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ORIGINAL ARTICLE

Bacillus subtilis vegetative isolate surviving chlorine dioxide exposure: an elusive mechanism of resistance

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Abstract

Aims: Oxidizing agents such as chlorine dioxide are widely used microbicides, including for disinfection of medical equipment. We isolated a *Bacillus subtilis* isolate from a washer-disinfector whose vegetative form demonstrated unique resistance to chlorine dioxide (0·03%) and hydrogen peroxide (7·5%). The aim of this study was to understand the mechanisms of resistance expressed by this isolate.

Methods and Results: A range of resistance mechanisms were investigated in the *B. subtilis* isolate and a reference *B. subtilis* strain (ATCC 6051) to include bacterial cell aggregation, the presence of profuse exopolysaccharide (EPS), and the expression of detoxification enzymes. The basis of resistance of the isolate to high concentrations of oxidizing agents was not linked to the presence of endospores. Although, the presence of EPS, aggregation and expression of detoxification enzymes may play a role in bacterial survival to low concentrations of chlorine dioxide, it is unlikely that the mechanisms helped tested to survive the bactericidal effect of higher oxidizer concentrations.

Conclusions: Overall, the mechanisms conferring resistance to chlorine dioxide and hydrogen peroxide remains elusive. Based on recent advances in the mode of action of oxidizing agents and notably hydrogen peroxide, we postulate that additional efficient intracellular mechanisms may be involved to explain significant resistance to in-use concentrations of commonly used high-level disinfectants.

Significance and Impact of Study: The isolation of a highly resistant vegetative Gram-positive bacterium to a highly reactive oxidizing agent is worrying. Understanding the mechanisms conferring such resistance is essential to effectively control such bacterial isolates. Here, we postulate that there are still mechanisms of bacterial resistance that have not been fully characterized.

Introduction

Bacterial resistance to biocides is a subject that has been highlighted by the European Commission (SCENIHR, 2009, 2010) and has culminated with a requirement of biocidal product manufacturers to provide information on the risks of resistance development with their products (Biocidal Product Regulation, 2013). The United States Food and Drug Administration (2013) recently required manufacturers of antimicrobial hand washes to provide evidence that their products have no effect on emerging bacterial resistance to antimicrobials. Reports of bacterial resistance (or tolerance) to biocides have not been limited to low level disinfection or preservatives (Scientific Committee on Consumer Safety 2012; Maillard *et al.* 2013), but also to highly reactive chemistries (including alkylating and oxidizing agents) used for high-level disinfection. Van Klingeren and Pullen (1993) were

the first to report isolates of atypical mycobacterial resistance to glutaraldehyde (2%) from endoscope washer-disinfectors. Similar glutaraldehyde-resistant isolates were later observed to be cross-resistant to other highly reactive biocides such as hypochlorite and oxidizers (Griffiths *et al.* 1997) but not to *ortho*-phthalaldehyde (Walsh *et al.* 1999; Fraud *et al.* 2001). More recently Duarte *et al.* (2009) reported a large outbreak with antibiotic resistant *Mycobacterium massiliense*, also resistant to glutaraldehyde (2%). A report has also described the development of mycobacterial (and other bacterial) resistance to glutaraldehyde and/or *ortho*-phthalaldehyde (Fisher *et al.* 2012).

In 2008, we reported the isolation of a number of Gram-positive vegetative bacteria from an endoscope washer-disinfector that used chlorine dioxide for high-level disinfection (Martin *et al.* 2008). Two isolates in particular, a *Bacillus subtilis* and a *Micrococcus luteus,* showed stable high-level resistance to the in use concentration of chlorine dioxide. The *Bacillus* isolate was also cross-resistant to hydrogen peroxide (7·5%) (Martin *et al.* 2008). This study reported that both strains were heavily embedded in a high concentration of exopolysaccharides (EPS). A more recent study on this *B. subtilis* isolate confirmed its ability to produce a very dense biofilm (Bridier *et al.* 2011). Resistance mechanisms to biocides expressed by bacteria can involve diverse mechanisms including a decrease in penetration of the biocide (e.g. through changes in membrane composition and regulation of porins), a decrease in the intracellular biocide concentration (e.g. expression of efflux pumps and detoxification enzymes) (Maillard 2007) and, in a few examples, changes in metabolic pathways (Webber *et al.* 2008) and DNA repair mechanisms (Maillard 2010). Other physical factors can include the production of EPS and cell aggregation, which can contribute to decreased available biocide concentration and cell protection respectively, and are of significant importance in bacterial biofilm resistance (Maillard and Denyer 2009).

In this study, we explore the possible resistance mechanisms in the *B. subtilis* endoscope washer-disinfector isolate (Martin *et al.* 2008) with high-level resistance to chlorine dioxide and hydrogen peroxide.

Materials and methods

Bacterial strains and culture

The endoscope washer-disinfector isolate of *B. subtilis* (48 RW) described in Martin *et al.* (2008) was investigated. The strain speciation was previously confirmed by 16S rRNA identification. *Bacillus subtilis* ATCC 6051, a reference strain sensitive to chlorine dioxide, was used as a 'negative' control (Martin *et al.* 2008). Bacteria were grown overnight in tryptone soya broth (TSB; Oxoid, Basingstoke, UK) at 37°C under constant agitation at 70 rev min^{-1}. The suspensions were then centrifuged at 7000 ***g*** and resuspended in tryptone sodium chloride (TSC; 1 g l^{-1} tryptone; Oxoid; 8·5 g l^{-1} sodium chloride; Fisher Scientific, Loughborough, UK). The absence of endospores in the test culture was scrupulously checked as described in Martin *et al.* (2008).

Removal of exopolysaccharide

Bacterial growth following a 24 h incubation at 37°C on tryptone soya agar (TSA; Oxoid) was recovered from the surface of the plate and added to a polypropylene copolymer centrifuge tubes (Fisher Scientific) containing 20 ml of sterile deionized water and vortexed. The sample was centrifuged at 13 500 ***g*** at 15°C for 30 min. The bacterial pellet was resuspended in 5 ml of sterile deionized water and centrifuge again at 13 500 ***g*** at 15°C for 30 min. The final pellet was resuspended in 5 ml TSC and check for the presence of EPS. Bacterial susceptibility to chlorine dioxide was measured in a carrier efficacy test (see below).

The presence of EPS was visualized by staining. An aliquot of the bacterial suspension was added to sterile glass cover slips (Fisher Scientific) prewashed in ethanol, then placed on a slide, dried and fixed. Slides were stained with a 2 : 1 mixture of aqueous Congo Red (Fisher Scientific) and 10% Tween 80 for 15 min, rinsed with distilled water, counter stained with Zeihl Carbol Fuschin (Fisher Scientific) for 6 min and finally washed with distilled water and air-dried. The slides were observed under 1000× magnification (Bx50 Microscope Olympus; Olympus, Southend-on-Sea, UK). Bacteria appear purple/pink and EPS if present as pale pink). Ten randomly selected fields of vision were investigated, and representative image were recorded and photographed.

Disruption of bacterial aggregates

Bacterial inocula were adjusted to 5×10^8 CFU ml^{-1} and 2·5 ml aliquots sonicated at a frequency of 23KHz with a with piezoelectric transducer sonicator (Soniprep 150). Sonication took place for 1 min prior to chlorine dioxide treatment with (3 g l^{-1} bovine serum albumin, BSA) or without organic load (0·3 g l^{-1} BSA) in a suspension test as described below.

Proteinase K pretreatment

The toxicity of proteinase K was investigated using the Bioscreen C Microbial Growth Analyser (Oy Growth

Curves Ab Ltd., Helsinki, Finland). Fifty microlitre of different concentrations (2, 10, 20, 50, 100, 200 μg ml^{-1}) of proteinase K was added to the wells of a 100-well honeycomb plate (Fisher Scientific) together with 300 μl of TSB and 50 μl of a washed bacterial inoculum. Plates were incubated in the Bioscreen for 14 h taking readings every 15 min. The plates were shaken for 15 s before each reading. Readings were recorded using the EZExperiment software (Oy Growth Curves Ab Ltd).

Washed overnight bacterial cultures were then pretreated with 200 μg ml^{-1} proteinase K (a nontoxic concentration for the bacteria) for 2 h and their susceptibility to chlorine dioxide (0·03%) tested in efficacy tests with or without organic load (see below).

Expression of detoxifying genes

We investigated two genes in particular, *sodA* and *katA*. In vegetative *B. subtilis*, oxidative stress caused by H_2O_2 induces the *katA* gene (Naclerio *et al.* 1995) notably when treated with sublethal concentrations of the oxidizing agents (Bol and Yasbin 1994). Likewise, the superoxide dismutase (SOD) encoded by *sodA* has been deemed to be essential in *B. subtilis* in response to oxidative stress (Inaoka *et al.* 1999).

Primers and DNA extraction

Oligonucleotide primers for catalase (*katA*) and superoxide dismutase (*sodA*) (Table 1) were obtained from Invitrogen (Paisley, UK). The sequence of the *gyrB* primer (which was used as control in reverse transcription polymerase chain reaction (RT-PCR)) (Table 1) was obtained from Prof. Colin Harwood (Newcastle University, UK) and supplied by Invitrogen. DNA was extracted from washed overnight bacterial cultures using Trizol® (Invitrogen) following the manufacturer's protocol.

RNA extraction

Bacterial samples were pretreated with chlorine dioxide 0·03% for 1 min in a suspension test based (see below), prior to RNA extraction. RNA extraction was carried out using the Ambion RiboPure™-Bacteria Kit (Applied Biosystems/Ambion, Austin, TX). Briefly, washed bacterial cells were adjusted to 5×10^8 CFU ml^{-1} and centrifuged at 13 000 ***g*** for 60 s at 4°C. The bacterial pellet was resuspending in RNAwiz into tubes containing zirconia beads (Applied Biosystems/Ambion). The tubes were then mixed for 10 min using a vortex mixer (Genie 2; Scientific Industries Inc., Thermo Fisher, Loughborough, UK) with adapter (Applied Biosystems/Ambion) at maximum speed. Zirconia beads were pelleted by centrifuging at 13 000 ***g*** for 5 min at 4°C. The bacterial lysate was transferred to a fresh tube. One millilitre of chloroform was added to 5 ml of lysate, shaken for 30 s and incubated for 10 min at room temperature. The sample was then centrifuged for 5 min at 4°C at 13 000 ***g***, and the aqueous phase was transferred to a fresh tube.

One millilitre of 100% ethanol was added to 1 ml of the sample and mixed thoroughly. A filter cartridge (Applied Biosystems/Ambion) was placed inside a 2 ml collection tube and the sample was transferred to the filter cartridge and centrifuged at 13 000 ***g*** for 1 min. The flow-through was discarded and the filter returned to the collection tube. The filter was then washed by adding 700 μl of wash solution-1 and centrifuged for 1 min at 13 000 ***g*** at 4°C. The flow-through was discarded and the filter returned to the collection tube. The filter was then washed with 500 μl of wash solution-2/3, centrifuged as stated earlier and the flow-through discarded. This step was repeated with 500 μl of wash solution-2/3. The filter was centrifuged for 1 min at 13 000 ***g*** at 4°C to remove excess wash solution and transferred to a fresh collection tube. The RNA elution step performed according to the manufacturer's instruction (Ambion RiboPure™). The elution step was repeated to retrieve the maximum total RNA. All RNA samples prepared showed an absorption ratio at 260/280 nm above 1·6, which indicated a high-quality nucleic acid was obtained. RNA samples were diluted when necessary with nuclease-free water (Fisher Scientific) to a maximum concentration of 1 μg μl^{-1}, treated with DNAse and stored at −80°C until used.

Table 1 Primers used in this study

Primer	Sequence
katA	F: GGCGTGAAAAACCTTGATGT
	R: TGACATCAAACGGATCGAAA
sodA	F: GCTTTACTCGCTGGGAATTG
	R: TCTCCGGCTAAAAGCACACT
gyrB	F: ACGGCATTACGGTTGAAGTG
	R: TCATCTCCGCTTAGGTTTGG

Bacterial treatment and RT-PCR

Reverse transcription was carried out with the Promega Improm-II Reverse Transcription System (Southampton, UK), using 1 μg RNA in a 20 μl reaction alongside a negative control omitting the enzyme AMV-reverse transcriptase. The RNA template, primer and nuclease-free H_2O initial 5 μl reaction mixture was run at 70°C for 5 min then held on ice until needed. The RT was run at 25°C for 5 min annealing time, 42°C for 60 min extension time and 70°C for 15 min to heat inactivate the AMV-reverse transcriptase, in a Techne Techgene thermal cycler (Jencons-PLS, Leighton Buzzard, UK). The cDNA product was stored at −20°C until used.

Amplification of the DNA and the cDNA from the RT reaction took place in 25 μl reactions using oligonucleotide primers. PCR was conducted using the GoTaq® Flexi DNA Polymerase kit (Promega) and control reactions were carried out either with sterile nuclease-free water instead of cDNA, or using the no-AMV-RT reaction samples instead of cDNA. PCR conditions were as follows; initial denaturation: 95°C for 10 min, denaturation: 95°C for 30 s (30 cycles), annealing: 55°C for 45 s, extension: 72°C for 1 min and final extension: 72°C for 10 min. The PCR amplification products were analysed by gel electrophoresis and stained with ethidium bromide, in 1× TAE buffer (40 mmol l^{-1} Tris-Acetate, 1 mmol l^{-1} EDTA) and visualized under UV light.

Enzyme assays

Catalase production was studied in each *B. subtilis* strain. A drop of washed bacterial cultures was added to a microscope slide followed by a drop of a 3% hydrogen peroxide solution. A positive reaction was observed by the formation of bubbles (oxygen release from catalase positive cells).

To quantify the production of catalase, a revised disc flotation method was used (Gagnon *et al.* 1959). Briefly, a dilution series of catalase (Bovine Liver, Sigma-Aldrich, US) in potassium phosphate buffer was made to obtain the following concentrations: 0·01, 0·0025, 0·001, 0·00025 and 0·0001%. Filter disc (Fisher scientific) were soaked in the different catalase solution for 30 s. The discs were then added to test tubes containing 5 ml of hydrogen peroxide (3%) and the time between the disc touching the surface of the hydrogen peroxide and the disc floating back to the surface was measured (Gagnon *et al.* 1959). This was repeated in triplicate for each concentration and a calibration curve was made. Bacterial supernatant samples obtained from a washed overnight bacterial culture were then used to soak the filter disc. Using the calibration curve, the production of catalase (% w/v) was calculated.

Suspension efficacy test

To understand the effect of EPS and proteinase K on the susceptibility of the bacterial strains to chlorine dioxide a suspension test based on the BS EN1276 (1997) was used. Briefly 1 ml of a washed bacterial test inoculum ($\approx 10^8$ CFU) was added to 8 ml of chlorine dioxide (0·03%) with 1 ml of TSC (containing 0·3 g l^{-1} BSA final concentration: clean condition) or 1 ml of soiling (3 g l^{-1} BSA final concentration: dirty condition). Following 30s, 1, 5, 10 and 30 min exposure, 1 ml of the test suspension was removed and diluted into 9 ml of neutralizer. A viable count was then performed using the pour plate method. Briefly, after a serial dilution, 1 ml of each dilution was added to a petri dish and covered with 20 ml of molten TSA at 40°C. Plates were incubated for 24 h at 37°C and colonies counted. Each experiment was performed in triplicate. A negative control consisted in using TSC instead of chlorine dioxide.

Carrier efficacy test

To understand the role of EPS and potential bacterial aggregation in the resistant strain, a carrier efficacy test based on the BS EN13697 (2001) was performed. Briefly sterile stainless steel discs (grade 2B finish; Goodfellows Cambridge Ltd., Huntingdon, UK) were inoculated with 20 μl ($\approx 10^8$ CFU) of the bacterial test inocula as prepared above. Organic load, when added to the test, consisted of 3 g l^{-1} BSA; clean condition consisted of 0·3 g l^{-1} BSA. The discs were then air-dried for 30 min in an incubator at 37°C before 100 μl freshly prepared chlorine dioxide (0·03%) was added. After 0·5, 1, 5, 10 and 30 min exposure at room temperature (approx. 20°C), a disc was removed and placed in a 100 ml bottle containing 10 ml of 5 g l^{-1} of sodium thiosulphate (Fisher Scientific; neutralizer) and 10 g glass beads. The bottles were then shaken on an orbital shaker for 1 min at 150 rev min^{-1}. A viable count was then performed as described above. Each experiment was performed in triplicate. A negative control consisted in using TSC instead of chlorine dioxide.

Transmission electron microscopy

Overnight broth *B. subtilis* cultures (1×10^8 CFU ml^{-1}) were washed and exposed to chlorine dioxide (0·01 and 0·03%; final volume 2 ml) for 30 s at room temperature (approx. 20°C). The concentrations and contact time used were shown not to cause a complete bacterial kill (Martin *et al.* 2008). Untreated bacteria were used as a control. Following treatment, bacterial samples were mixed with 2 ml of 2·5% glutaraldehyde in 0·1 mol l^{-1} sodium cacodylate buffer (pH 7·4) and centrifuged at 2200 ***g*** for 5 min. The bacterial pellet was then resuspended in 5 ml of 2·5% GTA in 0·1 mol l^{-1} sodium cacodylate buffer (pH 7·4). The sample was then left to fix for 1 h at room temperature, centrifuged as above, washed twice for 5 min in 0·1 mol l^{-1} cacodylate buffer and stained for 1 h in 1% osmium tetroxide in 0·1 mol l^{-1} of cacodylate buffer. This was followed by 3 × 5 min washes in 0·1 mol l^{-1} cacodylate buffer. The samples were then embedded in 3% agar and once set the samples were placed in holders, cut using razor blade and dehydrated progressively with 5 min washes with 30, 50, 70, 80, 90 and 100% ethanol. Samples were then washed in propylene oxide for 10 min. Resin (5 g

Araldite CY212, 5 g dodecyl succinic anhydride and 0·15 g n-benzyldimethylamine) infiltration was then carried out overnight at room temperature. Resin embedding then took place for 2 days at 60°C. Sections were then cut using a Reichert-Jung Ultracut Microtome (Wien, Austria) and placed onto copper grids. Samples were stained with 2% uranyl acetate for 10 min, rinsed twice in distilled water and then immersed in Reynolds lead citrate for 5 min. Grids were dried with filter paper between each step.

Transmission electron microscopy (TEM) samples were examined at 13 000×, 20 000×, 40 000×, 50 000×, 80 000× and 100 000× magnification with an EM 280 transmission electron microscope (Philips, Croydon, UK). Images were taken and examined visually for cellular differences between standard strains and washer-disinfector isolates, pre-exposed or not to chlorine dioxide (0·01% and 0·03%) for 30 s.

Statistical analysis

Statistical analysis was performed using Minitab® (release 14 software; Minitab Inc., State College, PA). One-way analysis of variance (ANOVA) was performed at a 95% level of significance to determine whether differences between the means of data sets were significant. Standard deviations (SD) were also calculated where appropriate.

Results

Role of EPS

Following scanning electron microscope evidence of the presence of large amounts of EPS in the resistant isolate from the washer-disinfector (Martin *et al.* 2008), EPS was removed by repeated washings and the EPS-free bacteria tested for their susceptibility to chlorine dioxide in a carrier test. The culture collection control was treated the same way. Observation of stained bacteria showed no appearance of any pink-stained EPS (data not shown). Figure 1 shows that the presence of EPS made little difference to *B. subtilis* (48 RW) and 6051 susceptibility to chlorine dioxide within short exposure time (<5 min). With longer contact times the presence of EPS contributed marginally (<1 $\log_{10}$) but statistically significantly ($P < 0{\cdot}05$) to a decreased susceptibility to the oxidizer.

The role of EPS in increasing resistance to chlorine dioxide through aggregation and better cohesion on surfaces was explored by using a carrier test (Fig. 2). When dried on surfaces there was no significant difference in chlorine dioxide efficacy between samples with or without EPS for the Washer disinfector (WD) isolate ($P = 0{\cdot}06$) or the ATCC standard strain ($P = 0{\cdot}85$).

Disruption of potential aggregates

The effect of possible bacterial aggregation on the efficacy of chlorine dioxide was investigated by the application of sonication prior to biocide exposure in clean (Fig. 3) and dirty conditions (data not shown). Sonication had no effect ($P > 0{\cdot}05$) on the susceptibility of the standard strain (6501) to chlorine dioxide in both clean (Fig. 3) and dirty conditions (data not shown). Sonication increased significantly ($P < 0{\cdot}05$) the susceptibility of *B. subtilis* (W48) after 1 min exposure when no organic load was present. However, the observed increase was minimal (between 1–2 $\log_{10}$) (Fig. 3). In the presence of organic load, chlorine dioxide (0·03%) failed to kill W48 (data not shown).

Proteinase K pretreatment

Proteinase K was used to digest proteins present in the EPS, including extracellular enzymes that may mop up or

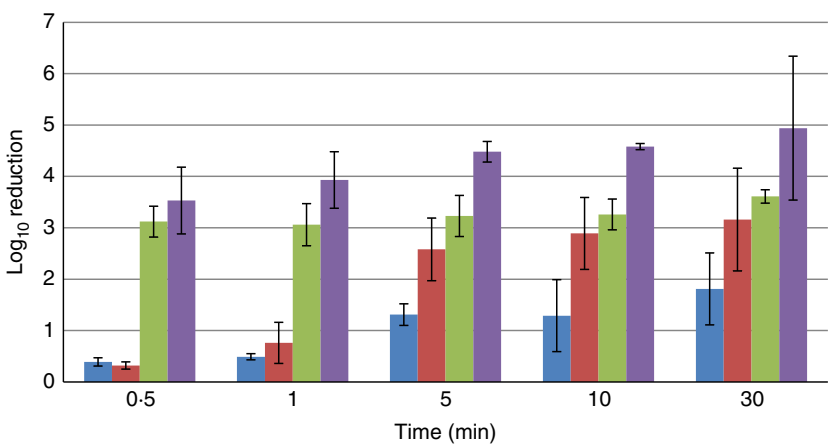

Figure 1 Effect of removal of exopolysaccharide (EPS) on the efficacy of chlorine dioxide (0·03%; no organic load: 0·3 g l^{-1} bovine serum albumin) against *Bacillus subtilis* (ATCC6051) and *B* isolate (48 RW) measured in a suspension test. (■) 48W with EPS, (■) 48W with no EPS, (■) 6051 with EPS, (■) 6051 without EPS.

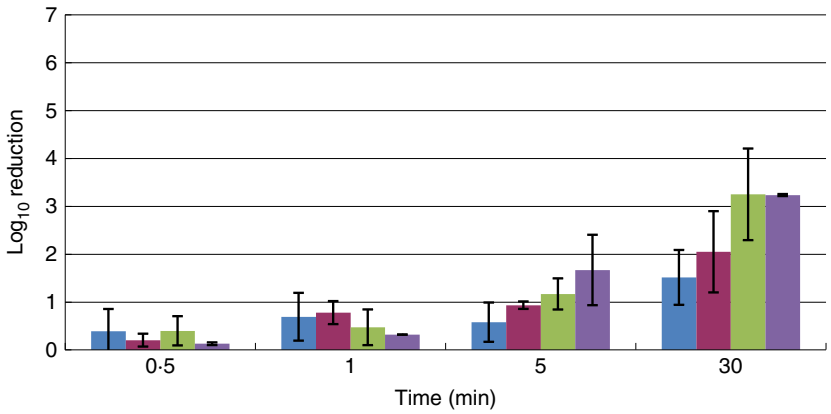

Figure 2 Effect of removal of exopolysaccharide (EPS) on the efficacy of chlorine dioxide (0·03%; no organic load: 0·3 g l^{-1} bovine serum albumin) against *Bacillus subtilis* (ATCC6051) and *B. subtilis* isolate (48 RW) measured in a carrier test. (■) 48W with EPS, (■) 48W with no EPS, (■) 6051 with EPS, (■) 6051 without EPS.

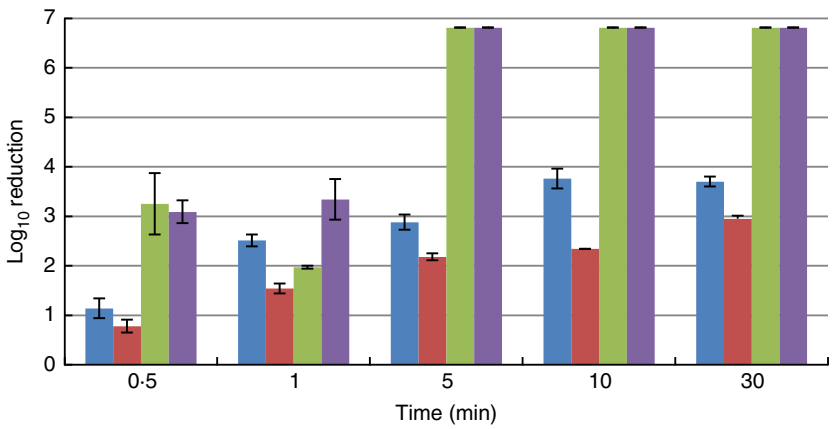

Figure 3 Effect of sonication (23 kHz for 1 min) on the efficacy of chlorine dioxide (0·03%) against *Bacillus subtilis* ATCC6051 and *B. subtilis* isolate (48 RW) in a suspension test in clean condition. (■) 48W with sonication, (■) 48W with no sonication, (■) 6051 with sonication, (■) 6051 without sonication.

degrade the oxidizing agents. Preliminary experiments with the Bioscreen C Microbial growth Analyser established that a proteinase K concentration of 200 μg ml^{-1} had no deleterious effect on the growth of the *B. subtilis* strains (data not shown). Bacterial pretreatment with proteinase K (with no soiling) did not affect ($P > 0·05$) bacterial susceptibility to chlorine dioxide 0·03% (Fig. 4).

Expression of detoxification enzymes following exposure to chlorine dioxide

The presence of catalase (*katA*) and superoxide dismutase (*sodA*) genes was confirmed by PCR (data not shown). Bacterial samples used for RT-PCR experiments were pretreated with chlorine dioxide 0·03% for 1 min prior to RNA extraction. Results are presented in Figs 5 and 6. Catalase (*katA*) and superoxide dismutase (*sodA*) genes were expressed after exposure. Both *katA* and *sodA* did not appear to be upregulated in either bacterial strain following a short exposure to chlorine dioxide. It has to be noted that gene expression was measured qualitatively by observing the brightness of the band signal, and not quantitatively as qPCR was not available at the time of the experiment. There was no evidence that the housekeeping gene *gyr*B was upregulated following exposure to the oxidizing agent.

Catalase activity

The isolate WD48 was shown to produce significantly ($P < 0·05$) more catalase than the standard strain; 0·0303 ± 0·004 and 0·0017 ± 0·0001 catalase production (% w/v), respectively.

Transmission electron microscopy

From the analysis of TEM micrographs (figures not shown) and the measurement of 50 individual bacterial cells for each strains, both *B. subtilis* strains were shown to have similar cell sizes; the average cell size for *B. subtilis* ATCC6051 and WD isolate were 1·41 ± 0·26 μm and 1·6 ± 0·27 μm, respectively. The difference in size was not significant ($P = 0·29$). More importantly, the micrographs also showed that there were no obvious differences in the fine structure of the cell wall between the two strains (data not shown).

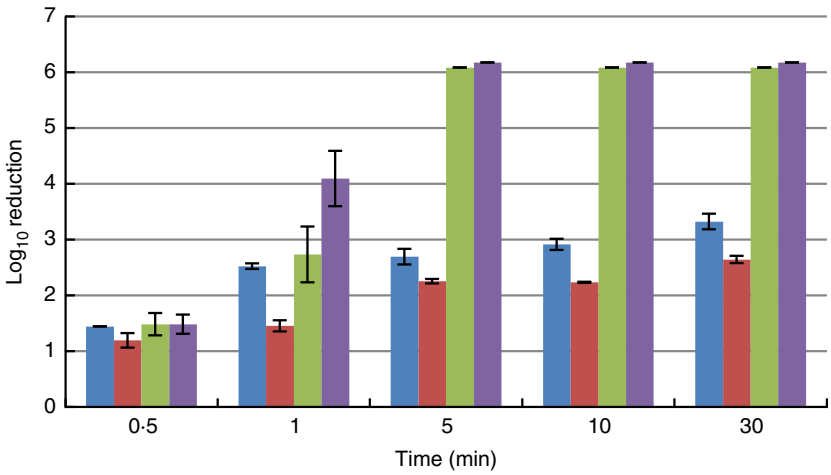

Figure 4 Effect of proteinase K pretreatment (200 μg ml^{-1} for 2 h) on the efficacy of chlorine dioxide (0·03%) against *Bacillus subtilis* ATCC6051 and *B. subtilis* isolate (48 RW) in a suspension test. (■) 48W before proteinase K treatment, (■) 48W after proteinase K treatment, (■) 6051 before proteinase K treatment, (■) 6051 after proteinase K treatment.

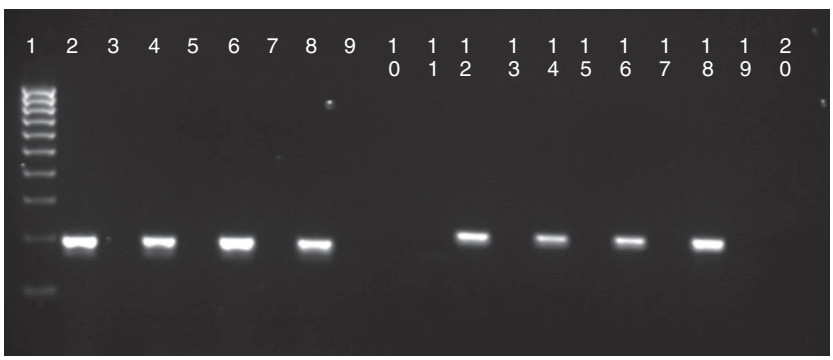

Figure 5 Expression of *gyrB* and *katA* in *Bacillus subtilis* following exposure or not to chlorine dioxide (0·03%). Lane 1: marker, Lane 2: *gyrB* isolate control, lane 3: *gyrB* isolate no RT control, Lane 4: *gyrB* isolate RT following ClO_2 exposure, lane 5: *gyrB* isolate no RT following ClO_2 exposure, Lane 6: *gyrB* 6051 RT control, Lane 7: *gyrB* 6051 no RT control, Lane 8: *gyrB* 6501 RT following ClO_2 exposure, Lane 9: *gyrB* 6501 no RT following ClO_2 exposure, Lane 10: water control, Lane 11: marker, Lane 12: *katA* isolate control, lane 13: *katA* isolate no RT control, Lane 14: *katA* isolate RT following ClO_2 exposure, Lane 15: *katA* isolate no RT following ClO_2 exposure, Lane 16: *katA* 6051 RT control, Lane 17: *katA* 6051 no RT control, Lane 18: *katA*6501 RT following ClO_2 exposure, Lane 19: *katA*6501 no RT following ClO_2 exposure, Lane 20: water control.

Chlorine dioxide 0·01% produced cell surface damage (blebs) in *B. subtilis* ATCC6051, which contrasts with the apparent absence of damage with the WD48 isolate (Fig. 7). At the higher concentration of 0·03%, a more diffused outer cell wall can be observed for both the standard strain and the WD48 isolate possibly indicating some membrane damage (Fig. 8).

Discussion

In a previous study, Martin *et al.* (2008) isolated a *B. subtilis* strain (W48) from a washer-disinfector using chlorine dioxide for endoscope disinfection. The vegetative isolate was shown to be resistant to the in use concentration of chlorine dioxide and cross-resistant to a hydrogen peroxide solution. Scanning electron microscopy of the isolate highlighted the presence of a large amount of EPS (Martin *et al.* 2008). A more recent study by Bridier *et al.* (2011) demonstrated the capacity of this isolate to produce a voluminous biofilm associated with a large amount of EPS. Based on these observations, it was reasonably assumed that the production of a high concentration of EPS and the apparent ability of the isolate to form aggregates might be responsible for its unique high resistance to oxidizing agents. Indeed, EPS has been implicated in biocide insusceptibility of bacterial biofilms (Gilbert *et al.* 2001; Mah and O'Toole 2001; Maillard 2007). It has been shown that EPS can prevent biocide penetration (Chen and Stewart 1996; Flemming and Wingender 2001; Maillard 2007), increase bacterial clumping (Wolfaardt *et al.* 1999) and decrease oxidizing biocide concentration by the production of extracellular detoxifying enzymes (Heinzel 1998; Wolfaardt *et al.* 1999; Stewart *et al.* 2000; Chapman 2003), although the importance of EPS in the penetration of biocides and their activity has recently been questioned (Araujo *et al.* 2014). Here, the

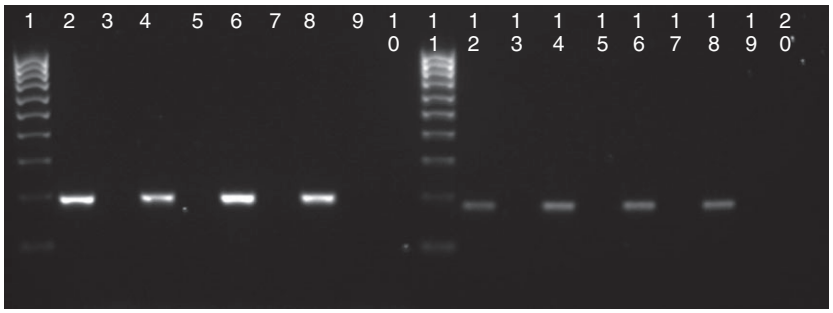

Figure 6 Expression of *gyrB* and *sodA* in *Bacillus subtilis* following exposure or not to chlorine dioxide (0·03%). Lane 1: marker, Lane 2: *gyrB* isolate control, lane 3: *gyrB* isolate no RT control, Lane 4: *gyrB* isolate RT following ClO_2 exposure, lane 5: *gyrB* isolate no RT following ClO_2 exposure, Lane 6: *gyrB* 6051 RT control, Lane 7: *gyrB* 6051 no RT control, Lane 8: *gyrB* 6501 RT following ClO_2 exposure, Lane 9: *gyrB* 6501 no RT following ClO_2 exposure, Lane 10: water control, Lane 11: marker, Lane 12: *sodA* isolate control, lane 13: *sodA* isolate no RT control, Lane 14: *sodA* isolate RT following ClO_2 exposure, Lane 15: *sodA* isolate no RT following ClO_2 exposure, Lane 16: *sodA* 6051 RT control, Lane 17: *sodA* 6051 no RT control, Lane 18: *sodA*6501 RT following ClO_2 exposure, Lane 19: *sodA*6501 no RT following ClO_2 exposure, Lane 20: water control.

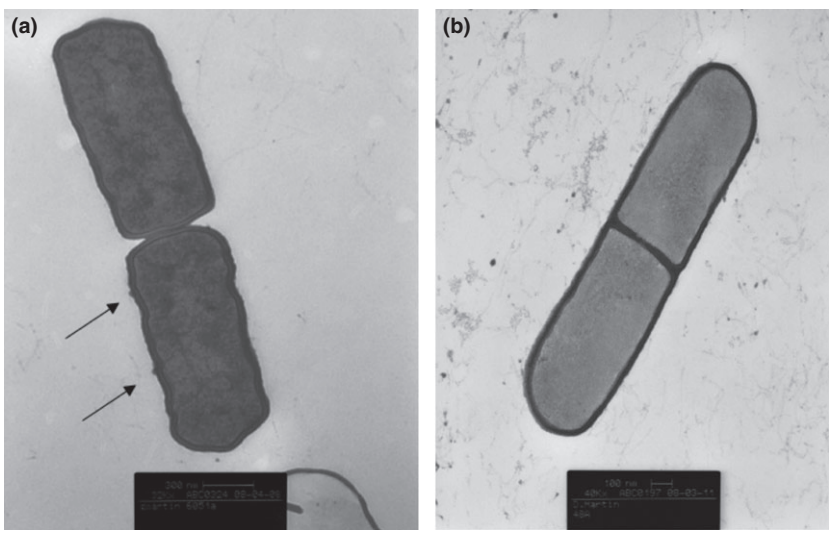

Figure 7 Electron micrographs of (a) *Bacillus subtilis* ATCC6051 and (b) WD isolate exposed to 0·01% chlorine dioxide (representative of 20 fields of view). Evidence of blebbing is shown by the arrows.

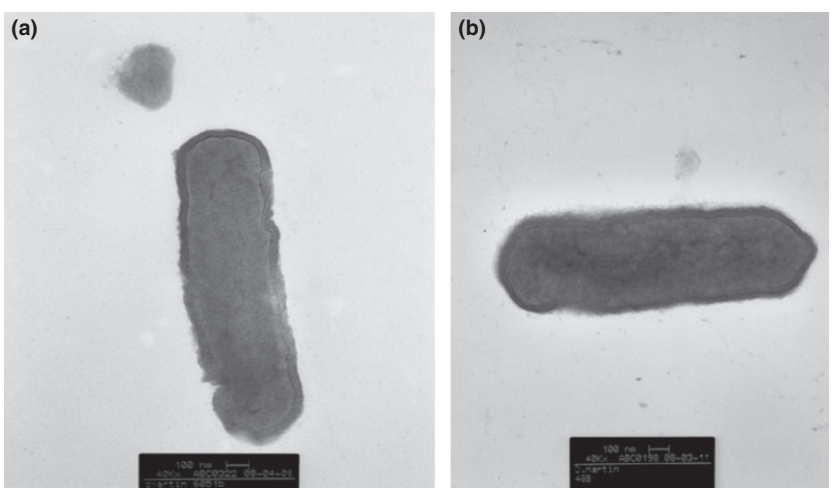

Figure 8 Electron micrographs of (a) *Bacillus subtilis* ATCC6051 and (b) WD isolate exposed to 0·03% chlorine dioxide (representative of 20 fields of view).

removal of EPS by successive washing steps altered modestly (by 1 $\log_{10}$) the susceptibility profile of the isolate but only after 5 min exposure as measured in a suspension test. A more significant experiment was the sonication of the bacterial suspension prior to treatment. It is known that sonication can increase the effectiveness of biocides by breaking up bacterial clumps (Joyce *et al.* 2003; Duckhouse *et al.* 2004). Here, sonication (23 kHz) of the isolate WD48 prior to disinfection marginally increased the efficacy of chlorine dioxide (approx. 1 $\log_{10}$). This effect was only evident with a 1 min exposure to the oxidizer. In addition, the presence of BSA (as an interfering factor in disinfection studies) completely negated the effect of sonication and the bactericidal activity of chlorine dioxide against WD48. It was also observed that the activity of the oxidizer against the standard strain decreased by 3 $\log_{10}$ in the presence of organic load.

It has been shown that proteinase K can disrupt biofilm formation through interfering and destroying the EPS produced by bacteria (Patterson *et al.* 2007; Boles and Horswill 2008). The use of proteinase K at 200 μg ml^{-1} (the highest concentration that was not detrimental for the growth of the *B. subtilis*) did not affect bacterial susceptibility to chlorine dioxide, possibly indicating that potential detoxification exo-enzymes might not be present outside the bacterial cells or play a significant role in resistance. The role of detoxification enzymes in bacterial resistance to oxidizing agents has been described, although it is unclear at present as to whether these enzymes can provide resistance to in use (high) concentrations of these biocides (Maillard 2007). The production of catalase is well known in the involvement in detoxifying oxidizing agents and a number of catalase genes have been identified (McDonnell 2007). The production or over-production of catalase has been shown to increase the survival of bacteria in the presence of hydrogen peroxide and peracetic acid (Engelmann and Hecker 1996; Elkins *et al.* 1999; Rochat *et al.* 2005), and affect the penetration of hydrogen peroxide into bacterial biofilms (Stewart *et al.* 2000). Here, the isolate WD48 was shown to produce more catalase than its culture collection counterpart. The low amount of catalase produced is unlikely to solely explain solely the resistance of the isolate WD48 to high concentration of both chlorine dioxide and hydrogen peroxide. Because of the inability to measure the production of catalase in the presence of oxidizing agent, the expression of catalase and superoxide dismutase was investigated. There was no evidence that *katA* and *sodA* genes were upregulated in the presence of chlorine dioxide in both the WD48 and standard *B. subtilis* (ATCC6051), although we recognized that in the absence of quantification of gene expression (qPCR) (which was not available at the time of the experiment), these data need to be taken with caution. In addition, other catalase and oxidase genes could have been expressed, although the sodA and *katA* genes have been shown to be the main detoxification enzymes in *B. subtilis* in response to oxidative stress (Bol and Yasbin 1994; Naclerio *et al.* 1995; Inaoka *et al.* 1999).

Although the presence of EPS aggregates and the production of catalase may contribute to the resistance of the isolate WD48 to the oxidizing agent, these mechanisms cannot explain solely the survival of the isolate to a high concentration of chlorine dioxide (0·03%). Remarkably, TEM imaging did not show any differences in the fine cell structure of WD48 compared to the susceptible standard strain. *Bacillus subtilis* (ATCC6051) was more prone to blebbing when exposed to chlorine dioxide at 0·01% than WD48. A concentration of chlorine dioxide (0·03%) seemed to produce the same damage (fuzzy outer wall) in both *B. subtilis*.

To hypothesize the mechanisms of resistance of WD48 to chlorine dioxide and hydrogen peroxide, one has to reflect on their mechanisms of action. Both oxidizing agents are highly reactive and will interact with proteins, although in different ways (Finnegan *et al.* 2010). The activity of oxidizing agents against lipid and specifically the plasma membrane is unclear and might not be significant in causing bacterial lethality. Ultimately both oxidizing agents are expected to interact with microbial DNA (Linley *et al.* 2012). With this in mind, one could speculate that the ability of a bacterium to repair its DNA could provide some level of resistance to oxidizers. For example, *Deinococcus radiodurans* is well known for its extreme resistance to DNA-damaging agents, including ionizing radiation, UV radiation, and mitomycin C (Slade and Radman 2011). Concerning oxidizing agents, it is possible that a gross change in individual lipid or protein structures at the bacterial surface can explain resistance to high concentrations of both chlorine dioxide and hydrogen peroxide. The TEM observation of the treated W48 isolates, and comparison with susceptibility data to chlorine dioxide may indicate that the surface overall has become somewhat more resistant to the effects of the oxidizing agent, which may be related to the types and arrangement of structures on the surface. This has been observed with other biocides Tattawasart *et al.* (2000a) observed a change in lipid and protein composition in *Pseudomonas stutzeri* that were resistant cationic biocides. In this case, the strain size and appearance as observed by scanning electron microscopy was profoundly altered when compared to a susceptible isolate from a culture collection Tattawasart *et al.* (2000b). Another study linked subtle changes in the surface expression or availability of membrane proteins (i.e. porins) with aldehyde-

based biocide resistance in mycobacteria (Svetlikova *et al.* 2009). The resistance mechanism in this case appears to be linked to the lack of availability of surface protein and cell wall rearrangement. There are marked differences in mechanisms of action between aldehyde and oxidizing agents, but overall structural difference may provide greater resistance to biocides in general as shown in the comparison of Gram-negative and Gram-positive bacteria (Maillard 2007, 2010). To resolve the resistance mechanisms expressed in the chlorine dioxide resistant isolate, further work on the surface structure, including protein composition as well as intracellular interactions (such as oxidizer-DNA interaction) are needed. It is fascinating that such an isolate can survive disinfection activity. The obvious resistance mechanisms including the presence of EPS, aggregation and the production of catalase, cannot explain resistance to such a high concentration of oxidizers. However, it does appear that the culmination of novel mechanisms have been acquired by this isolate to enable it to survive overtime in adverse environmental conditions.

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Conflict of Interest

Dr G McDonnell has shares in Steris Corporation.

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