

Oxidative stress response in *Clostridium perfringens*

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Clostridium perfringens, a strictly anaerobic bacterium, is able to survive when exposed to oxygen for short periods of time and exhibits a complex adaptive response to reactive oxygen species, both under aerobic and anaerobic conditions. However, this adaptive response is not completely understood. *C. perfringens* possesses specialized genes that might be involved in this adaptive process, such as those encoding superoxide dismutase (SOD), superoxide reductase and alkyl hydroperoxide reductase, but their contribution to the oxidative stress response and their control mechanisms are unknown. By a combination of functional complementation of *Escherichia coli* strains impaired in either SOD, alkyl hydroperoxide reductase (AhpC) or catalase activity (Cat), transcription analysis and characterization of mutants impaired in regulatory genes, it was concluded that: (i) the product of the *sod* gene is certainly essential to scavenge superoxide radicals, (ii) the *ahpC* gene, which is fully induced in all oxidative stress conditions, is probably involved in the scavenging of all intracellular peroxides, (iii) the three rubrerythrin (*rbr*) genes of *C. perfringens* do not encode proteins with *in vivo* H₂O₂ reductase activity, and (iv) the two rubredoxin (*rub*) genes do not contribute to the hypothetical superoxide reductase activity, but are likely to belong to an electron transfer chain involved in energy metabolism.

INTRODUCTION

Clostridium perfringens is a ubiquitous, Gram-positive, oligosporogenous pathogen, present in the air, soil and water and in the intestinal tracts of humans and animals. Although *C. perfringens* is strictly anaerobic, it is considered to be an aerotolerant anaerobe. Indeed, vegetative and stationary-phase cells can survive in a growth-arrested state in the presence of oxygen and/or low concentrations of superoxide- or hydroxyl-radical-generating compounds (Trinh *et al.*, 2000; Briolat & Reysset, 2002). Like many other bacteria, *C. perfringens* exhibits a typical adaptive response to oxidative stresses: cells treated with a sublethal dose of a stress agent can subsequently withstand higher doses of the same agent or products generating identical reactive oxygen species (for a review see Sies, 1986). These properties may explain why *C. perfringens* can be easily found in and recovered from environmental samples in a wide variety of biotopes. This aerotolerance and the fact that vegetative cells are not rapidly killed by the main reactive oxygen species might also explain how *C. perfringens* survives during the initial stage of the infectious process. O'Brien & Melville (2000) have shown that although human polymorphonuclear leukocytes and monocytes are able to

phagocytose and kill *C. perfringens* cells during the early stages of infection, some bacteria are phagocytosed by macrophages, but can survive in the cytoplasm. This suggests that *C. perfringens* cells are protected from the oxidative burst of the macrophages. According to Voyich *et al.* (2003), upregulation of oxidative stress genes, virulence genes, cell-wall biosynthesis genes and related regulatory genes is observed when group A streptococci evade polymorphonuclear leukocyte phagocytosis. The genes encoding the glutathione peroxidase, alkyl hydroperoxide reductase (etc.) proteins, which detoxify cell-damaging reactive oxygen species, and the genes encoding proteins involved in DNA repair are fully induced 180 min after phagocytosis. By analogy, *C. perfringens* within macrophages probably elicit such protective responses.

The genes involved in the oxidative stress response of anaerobic Gram-positive bacteria have not all been identified. However, rubrerythrin (*rbr*) and superoxide dismutase (*sod*) genes have been identified in *C. perfringens* strain NCIMB8875 (Lehmann *et al.*, 1996; Geissmann *et al.*, 1999), but it was shown that neither *sod* nor *rbr* transcription was influenced by oxidative stress, and these results did not lead to any conclusions about the role of the two genes in the defence against oxygen in *C. perfringens*. In parallel, studies of sulfate-reducing *Desulfovibrio* species have recently given new insight in the area of stress resistance in anaerobic bacteria (Pianzola *et al.*, 1996;

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Tc, tetracycline; CRP, cAMP-binding protein; Pl, plumbagin; PQ, paraquat; SOD, superoxide dismutase; t-BT, ter-butylhydroperoxide.

Lumppio *et al.*, 1997; Voordouw & Voordouw, 1998; Coulter *et al.*, 1999; Jenney *et al.*, 1999; Lumppio *et al.*, 2001; Emerson *et al.*, 2002). Homologues of some of the oxidative stress response genes found in *Desulfovibrio vulgaris* have thus been sought in *C. perfringens*. Analysis of the genome sequence of strain 13 shows that *C. perfringens* possesses genes potentially encoding (i) a classical Mn-SOD (*sod*), (ii) an alkyl hydroperoxide reductase (*ahpC*), (iii) two rubredoxins (*rub*) suspected to belong to two superoxide reductase systems and (iv) three non-haem iron rubrerythrins (*rbr*), with hypothetical H₂O₂ reductase activity.

We used various strategies to evaluate the role of these genes in the oxidative stress response of *C. perfringens*. First, we carried out functional complementation experiments in *Escherichia coli* strains impaired in either SOD, alkyl hydroperoxide reductase or catalase activity to evaluate the catalytic properties of these proteins. Second, we compared the level of transcription of these genes in stressed and unstressed cells of *C. perfringens*. Finally, we studied the physiological properties of regulatory mutants constructed by gene inactivation, to confirm whether inactivated genes were indeed involved in the oxidative stress response.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *E. coli* strains were routinely grown aerobically at 37 °C in Luria–Bertani (LB) broth containing ampicillin (Ap, 100 µg ml⁻¹), chloramphenicol (Cm, 25 µg ml⁻¹) or erythromycin (Em, 300 µg ml⁻¹) when needed. Physiological assays were carried out in M63 minimal medium (Miller, 1972) or in this medium supplemented with 5 g Casamino acids l⁻¹ (M63casa medium). *C. perfringens* strains were routinely grown and stored under anaerobic conditions in TGY medium (Privitera *et al.*, 1979), and in MCP or MMP media (Trinh *et al.*, 2000) for oxidative stress challenges. The media were supplemented with Cm (25 µg ml⁻¹) or Em (25 µg ml⁻¹) when required. Anaerobic cultures were incubated at 37 °C in an anaerobic chamber (Jacomex) in a 90% N₂, 5% CO₂ and 5% H₂ atmosphere. Growth was assessed by measuring the optical density at 440 or 520 nm. For cell counts, aliquots from liquid cultures were serially diluted in anaerobically prepared 90 mM potassium phosphate buffer (PB, pH 7.0) and plated onto pre-reduced MCP (containing 0.5% cysteine/HCl). Colony-forming-units (c.f.u.) were counted after 24–48 h at 37 °C. The number of c.f.u. obtained was not affected by prior incubation for more than 6 h in PB. Sensitivity to air and to other oxidative stress conditions was described previously (Briolat & Reysset, 2002).

Kinetics of *C. perfringens* cell survival under aerobic conditions. Bacteria were grown to mid-exponential phase in MMP medium supplemented with 0.01% yeast extract at 37 °C in an anaerobic chamber. The bacterial cultures supplemented without and with 2.5 µM plumbagin (Pl) were then transferred to aerobic conditions at 37 °C with shaking in a rotary incubator (250 r.p.m.), in flasks with a capacity 5–10 times greater than the volume of the culture. At various times, aliquots were returned to the anaerobic chamber and viability was measured. The rate of cell survival was

calculated as the number of viable cells after exposure to air divided by the total number of viable cells before treatment.

Inhibition of *E. coli* growth with paraquat (PQ), H₂O₂ and ter-butylhydroperoxide (t-BT) on solid medium. *E. coli* cells were grown overnight in M63casa liquid medium and then spread onto M63casa solid medium, with or without Ap (50 µg ml⁻¹). Then, 5 µl of 25, 50 or 25 mM PQ, H₂O₂ or t-BT solutions, respectively, was spotted onto small circles (diam. 6 mm) of blotting paper that had been placed on the agar plate. The plates were then incubated for 24 h at 37 °C and the diameter of inhibition was measured. Means of four to eight inhibition zones were determined for each strain.

Peroxidase activity gels. Peroxidase activity was detected in native polyacrylamide gels as described by Heym & Cole (1992). A crude extract of *E. coli* cells was prepared as follows. The strains were cultivated in LB medium until stationary phase, harvested by centrifugation and washed twice in 50 mM PB buffer, pH 7.0. The cells were then resuspended in 1/25 of the initial volume of PB buffer and disrupted by sonication (3 × 3 min with 5 min cooling intervals). Cell debris was removed by centrifugation (20 min at 15 000 g) and the supernatant was stored frozen in aliquots. Proteins were quantified using the method of Bradford (1976).

Molecular and genetic techniques. Unless otherwise stated, all DNA manipulations were performed by standard techniques (Sambrook *et al.*, 1989). DNA preparation and *C. perfringens* electroporation were done as described by Briolat & Reysset (2002).

Cloning of genes or genetic regions of *C. perfringens*. The genes encoding the alkyl hydroperoxide reductase (*ahpC*), the SOD (*sod*), the rubrerythrins (*rbr1*, *rbr2* and *rbr3*), the rubredoxins (*rub1* and *rub2*) and the genetic regions GRI (from *CPE0776* to *CPE0778*) and GRII (from *CPE0779* to *CPE0781*) (see Fig. 1) were amplified by PCR using specific sets of primers (Table 2). After purification, the PCR products were cloned into the pGEMTeasy vector (Promega) and introduced into *E. coli* DH5α by electroporation. The multiple cloning sequence of the pGEMTeasy vector is flanked by promoters recognized by the T7 and SP6 RNA polymerases, respectively. However, as the two polymerases are both absent from the *E. coli* strains used in this study, the *C. perfringens* genes are likely to be expressed from their own promoter.

Gene inactivation. The *CPE0776* and *CPE0779* genes were disrupted by single crossover insertion of pKNT21DJ776 and pKNT21DJ779, respectively. The Cm^R transformants obtained after electroporation of the 13R strain were reisolated and total DNA was prepared. PCR and Southern hybridization using pUC19 as a probe validated the disruption of the two genes.

Preparation and quantification of RNA. *C. perfringens* (50 ml) was harvested 0, 20, 40 and 60 min after the onset of each stress condition and total RNA was extracted using the trizol reagent (Gibco-BRL) (Dupuy & Sonenshein, 1998). RNA concentration was determined by the orcinol reaction (Schneider, 1957). Various concentrations (5, 2.5, 1 and 0.5 µg) of the denatured DNA-free RNA preparations from each time point were blotted onto two nylon filter membranes. The filters were subjected to Northern hybridization with an internal 10⁶ d.p.m. [α -³²P]dCTP (Amersham) radiolabelled probe of the gene of interest and 10⁶ d.p.m. of a specific *C. perfringens* 16S RNA probe. Filters were hybridized overnight at 55 °C in rapid-hyb buffer (Amersham) and then washed once for 15 min in 1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate)/0.1% SDS, and three times for 10 min each in 0.2 × SSC/0.1% SDS at 55 °C. Radioactivity was quantified using the Storm Phospho-Imager (Molecular Dynamics). Standardization was done by dividing the value obtained for a given probe by the corresponding

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
QC1301	Wild-type (=MG1655)	Genetic Center
QC1799	MG1655 F ⁻ Δ (<i>argF-lac</i>) <i>U169rpsL</i> Δ <i>sodA3</i> Φ (<i>sodB-kan</i> Δ 2)	Touati <i>et al.</i> (1995)
QC2476	MG1655 <i>katE::Tn10 katG::Tn10</i> Tc ^R	Bebien <i>et al.</i> (2002)
QC2481	MG1655 Δ <i>ahpC</i> (=DA103)	H�erouart <i>et al.</i> (1996)
<i>C. perfringens</i>		
13	Toxinotype A (wild-type)	Mahony & Moore (1976)
13R	Rifampicin-resistant derivative of strain 13	Trinh <i>et al.</i> (2000)
Plasmid		
pGEMTeasy	Cloning vector, Ap ^R	Promega
pACYC184	Cloning vector, Cm ^R	Biolabs
pHSG576	Low-copy-number cloning vector, Cm ^R	Takeshita <i>et al.</i> (1987)
pUC19	Cloning vector, Ap ^R	Yanisch-Perron <i>et al.</i> (1985)
pKNT19	Shuttle vector, Ap ^R Em ^R	Azeddoug <i>et al.</i> (1992)
pKNT21	Suicide vector, Ap ^R Cm ^R	Briolat & Reysset (2002)
pGC <i>sod</i>	Cloning of the 1634 bp PCR fragment containing the <i>sod</i> gene into pGEMTeasy (primers 1236L/R)	This study
pG <i>sod::catP</i>	Cloning of a 904 bp <i>Sma</i> I fragment containing the <i>catP</i> gene into the blunt-ended <i>Hind</i> III restriction site of pG <i>sod</i>	This study
pGrbr1	Cloning of a 1259 bp PCR fragment containing the <i>CPE0135</i> gene into pGEMTeasy (primers 0135L/R)	This study
pGrbr2	Cloning of a 835 bp PCR fragment containing the <i>CPE0855</i> gene into pGEMTeasy (primers 0855L/R)	This study
pGrbr3	Cloning of a 714 bp PCR fragment containing the <i>CPE0135</i> gene into pGEMTeasy (primers 1331L/R)	This study
pGRI	Cloning of a 3227 bp PCR fragment containing the <i>CPE0776-CPE0778</i> genes into pGEMTeasy (primers GRI L/R)	This study
pGRII	Cloning of a 2777 bp PCR fragment containing the <i>CPE0779-CPE0781</i> genes into pGEMTeasy (primers GRII L/R)	This study
pGRI Δ 0776	Cloning of a PCR fragment containing the <i>CPE0777-CPE0778</i> genes into pGEMTeasy (primers 0777L/0778R)	This study
pGRII Δ 0779	Cloning of a PCR fragment containing the <i>CPE0780-CPE0781</i> genes into pGEMTeasy (primers 0780L/0781R)	This study
pGC <i>CPE0776</i>	Cloning of a 1200 bp PCR fragment containing the <i>CPE0776</i> gene into pGEMTeasy (primers 0776L/0776R)	This study
pGC <i>CPE0779</i>	Cloning of a PCR fragment containing the <i>CPE0779</i> gene into pGEMTeasy (primers 0779L/0779R)	This study
pG <i>ahpC</i>	Cloning of a PCR fragment containing the <i>CPE0782</i> gene into pGEMTeasy (primers 0782L/0782R)	This study
pKNT21DJ776	Cloning of an internal 519 bp <i>Hind</i> III fragment from <i>CPE0776</i> into pKNT21	This study
pKNT21DJ779	Cloning of an internal 520 bp <i>Hind</i> III fragment from <i>CPE0779</i> into pKNT21	This study

16S rRNA value. The results are expressed as the means of the various RNA concentrations used.

Computer methods. The whole-genome sequence of *C. perfringens* was visualized and analysed using Artemis software (Rutherford *et al.*, 2000). *C. perfringens* gene sequences were compared with sequences present in public databases using FASTA, BLASTN and BLASTX (Altschul *et al.*, 1994). Protein and regulatory or consensus sequences were determined using the MOTIF programs of the Staden package (Staden *et al.*, 2003).

RESULTS

***In silico* identification of *C. perfringens* genes possibly involved in the scavenging of H₂O₂, organic peroxide and superoxide radicals**

The publication of the complete sequence of *C. perfringens* strain 13 by Shimizu *et al.* (2002) allowed us to identify genes that might scavenge H₂O₂, t-BT and/or superoxide radicals.

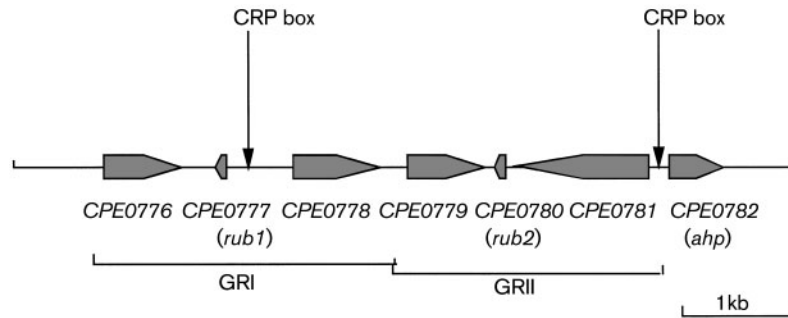


Fig. 1. Genetic organization of the chromosomal region containing the two rubredoxin (*rub*) genes of *C. perfringens*. The GRI and GRII genetic regions are indicated. The putative locations of the CRP boxes are indicated by arrows.

Some of them were selected because they encoded proteins with similar sequences to oxidative stress proteins from anaerobic bacteria, particularly *Desulfovibrio* species, which have been shown to participate in the oxidative stress response. These included:

- (i) The *sod* gene potentially encoding a SOD showing extensive similarity to amino acid sequences typically conserved in Mn-SOD from both Gram-positive and Gram-negative bacteria. An orthologous SOD gene has previously been cloned from *C. perfringens* strain NCIMB8875 by functional complementation of an *E. coli* strain lacking superoxide activity (Geissmann *et al.*, 1999).
- (ii) The *rbr1*, *rbr2* and *rbr3* genes, potentially encoding three rubrerythrin proteins. These proteins, which are 39 to 48 % identical, are closely related to the well characterized rubrerythrin of *Desulfovibrio vulgaris* (GenBank accession no. AAB39991-1) and to other rubrerythrins from strictly anaerobic bacteria and archaea. The rubrerythrin and related nigerythrin protein from the anaerobic sulphate-reducing bacterium *D. vulgaris* are suspected to possess NADH peroxidase activity at least in an *in vitro* assay system (Coulter *et al.*, 1999).
- (iii) The *rub1* and *rub2* genes, both of which encode highly conserved rubredoxin proteins. Such non-haem iron

Table 2. Primers used to amplify the genes or genetic regions of interest

Gene (length in bp)	Left (L) and right (R) primers (5'-3')	Length of the PCR product (bp)
<i>CPE1236</i> (<i>sod</i> , 683)	L: d(GGAAAATAAGTTACATCAAGGGAG) R: d(ATATTGCGCCATAATTCCCCC)	1634
<i>CPE0135</i> (<i>rbr1</i> , 587)	L: d(TTAAGCCTGCTGCTCAGCCAGAG) R: d(TGGTAGTGTTTAGCTACTCACTAC)	1259
<i>CPE0855</i> (<i>rbr2</i> , 539)	L: d(CCTTACTATGTATTTATTAATGAG) R: d(AATCTTAAAACCTAAAACCTTGATC)	835
<i>CPE1331</i> (<i>rbr3</i> , 536)	L: d(AACTAAAAGTCTATAGGTATTAGC) R: d(TGTGATGAATAGGAAAAACTTTC)	714
GRI	L: d(GTTATGAACATGAAGCTGCTGG) R: d(CATATTTTCACTTCTCCCTCTC)	3227
GRII	L: d(GAAATAGTTTAAAAGATTTAGGCG) R: d(CAGTGCCTTTTAAATCCTCTG)	2777
<i>CPE0776</i> (702)	L: d(GTAATGAACATGAAGCTGCTGG) R: d(AATTTGATTGAATCTATAACCACT)	1200
<i>CPE0777</i> (<i>rub1</i> , 161)	L: d(TAGGTTGAAAATTTTAAACAAGGC) R: d(TCCTAAAATATGACTGGCATCAC)	629
<i>CPE0778</i> (746)	L: d(AAAGTCAAAGACTGTGTTTGGG) R: d(CATATTTTCACTTCTCCCTCTC)	1591
<i>CPE0779</i> (711)	L: d(GAAATAGTTTAAAAGATTTAGGCG) R: d(ATGATCCAGCTGTAGGAGATCC)	1200
<i>CPE0780</i> (<i>rub2</i> , 161)	L: d(AACAAATGGCAAACACTACTGTGG) R: d(TGTTTTTGGTTCATTTGGATGG)	601
<i>CPE0781</i> (1220)	L: d(TTATCTACTCCACAAAGAGGGC) R: d(CAGTGCCTTTTAAATCCTCTG)	1696
<i>CPE0782</i> (<i>ahpC</i> , 567)	L: d(TTTTGTGAAAATCTACAACCTTAG) R: d(ATTTACGTCTCCTCCTTAGTCC)	1000

proteins have been found in numerous species of anaerobic eubacteria and archaea. These two *rub* genes, which encode polypeptides of 53 and 54 aa, respectively, are located in the same chromosomal region of *C. perfringens* strain 13 and share a similar genetic organization. Both genes are associated with a gene encoding a flavoprotein and a gene that encodes a product displaying similarity with transcriptional regulators. The *rub1* gene is located 664 bp upstream of *CPE0778*, which encodes a probable flavohaemoprotein (haemoglobin-like protein) sharing similarity to other proteins possessing a probable NADH:rubredoxin-oxidoreductase (Rbo) activity. Likewise, *rub2* forms an operon with the *CPE0781* gene, encoding a hypothetical flavoprotein. The product shares 29% identity and 47% similarity with the rubredoxin-oxygen-oxidoreductase (Roo) from *Desulfovibrio gigas*, which is believed to catalyse the reduction of dioxygen to water (Gomes *et al.*, 1997; Kurtz & Coulter, 2002). Two putative regulatory genes (*CPE0776* and *CPE0779*) are located upstream of two rubredoxin-encoding genes. The two corresponding proteins, share 76% identity at the amino acid level and belong to the cAMP-binding protein (CRP)/FNR superfamily of transcriptional factors which includes the CRP, FNR and FLP (FNR-like protein) regulatory proteins (Green *et al.*, 2001). The genetic region including *CPE0776*, *rub1* and *CPE0778* is designated GRI and that including *CPE0779*, *rub2* and *CPE0781* is designated GRII (Fig. 1).

The sequence data also show that the *rub2-CPE0781* operon is separated by 208 bp from the *ahpC* gene of *C. perfringens*. Associated with the specific electron transporter (AhpF), AhpC is suspected of being the main scavenger of both endogenous alkyl peroxide and H₂O₂ in *E. coli* (Seaver & Imlay, 2001). No clear orthologue of the *ahpF* gene was found in the entire *C. perfringens* genome. However, a BLAST search identified three genes (*CPE0783*, *CPE2440*, *CPE2354*) sharing about 30% identity with *ahpF*, but annotated as probable thioredoxin reductase proteins. Such similarity with a family of thioredoxin reductase proteins has already been pinpointed for the AhpF polypeptide of *Salmonella typhimurium* (Tartaglia *et al.*, 1990). Notice that one of these genes (*CPE0783*) is located just downstream (247 bp) of the *ahpC* gene of *C. perfringens* (Fig. 1).

Functional complementation of a Δ *sodA* Δ *sodB* *E. coli* strain

To evaluate the superoxide scavenging activity of the *sod*, *rub* and *rbr* genes of *C. perfringens*, the recombinant plasmids carrying these genes were introduced into *E. coli* strain QC1799 deficient in both *sodA* and *sodB* genes, encoding manganese- and iron-SODs, respectively. This strain was very sensitive to superoxide-generating compounds (e.g. PQ, PI) and unable to grow in minimal medium in the absence of amino acids. This last property was due to superoxide damage of iron-sulphur enzymes in the biosynthetic pathway of branched-chain amino acids (Touati, 2000). The diameter of growth inhibition of the transformants with 25 mM PQ revealed that all the *C. perfringens* genes tested in strain QC1799 provided partial (*rbr2*, *rbr3*, GRI and *sod*) or complete (*rbr1* and GRII) protection against this agent (Fig. 2). These results were in good agreement with those obtained in liquid minimal medium (Fig. 3). Plasmids carrying the *rbr1* gene and the GRII region of *C. perfringens* fully restored the aerobic growth of strain QC1799, whereas the expression of *sod*, *rbr2* and *rbr3* genes or the entire GRI region were less efficient.

To evaluate the potential transcriptional regulatory properties of *CPE0776* and *CPE0779* polypeptides, the corresponding genes were deleted from the GRI and GRII regions, generating pGRI Δ *CPE0776* and pGRII Δ *CPE0779*, respectively. These plasmids were introduced into strain QC1799 and the transformants were assayed. Inactivation of the *CPE0776* gene completely abolished the ability of the Δ *sodA* Δ *sodB* *E. coli* strain to grow in minimal medium and did not enhance resistance to PQ (Figs 2 and 3c). These results suggested that *rub1* and/or *CPE0778* genes were no longer expressed by pGRI Δ *CPE0776*. In contrast, inactivation of *CPE0779* from the GRII region only slowed down the growth rate of the transformants in minimal medium and only partially protected the cells from PQ. The results suggested that *CPE0779* can activate the transcription of the *rub2-CPE0781* operon, but that the operon can also be transcribed, albeit less efficiently, from a promoter not regulated by the *CPE0779* protein. Moreover, although the amino acid sequences of *CPE0776* and *CPE0779* are 76% identical, the two regulatory proteins are very specific. When

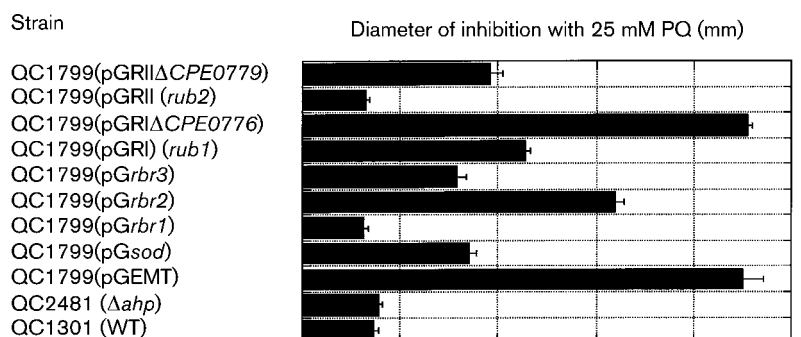


Fig. 2. Diameter of growth inhibition with 25 mM PQ of *E. coli* QC1799 (Δ *sodA* Δ *sodB*) complemented with the *sod*, *rbr*, *rub* genes of *C. perfringens*. The mean inhibitory diameter was measured after 24 h on M63 solid medium supplemented with Casamino acids. The wild-type *E. coli* QC1301 and QC2481 (Δ *ahp*) strains were used as controls.

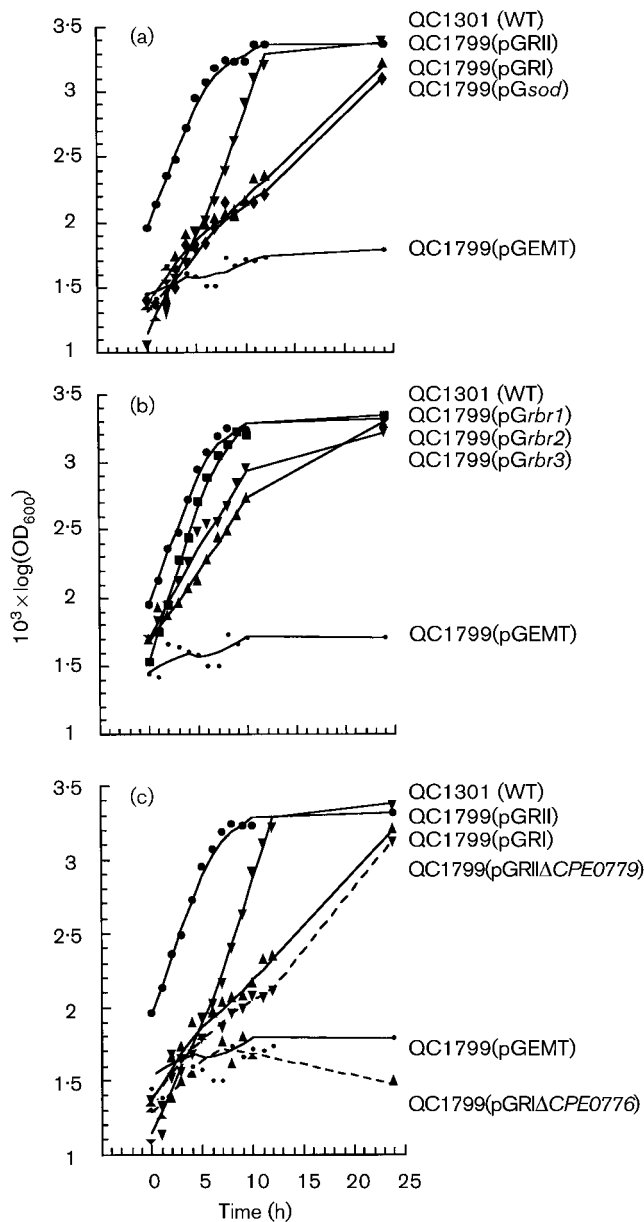


Fig. 3. Growth of *E. coli* strain QC1799 (Δ sodA Δ sodB) complemented with the *sod*, *rbr*, *rub* genes from *C. perfringens* in minimal M63 liquid medium. The cells were pre-grown overnight aerobically in M63 supplemented with amino acids at 37 °C, and then diluted at 1/100 (v/v) in pre-warmed minimal M63 medium. The wild-type *E. coli* QC1301 and QC1799 (Δ sodA Δ sodB) strains were used as positive and negative controls, respectively. (a) Growth of QC1799 complemented with the pGRI (*rub1*), pGRII (*rub2*) and *sod* genes of *C. perfringens*; (b) growth of QC1799 complemented with the three ruberythrin (*rbr*) genes of *C. perfringens*; (c) growth curves of QC1799 complemented with the pGRI and pGRII genetic regions deleted or not of their regulatory genes *CPE0776* and *CPE0779*, respectively.

cloned into low-copy-number plasmid pHSG576, *CPE0779*, but not *CPE0776*, restored the resistance to PQ of QC1799(pGRII Δ CPE0779) in minimal medium (diameter of inhibition = 15.0 ± 1.15 vs 36.5 ± 2.89 mm for 25 mM PQ). These results strongly suggested that *CPE0776* and *CPE0779* are positive regulatory proteins. Based on the protein motif (COG0664), *CPE0776* and *CPE0779* are more specifically related to the CRP family, and possessed the specific CRP DNA-binding motif **RE(T)(V)(S)R** located at the C-terminal end of the proteins. Two putative consensus CRP binding sites containing the imperfect palindrome TGTGAN₆TCACA (see Green *et al.*, 2001) were identified upstream of the two rubredoxin genes. Our working hypothesis is that *CPE0776* and *CPE0779* belong to the CRP subfamily, which controls the response to glucose starvation in a broad spectrum of bacterial species

Functional complementation of a catalase-deficient *E. coli* strain

The recombinant plasmids described above were also introduced into *E. coli* QC2476 devoid of catalase activity. Whatever the constructions used, the inhibition concentrations of H₂O₂ were identical to that of the *E. coli* mutant strain in either aerobic or anaerobic conditions (data not shown). However, a peroxidase activity was detected in a protein crude extract of strain QC2476(pGrbr1) (Fig. 4). This suggests that only one of the three ruberythrin genes (*CPE0135*) possesses an *in vitro* peroxidase activity and that this activity is not sufficient to protect *in vivo* a catalase-negative strain of *E. coli* from the toxic action of H₂O₂.

Functional complementation of a Δ ahp *E. coli* strain with the *ahpC* gene of *C. perfringens*

The *ahpC* gene of *C. perfringens* was cloned into both pGEMTeasy, a multicopy plasmid, and pACYC184, a low-copy-number vector and the resulting constructs were introduced into *E. coli* QC2481 (Δ ahp). Whatever the plasmid used, the *ahpC* gene increased resistance to t-BT. The diameter of growth inhibition for 200 mM t-BT was ≥ 50 , 12.3 and 14.5 mm for strains QC2481, QC2481(pGahpC) and QC2481(pACYCahpC), respectively, versus ≤ 6 mm for the wild-type strain QC1301. As the alkyl hydroperoxide reductase of *E. coli* is also active against H₂O₂, we studied the ability of the AhpC protein of *C. perfringens* to restore H₂O₂ resistance. The H₂O₂ inhibition concentrations were similar for strains QC2481 (Δ ahp) and QC2476 (*katE*::Tn10 *katG*::Tn10) complemented or not complemented with the *ahpC* gene of *C. perfringens* (data not shown).

Transcriptional analysis of the *sod*, *rub*, *rbr* and *ahpC* genes of *C. perfringens*

To evaluate the roles of the *sod*, *rub*, *rbr* and *ahpC* genes in the oxidative stress response, we monitored the transcriptional level of these genes in stressed *C. perfringens* cells. Induction (-fold difference) was determined by comparing

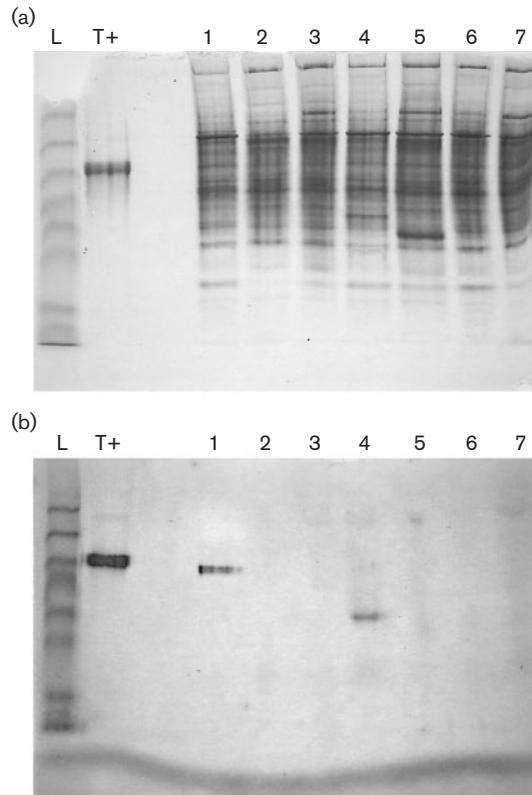


Fig. 4. Peroxidase activity in crude protein extracts of strain QC2476 (*katE*::Tn10 *katG*::Tn10) complemented with the rubrerythrin (*rbr*) and rubredoxin (*rub*) genes of *C. perfringens*. (a) Non-denaturing polyacrylamide gel of crude protein extracts (50 μ g) stained with Coomassie blue. (b) Activity staining of a native polyacrylamide gel for peroxidase activity with 50 μ g crude protein extract. Lanes: L, molecular mass marker; T+, 25 ng purified KatG from *Mycobacterium tuberculosis* used as a positive control; 1, QC1301 (wild-type); 2, QC2476(pGEMT); 3, QC2476(pGrbr3); 4, QC2476(pGrbr1); 5, QC2476(pGrbr2); 6, QC2476(pGRI) (*rub1*); 7, QC2476(pGRII) (*rub2*).

stressed samples to the $t=0$ control sample. The cells were grown to mid-exponential phase in MMP medium under anaerobic conditions and then maintained under anaerobic conditions after the addition of subinhibitory H_2O_2 (50 μ M) or t-BT (25 μ M) to the medium, or transferred into the normal atmosphere with vigorous shaking with or without PI (2.5 μ M), H_2O_2 (50 μ M) and t-BT (25 μ M). Samples were harvested after 0, 20, 40 and 60 min. RNA was then extracted and hybridized either with radiolabelled internal probes corresponding to the various genes or with a 16S rRNA probe used as an internal standard. Some of the data are illustrated in Fig. 5. The main results may be summarized as follows.

(a) The *rub1* and *CPE0778* genes of the GRI region were not induced when *C. perfringens* cells were shaken in air with or without PI. In contrast, under the same aerobic conditions,

the *CPE0781* gene of the GRII region was induced three- to sevenfold, the lowest value being obtained with 2.5 μ M PI. These results suggested that the genes of the GRII region, which are fully activated under aerobic conditions but not activated under anaerobic conditions, participate one way or another in the stress response.

(b) The *sod* gene was induced three- to fourfold under aerobic conditions when the intracellular superoxide-producing drug PI was present. The induction was detected 20 min after addition and remained maximal for at least 60 min. This suggested that the *sod* gene also takes part in the superoxide stress response. When H_2O_2 or t-BT was added to the medium in both aerobic and anaerobic conditions, the *sod* gene was not significantly induced. It is nevertheless possible that the basal level of transcription was sufficient to produce superoxide activity in the presence of oxygen, H_2O_2 or t-BT, as already suggested by Geissmann *et al.* (1999).

(c) The mRNA induction level of the three rubrerythrin-encoding genes (*rbr1*, *rbr2* and *rbr3*) was only significant (three- to fourfold) when the cells were shaken in air in the presence of t-BT.

(d) The *ahpC* gene of *C. perfringens* was highly induced (up to 50-fold) whatever the aerobic stress conditions used. Under anaerobic conditions, the induction was less important, about eightfold in cells challenged with H_2O_2 and between 10- and 20-fold in cells challenged with t-BT.

Physiological properties of mutants impaired in the potentially regulatory genes of GRI and GRII of *C. perfringens*

To confirm the physiological role of the genes studied in the oxidative stress response of *C. perfringens*, we attempted to create insertional mutants of the genes of interest. Unfortunately, despite numerous experiments, we failed to inactivate the *sod* gene. However, we successfully disrupted the regulatory genes of the GRI and GRII regions (*CPE0776* and *CPE0779*, respectively). The survival of the *CPE0776*::*catP* and *CPE0779*::*catP* mutants was monitored under aerobic conditions with and without PI. The survival curves were identical to those obtained with the wild-type strain, but the sizes of the colonies were considerably smaller compared to those of strain 13R (data not shown). The results suggested that even if the activity of Rub1 and *CPE0778* is not essential for the survival of *C. perfringens* under aerobic conditions, and that lowering the activity of the *CPE0781-rub2* operon was not sufficient to impair survival at least during the time-course (6 h) of the experiment, they might be both required when the cells are maintained for a longer period of time under aerobic conditions.

DISCUSSION

The functional complementation of *E. coli* strains lacking SOD showed that the SOD protein, the two rubredoxin/haemoprotein and the three rubrerythrin polypeptides of

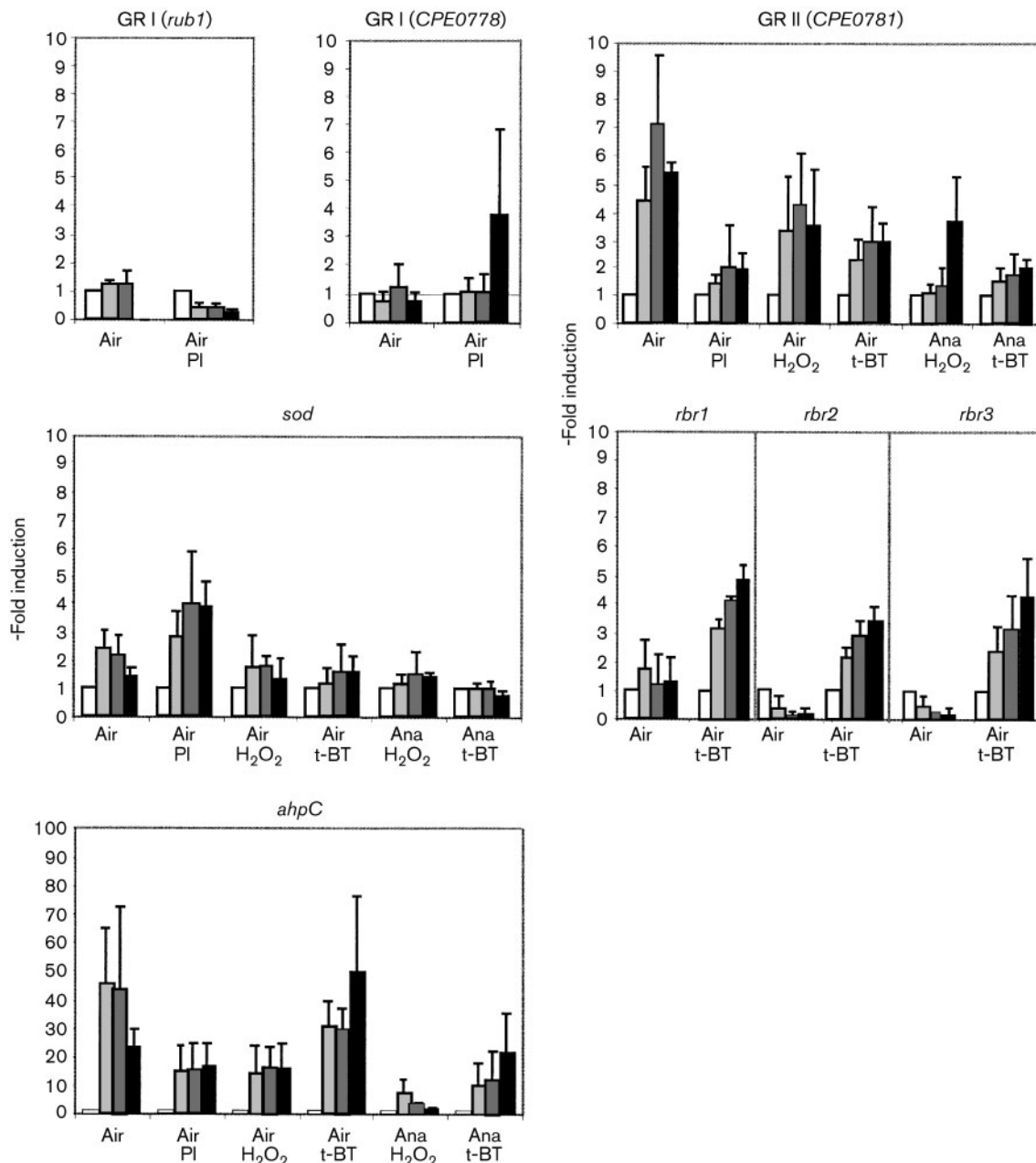


Fig. 5. Levels of *rub*, *rbr*, *sod* and *ahpC* transcripts in *C. perfringens* under various oxidative stress conditions. Mid-exponential-phase *C. perfringens* strain 13 cells were maintained under anaerobic (Ana) conditions after addition at $t=0$ of 50 μM H_2O_2 or 25 μM t-BT or transferred into normal atmosphere (Air) after addition or not of 2.5 μM PI, 50 μM H_2O_2 or 25 μM t-BT. Aliquots were harvested at 0 (white bars), 20 (light grey bars), 40 (dark grey bars) and 60 min (black bars) and total RNA was prepared. Northern blotting was performed with an internal probe for *rub1* and *CPE0778* from the GRI region, *CPE0781* from the GRII region, *sod*, *rbr1*, *rbr2*, *rbr3* and *ahpC*. Results were normalized using a specific 16S rRNA probe as internal standard. The ratios of specific mRNAs/16S rRNAs at $t=0$ in air were: 0.064 ± 0.013 (*rub1* probe); 0.217 ± 0.076 (*CPE0778* probe); 0.034 ± 0.01 (*CPE0781* probe); 0.287 ± 0.051 (*sod* probe); 0.035 ± 0.005 (*rbr1* probe); 0.137 ± 0.03 (*rbr2* probe); 0.367 ± 0.129 (*rbr3* probe); 0.070 ± 0.027 (*ahpC* probe).

C. perfringens strain 13 protect the mutant strain from the adverse effect of superoxide to some extent. In contrast, they were unable to protect an *E. coli* mutant lacking catalase activity from H_2O_2 . However, physiological studies of *C.*

perfringens mutants and mRNA expression levels showed that these results were not sufficient to conclude that these genes are definitely involved in the oxidative stress response of this species.

Geissmann *et al.* (1999) have shown that the *sod* mRNA level of *C. perfringens* strain NCIMB8875 increases during exponential growth and peaks upon entry into stationary phase, independently of whether *C. perfringens* is grown under anaerobic or partially aerobic conditions. In the more drastic stress conditions used in this study, exposure to oxygen induced the *sod* gene by a maximum of threefold. Due to the rather high level of SOD still present under anaerobic conditions, this response is undoubtedly sufficient to protect cells against superoxide generated under aerobic conditions. The fact that we failed to generate a *sod* mutant of *C. perfringens* strain 13R suggests that the *sod* gene is essential for survival. However, superoxide-deficient mutants of aerobic organisms [e.g. *E. coli* (Carlioz & Touati, 1986), *Candida albicans* (Hwang *et al.*, 2002), *Streptococcus agalactiae* (Poyart *et al.*, 2001)], or of microaerophilic bacteria exemplified by *Helicobacter pylori* (Seyler *et al.*, 2001), have been readily obtained. These mutants are generally more sensitive to oxygen or to oxidative stress than the wild-type and display an increased susceptibility to bacterial killing by macrophages. No such mutants have been obtained in strictly anaerobic Gram-positive bacteria, but *sod* mutants have been reported in *Porphyromonas gingivalis* (Nakayama, 1994; Lynch & Kuramitsu, 1999).

C. perfringens 13R possesses three genes encoding a non-haem iron protein belonging to the rubrerythrin family. Some members of this family possess NADH peroxidase activity in an *in vitro* system containing NADH and H₂O₂, and a bacterial NADH oxidoreductase (Coulter *et al.*, 1999). However, the specific activity is very low and the activity detected *in vitro* may be different from that expressed *in vivo*. As some of these proteins can complement *E. coli* strains devoid of SOD, they may possess SOD-like activity (Lehmann *et al.*, 1996). However, according to Lumppio *et al.* (1997), this activity was one or two orders of magnitude lower than that reported for classical iron SOD. They thus suggested that these proteins protect cells against oxidative stress by both scavenging iron from and donating iron to oxidatively damaged [4Fe–4S] proteins. The proteins may also have peroxidase activity. A mutant of *P. gingivalis*, an obligate anaerobe, devoid of rubrerythrin-like proteins, was more sensitive to dioxygen and H₂O₂ than the wild-type strain (Sztukowska *et al.*, 2002). Our data indicated that the three rubrerythrin genes were only significantly induced when the cultures were shaken in air in the presence of t-BT. Thus, a possible role of the rubrerythrin proteins in the redox reaction required to eliminate organic peroxides under aerobic conditions is not excluded, but an efficient hydrogen peroxidase activity of these proteins *in vivo* remains questionable. This latter hypothesis was also suggested by Geissmann *et al.* (1999), who showed that in *C. perfringens* NCIMB8875, rubrerythrin (*rbr*) gene transcription was not influenced by oxidative stress.

The *C. perfringens* genes or operons able to restore the growth of a *sodA sodB E. coli* mutant on minimal medium

included the two rubredoxin genes associated with flavoprotein- or flavohaemoprotein-encoding genes (*CPE0778* and *CPE0781*, respectively). The *rbo* genes from *Desulfovibrio* species have the same properties (Pianzola *et al.*, 1996). It has been proposed that the Rbo protein catalyses the reduction of the superoxide anion to H₂O₂ at the expense of cellular reducing agents such NAD(P)H (Liochev & Fridovich, 1997; Emerson *et al.*, 2002; Kurtz & Coulter, 2002). It has also been proposed that the flavohaemoprotein is a rubredoxin-oxygen oxidoreductase (Roo), the final component of a soluble electron transfer chain that produces energy by recycling NAD(P)⁺ from carbon reserves in an aerobic environment (Santos *et al.*, 1993). The fact that in *C. perfringens* these genes might be regulated by a CRP-like protein, which controls the response to glucose starvation, and that the size of the colonies of the mutants impaired in these regulatory genes is smaller than those of the wild-type strain when plated after oxidative stress, favour the latter hypothesis. Moreover, orthologues of putative NADH:rubredoxin-oxidoreductase and rubredoxin-oxygen-oxidoreductase genes are widespread in anaerobic micro-organisms, even though the genetic organization may be different (Das *et al.*, 2001; Lumppio *et al.*, 2001)

The *ahpC* gene was rapidly and fully induced when exponentially growing wild-type *C. perfringens* was exposed to air or to various oxidative stress under anaerobic conditions. As a general rule, anaerobic bacteria do not possess catalase activity. It is thus possible that in strict anaerobic bacteria AhpC plays a pivotal role in scavenging the various peroxide species, and accumulates the activity of the two major scavenging enzymes, alkyl hydroperoxide reductase and catalase found in aerobic bacteria (Seaver & Imlay, 2001).

Thus, taken together this specialized enzymic equipment may explain how *C. perfringens* and other strictly anaerobic bacteria can survive occasional oxidative stress, despite the fact that the key metabolic enzymes required for normal growth are promptly damaged by oxygen or superoxide (Imlay, 2002; Pan & Imlay, 2001).

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