates the biochemical role of Dps–ferrous ion
combination-based enzymic antioxidants. Comparing
Fig. 7(a) with Fig. 7(b), it can be seen that only the
bundled shell-like oligomeric assembly of Dps (different
oligomers in lanes 3–6 in Fig. 7b) has the ability to
incorporate iron in vitro.

Dps ferroxidase activity was also assayed by Fe3+-
ferrozine (a ferrous indicator) complex formation (Fig.
8): the higher the concentration of Fe3+, the higher the
A570. As shown in Fig. 8(a), the A570 demonstrated a
sharp drop under acidic (pH 5.0) conditions, indicating a
high ferroxidase activity of Dps. Fe3+ absorbs at 305 nm,
while Fe2+ does not. As shown in Fig. 8(b), ferrous
ammonium sulfate did not absorb under acidic (pH 5.0)
conditions. Within 5 min of Dps addition, the Fe3+ level
increased sharply. These results illustrate that Dps
catalyses the formation of Fe3+ via the oxidation of
Fe2+ using O2 in the air, i.e. it exhibits ferroxidase
activity at pH 5.

DISCUSSION
In the present work, the responses of B. longum subsp.
longum BBMN68 to 3 % O2 stress at the proteomic level
under short-term (conditions I and III) and long-term
(condition II) O2 stress were found to differ. Proteins
involved in protecting proteins, DNA and RNA from O2
damage may play important roles in the oxidation-
protective mechanisms in BBMN68.

Proteins and the redox system against O2
exposure-derived stress
AhpC has two isoforms, as shown in Fig. 4. Based on an
earlier report (Wolf et al., 2008), we assume that the lower
pI spot is the oxidative isoform of AhpC and the higher pI
spot is its reductive isoform. Short O2 exposure induced a
Table 2. Identification of differentially regulated cellular proteins (>1.7-fold) of *B. longum* subsp. *longum* strain BBMN68 under oxidative stress conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spot no.</th>
<th>Protein name</th>
<th>Accession no.</th>
<th>Locus</th>
<th>Gene</th>
<th>MM*</th>
<th>pI*</th>
<th>Protein score</th>
<th>Sequence coverage (%)</th>
<th>Fold change†</th>
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<td>BL0615</td>
<td>ahpC</td>
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<td>nrdA</td>
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<td>BL1626</td>
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<td>dps</td>
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<td>81780</td>
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<td>BLD_0536</td>
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<td>4.66</td>
<td>121</td>
<td>49</td>
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<td>Molecular chaperone (small heat-shock protein)</td>
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<td>BL0576</td>
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<td>57377</td>
<td>5.11</td>
<td>239</td>
<td>52</td>
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shift in AhpC to its oxidative form, but after prolonged exposure to O$_2$, a certain equilibrium was established between its reductive and oxidative isoforms (Fig. 3c, d and Fig. 4, AhpC, condition II). In B. longum subsp. longum NCC2705, AhpC, together with NADH-oxidase, thiol peroxidase and the peptide methionine sulfoxide reductase, have been found to serve as the main proteins reversing oxidative damage in proteins and lipids, generating a moderate level of aerotolerance (Schell et al., 2002).

AhpC is a key H$_2$O$_2$-degrading enzyme in the superoxide reductase-AhpC-rubredoxin pathway (Mukhopadhyay et al., 2007); however, in the B. longum subsp. longum genome, sequences homologous to superoxide reductase and rubredoxin are not found (KEGG data). Bryk et al. (2000) have proposed that in addition to the above pathway, Mycobacterium tuberculosis has a dihydrolipoamide dehydrogenase (Lpd) and dihydrolipoamide succinyltransferase (SucB) pathway for antioxidant defence. In this pathway, Lpd, AhpCox and AhpCred play key roles in peroxidase activity, protecting against both hydrogen and alkyl peroxides. In BBMN68, Lpd (BLD_0826) showed constitutive expression linked with sugar metabolism, and only a change in AhpC was observed in the 2-DE maps.

The proteins of the PNDR superfamily include one NAD$^+$-binding motif and two FAD-binding motifs; they catalyse the electron transfer between pyridine nucleotides and disulfide compounds, and play important roles in protein functions and folding (Avila et al., 2007). The gene BL1626, encoding a class I pyridine nucleotide-disulfide oxidoreductase in B. longum subsp. longum NCC2705, is highly induced after challenging the cells with H$_2$O$_2$ (Klijn et al., 2005). Here we found that spot 4, a class I PNDR, and its transcription level were both upregulated after oxygen treatment under condition I, which was in good agreement with NCC2705 under H$_2$O$_2$ stress.

### Proteins involved in protecting against oxidative damage to DNA and RNA

MutT1 was induced at both the protein and transcriptional levels under condition I (Fig. 4). MutT1 is a key factor in DNA-damage protection, excision repair and error avoidance (David et al., 2007; Saumaa et al., 2007; Sekiguchi, 1996). The best-studied E. coli MutT1 protein hydrolysates 8-oxo-dGTP to 8-oxo-dGMP, preventing misincorporation of 8-oxoguanine-containing nucleotides into DNA (Maki & Sekiguchi, 1992) and mRNA (Ishibashi et al., 2005). MutT1 protection of vegetative cells of Bacillus subtilis from oxidative stress has been observed (Castellanos-Juárez et al., 2006).

MutT1 belongs to the nudix superfamily of NTP pyrophosphatases, which can hydrolyse a wide range of substrates, including dinucleoside polyphosphates, nucleotide sugars, capped RNA and dinucleotide coenzymes to sanitize non-canonical NTPs in the nucleotide pool; they can also
Fig. 4. Enlargement of AhpC, NrdA, BL1626, Dps and MutT1 protein spots on 2-DE gels, and sqRT-PCR analysis of mRNA expression of the encoding genes. For abbreviations of protein names and gene symbols, refer to Table 2. Spots indicated by arrows and numbered represent the proteins that showed significant variations, and those with an arrow but no number represent proteins that did not change relative to their respective controls. Asterisks indicate a statistically significant difference from the normal condition ($P<0.05$). White bars, relative gene expression under non-oxidative conditions (control); hatched bars, relative gene expression under 3% O$_2$ stress conditions (stress). (I) Cultures in the mid-exponential phase were treated with 3% O$_2$ (stress) or no O$_2$ (control) for 1 h. (II) Cultures in the mid-exponential phase were treated with 3% O$_2$ (stress) or no O$_2$ (control) for 9 h. (III) Cultures in the stationary phase were treated with 3% O$_2$ (stress) or no O$_2$ (control) for 1 h. Data shown are the means ± SD of three independent experiments.
perform a housekeeping function by removing various end products of cell metabolism and/or regulation (Galperin et al., 2006). In the NTP pyrophosphohydrolase family, MazG has been shown to play a role in the oxidative stress response: deletion of mazG in Mycobacterium smegmatis renders the mycobacterium defective in its response to oxidative stress, and expression of wild-type MazG restores cell viability under those conditions (Lu et al., 2010).

Fig. 5. Dps binding and protection of DNA. (a) Dps at different concentrations (0, 5, 10 and 15 μg; lanes 2–5) incubated with pUC19 DNA (0.1 μg) was resolved by agarose electrophoresis and photographed under UV illumination. (b) Dps at different concentrations (0, 0 and 15 μg; lanes 1–3) was incubated with pUC19 DNA (0.1 μg), and Fe(II) (SO₄)₂ (final concentration 100 μM) and H₂O₂ (final concentration 5 mM) were added (except for lane 2). Following resolution by agarose electrophoresis, the gel was photographed under UV illumination.

Fig. 6. Dps protection against H₂O₂ stress as determined by cell growth. (a) E. coli bearing plasmid pET-31b(+) (negative control) and E. coli bearing pET-31b(+) -dps were induced by 0.5 mM IPTG. The diameters of the growth-inhibition zones generated by addition of different H₂O₂ concentrations were recorded. H₂O₂ was loaded at 20.6, 41.2, 82.5, 165, 330 and 660 μg on disks 1–6, respectively. (b) Diameters of the inhibition zones for each concentration of H₂O₂ with the negative control [E. coli with pET-31b(+), dark-grey bars] and with the Dps-overexpressing strain [carrying pET-31b(+) -dps, light-grey bars] corresponding to disks 1–6. Results are given as mean ± SD of six independent measurements.
In addition, we found enolase to be greatly upregulated and PNPase to be downregulated under condition I. PNPase, enolase, endoribonuclease, RNase E, ATP-dependent RNA helicase and RhlB, existing in a multicomponent ribonucleolytic complex (RNA degradosome), are essential for RNA degradation (Carpousis, 2007). Enolase is related to RNA degradation (ko03018), as well as to glycolysis and gluconeogenesis (blj00010). Significantly increased expression of enolase has been found in \textit{B. bifidum} 4549dOx, the bile-resistant derivative of \textit{B. bifidum} CECT4549 and \textit{B. longum} subsp. \textit{longum} NCIMB 8809, upon exposure to bile (Sánchez et al., 2004; Ruiz et al., 2009). PNPase is a major RNA-degrading enzyme in \textit{E. coli} cells and protects \textit{E. coli} against oxidative stress (Wu et al., 2009). PNPase protein decreased rapidly under oxidative stress in BBMN68, consistent with the behaviour of its human counterpart when human cells are exposed to H2O2 and menadione (Hayakawa & Sekiguchi, 2006).

Dps has been found to be induced by oxidative stress in eubacterial and archaeal species, and is a key factor in the protection of prokaryotic cells against oxidative damage. Dps binds nonspecifically to genomic DNA, forming a crystalline-like structure, and the Dps–DNA complex

![Fig. 7. Iron-incorporation ability of Dps. Lanes: 1, BSA (30 μg); 2, horse spleen ferritin (30 μg); 3–6, Dps (30, 60, 90 and 120 μg). BSA, horse spleen ferritin and Dps were incubated with 1 mM Fe(NH4)2(SO4)2, then subjected to native PAGE. The ferrous ion zone was detected as a royal blue band using K3[Fe(CN)6] stain (a) (arrows indicate proteins with ferrous binding), and protein bands were stained with CBB G250 (b).](image)

![Fig. 8. Ferroxidase activity of Dps. Dps (final concentration 2 μg μl\(^{-1}\)) was added to 100 μM ferrous ammonium sulfate to start the reaction. At the indicated time points, a 250 μl aliquot of the reaction was removed, the reaction was stopped with 25 μl 30 mM ferrozine, and the absorbance was determined at 570 nm (a) and 305 nm (b). (○) Control blank (100 μM ferrous ammonium sulfate in acetate buffer, pH 5.0), (■) Dps and ferrous ammonium sulfate in the same buffer. Values are means of three replicates.](image)
protects DNA from oxidative damage physically by compacting chromatin, and chemically by sequestering iron and limiting the Fenton reaction (Haikarainen & Papageorgiou, 2010; Halsey et al., 2004; Zhao et al., 2002). In recent years, other functions of Dps have been reported, including iron binding in several bacterial species; BL0618 has been identified in the genome of B. longum subsp. longum as potentially encoding Dps and possessing conserved iron-binding sites. Hydroxyl radicals are the third type of ROS formed when H₂O₂ is taken up with free iron in the Fenton reaction. This reaction can be prevented by quenching H₂O₂ or sequestering free iron (Klijn et al., 2005).

The role of Dps in oxidative-stress protection in E. coli and M. smegmatis strain mc² 155 has been thoroughly studied and confirmed (Ceci et al., 2005; Choi et al., 2000; Nair & Finkel, 2004). Dps from BBMN68 shares 57 and 67 % homology with Dps from E. coli and M. smegmatis strain mc² 155, respectively, at the amino acid level. At the protein level, Dps was only upregulated in BBMN68 in the active growth phase (condition I); it was also clearly upregulated at the mRNA level under conditions I and II. This suggests that Dps in BBMN68, like its counterparts in other bacteria, plays a role in protecting cells against oxidative stress.

Transcription factors also play crucial roles in regulating stress-response gene expression. dps expression is regulated by many transcription activators, such as SoxRS, OxyR, σ³, σ⁵, SlyA and RecA in Salmonella enterica (Halsey et al., 2004). In E. coli, dps is regulated by RpoS (σ³⁶), OxyR, integration host factor (IHF), Fis and H-NS (Halsey et al., 2004). B. longum subsp. longum lacks homologues of most of these genes; it only has the σ³⁶- and σ³⁰ (RpoD)-homologous protein HrdB, which is encoded by BL1428, and recA (BL1415) (KEGG data) in its genome. As already noted, Dps was increased at the protein level in BBMN68 only in the active growth phase (condition I), and upregulated at the mRNA level under conditions I and II. We also observed recA to be significantly upregulated under conditions I, II and III at the transcriptional level in BBMN68 (Fig. 9), as reported for all eubacteria, in which it is involved in the DNA recombination process (Sakai & Cox, 2009). Thus, recA may be involved in dps activation.

An increase in the ribonucleotide reductase protein NrdA and its mRNA levels was observed in oxygen-treated BBMN68 (Fig. 4). In bacteria, different classes of ribonucleotide reductase existing under various conditions can be induced and overexpressed in the presence of H₂O₂ and aerobic/strict anaerobic growth conditions, respectively. Ribonucleotide reductases play pivotal roles in DNA replication and repair (Nordlund & Reichard, 2006), and are known to decrease the deleterious effect of oxidative stress.

**Fig. 9.** sqRT-PCR analysis of mRNA expression of the recA and ndrHIR genes. Asterisks indicate a statistically significant difference (P<0.05) between the control (white bars) and 3 % O₂ stress conditions (hatched bars).
stress in *E. coli* cells (Monje-Casas et al., 2001). In *B. longum* subsp. *longum*, glutaredoxin-like protein (BLD_1460, NrdH), ribonucleotide reductase stimulatory protein (BLD_1461, NrdI) and NrdA (BLD_1462) are organized on the ribonucleotide reductase operon (KEGG data). Overexpression of *nrdH* and *nrdI* was observed (Fig. 9), in accordance with the increase in NrdA (Fig. 4, NrdA). BLD_0169 encodes the transcriptional regulator NrdR in *B. longum* subsp. *longum*, and its expression was also increased under oxidative stress (Fig. 9). In *Streptomyces coelicolor*, NrdR is a zinc-ATP/dATP-containing protein that binds to the promoter region of ribonucleotide reductase operons (Grinberg et al., 2006). NrdR represses the transcription of ribonucleotide reductase genes in most bacteria via *nrdR* regulation. Therefore, the upregulation of ribonucleotide reductases may accelerate replication and repair of oxidatively damaged DNA, in line with the increase in *nrdHIA* and *nrdR* in BBMN68 (Fig. 9).

To confirm oxidative stress protection by Dps in BBMN68, the functions of Dps were studied in *vitro* and in *vivo*. It was shown that Dps can bind DNA to protect DNA against oxidative degradation in *vitro* (Fig. 5). Overexpression of BBMN68 Dps increased the survival of *E. coli* cells in *vivo* under oxidative challenge (Fig. 6). The results demonstrated that Dps can incorporate and oxidize iron to prevent the formation of ROS by the Fenton reaction in *vitro* (Figs 7 and 8). These results demonstrate that changes in the Dps protein level in BBMN68 have cellular, physiological and biochemical functions in DNA protection and increasing cell survival under oxidative stress.

The above detoxification proteins were distinctly different from those of other anaerobes under oxidative stress conditions. A large number of detoxification proteins are induced by oxidative stress in other anaerobes, such as catalase, SOD and cytochrome c peroxidase, to name a few (Baughn & Malamy, 2004; Das et al., 2005; Kawasaki et al., 2009b; Le Fourn et al., 2008; Miyoshi et al., 2003; Talwalkar & Kailasapathy, 2003). These proteins were not detected in *B. longum* subsp. *longum* strain BBMN68.

In conclusion, a short exposure to O2 in the mid-exponential phase resulted in the largest number of differentially expressed proteins relative to controls, while the same O2 treatment in the stationary phase led to the lowest number of differentially expressed proteins. The differentially expressed proteins under conditions I, II and III all demonstrated the same regulatory trends. Pivotal players in protecting proteins from oxidative stress, such as AhpC, PNDR and S30EA, were upregulated, as were key proteins involved in housekeeping functions that protect DNA, RNA and nucleotide pools from oxidative damage, i.e. Dps, NrdA, MutT1 and enolase. Our findings provide a systematic view of the response of bifidobacteria to short- and long-term moderate O2 exposure, which might partially explain the mechanism in *B. longum* subsp. *longum* that controls oxidative damage and support the potential use of BBMN68 in the food industry.

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