Isolation and characterization of a hydrogen peroxide resistant mutant of *Bacillus subtilis*

Orla M. Hartford† and Barbara C. A. Dowds

Author for correspondence: Barbara C. A. Dowds. Tel: +353 1 6285222. Fax: +353 1 7083845.
e-mail: BDOWDS@VAX1.MAY.IE

Department of Biology, St Patrick's College, Maynooth, Co. Kildare, Ireland

A mutant of *Bacillus subtilis* has been isolated by continuous selection in increasing concentrations of H₂O₂. It grew with a doubling time of 85 min in minimal medium containing 150 mM H₂O₂, whereas the wild-type parent lysed in 100 mM H₂O₂. The mutant was also more resistant to organic peroxides than the wild-type. Further resistance to H₂O₂ could not be induced by pretreatment with low concentrations of the oxidant. The mutant synthesized a number of proteins at a much higher rate than the wild-type, including constitutive synthesis of all of the proteins which were induced by H₂O₂ in the wild-type. Four of these proteins were sequenced; three were identified as catalase and two subunits of alkyl hydroperoxide reductase. Two proteins whose synthesis was repressed in the mutant were sequenced, and one was identified as flagellin. The mutant grew as non-flagellated, partially septate, filaments of cells, and fragments of flagella were seen in the surrounding medium.

Keywords: *Bacillus subtilis*, hydrogen peroxide resistant mutant

INTRODUCTION

All aerobic organisms generate oxygen free radicals as a result of oxygen metabolism. These include the superoxide radical, H₂O₂ and the hydroxyl radical, which are highly reactive with DNA, lipids and proteins. Cells protect themselves against oxidant toxicity either by scavenging the oxidants with enzymes such as catalase, superoxide dismutase and reductases or, in the case of DNA damage, by repairing the damage after it has taken place (Farr & Kogoma, 1991).

Oxidative stress may be generated experimentally by treating cells with H₂O₂. Treatment with high concentrations of this oxidant kills cells, whereas pretreatment with low concentrations can protect the cells against the otherwise lethal effects of high concentrations. This phenomenon has been studied in detail in *Salmonella typhimurium*, where a 60 µM H₂O₂ pretreatment protects the cultures against the effects of a 10 mM treatment and causes the induction of at least 30 proteins, as determined

on two-dimensional gels (Christman *et al.*, 1985). The induction of at least nine of these proteins is positively regulated by the OxyR protein (Christman *et al.*, 1985), which is activated directly by oxidation (Storz *et al.*, 1990). The OxyR-regulated proteins include catalase, alkyl hydroperoxide reductase and glutathione reductase as well as some heat-shock proteins (Christman *et al.*, 1985). *Escherichia coli* mounts a similar response to H₂O₂ (Farr & Kogoma, 1991). In *E. coli*, the increased survival after pretreatment with low concentrations of H₂O₂ results partly from an increased capacity for DNA repair (Demple & Halbrook, 1983), but this appears not to be controlled as part of the *oxyR* regulon (Farr & Kogoma, 1991). A different but overlapping set of proteins are induced by superoxide-radical-generating agents (Greenberg & Demple, 1989), and the induction of at least six of these proteins is positively regulated by the SoxR and SoxS proteins in *E. coli* (Wu & Weiss, 1991).

We have studied the effects of H₂O₂ on *Bacillus subtilis*. These bacteria are Gram-positive obligate aerobes which sporulate in response to some stresses. Thus they might be expected to display a different response to oxidative stress from *E. coli* and *S. typhimurium*. In the case of *B. subtilis* JH642, there is a 0.01–0.1 % survival after a 10 min treatment with 10 mM H₂O₂ during the exponential phase of growth. Pretreatment of cultures with 50–100 µM H₂O₂ results in an approximately 100-fold increase in survival (Dowds & Hoch, 1991; Murphy *et al.*,

† Present address: Department of Microbiology, Trinity College, Dublin 2, Ireland.

The PIR accession numbers for the amino acid sequence data reported in this paper are shown in parentheses after the proteins: 78 kDa (A44519), 39 kDa (B44519), 113 kDa (D44519), 54 kDa (E44519), 59.5 kDa (F44519) and 23 kDa (G44519).

1987). During the pretreatment period at least eight proteins are transiently induced, as determined on one-dimensional gels (Murphy *et al.*, 1987). Cells with null mutations at the *spo0A* locus are unable to respond to changes in the environment which would normally lead to sporulation, and are defective in initiating a wide range of stationary-phase events (Trach *et al.*, 1990). These mutants are also about 100-fold more resistant to the effects of 10 mM H₂O₂ than the wild-type (Dowds *et al.*, 1987); this effect is mediated by the *hpr* pathway as shown by the suppression of the resistance phenotype in *spo0A* mutants by a second mutation in the *hpr* gene (Dowds & Hoch, 1991). The *spo0A* mutation does not affect the inducible phenotype, despite the high level of uninduced resistance to H₂O₂ of strains bearing this mutation (Dowds & Hoch, 1991).

In this study we set out to generate a constitutively resistant mutant of *B. subtilis* which could be used to define the gene regulating induced protection against H₂O₂. In addition, it was hoped that the mutant would help us to clone the genes coding for the induced proteins. This task has been difficult due to the non-abundant nature of these proteins and the transience of their induction. Screening of gene fusions for induction by H₂O₂ yielded two clones that were induced late (around 1 h) following H₂O₂ addition. One of these was induced as a consequence of induction of the defective prophage PBSX by H₂O₂ (Hartford & Dowds, 1992). For this reason, the mutant described here was generated from strain YB886, which is uninducible for PBSX and has been cured of another defective prophage SPβ (Yasbin *et al.*, 1980). The mutant was found to be constitutively resistant to high concentrations of H₂O₂ and accumulated high levels of several proteins, including those induced by H₂O₂ in the wild-type.

METHODS

Mutant selection. The *B. subtilis* strain used in this work was YB886 (*trpC2 metB5 xin-1 SPβ*) (Yasbin *et al.*, 1980). The mutant strain MA991 was derived from YB886 as follows. A culture of YB886 was subjected to continuous selection over 16 growth cycles in increasing concentrations of H₂O₂, taking the approach of Sauret *et al.* (1979). The culture was grown in minimal medium (Hartford & Dowds, 1992) supplemented with Casamino acids (0.08%) (MM+CAA) in 20 ml volumes. In each cycle, the culture was grown for 2 h before the addition of H₂O₂, which was then present for one growth cycle (10 h). A volume of 0.3 ml was then diluted into 20 ml medium as before, grown for 2 h, and a higher concentration of H₂O₂ was added for 10 h as before. This continued for 16 growth cycles with the following concentrations (mM) of H₂O₂ being added to successive cycles of growth: 0.2, 1, 2, 2.5, 3.5, 5, 7.5, 11, 15, 21, 28, 36, 44, 58, 65 and 74. After each cycle, cells were plated onto MM+CAA agar (MM+CAA with 1.5% agar) to check for obvious contamination. After the last cycle, the stationary-phase culture was diluted and plated on MM+CAA agar and six colonies were picked. Each was grown in MM+CAA containing 50 mM H₂O₂. All had the same growth rate, which was significantly higher than that of YB886 under the same conditions. One was used for further experiments and named MA991.

Assessment of survival after short-term treatments with H₂O₂. Cultures were grown to an OD₅₅₀ of 0.15. One millilitre amounts were placed in test-tubes containing 0–1 M H₂O₂ and the tubes were shaken at 200 r.p.m. for 15 min at 37 °C. When cells were subjected to two treatments, the two were sequential and lasted for 15 min each. Survival of the cells was assessed by plating dilutions on LB agar and comparing the viable titre with that of an untreated control tube of culture shaken for the same period of time.

Metabolic labelling of proteins after H₂O₂ treatment and SDS-PAGE. The procedures used were those described by Murphy *et al.* (1987).

Enzyme activity. Cultures were grown in MM+CAA medium to an OD₅₅₀ of 0.2. The cells were lysed with lysozyme (Murphy *et al.*, 1987), and catalase and superoxide dismutase activity were determined by the methods of Aebi (1984) and Darr *et al.* (1987), respectively. Protein concentration was determined using a Bio-Rad protein assay kit.

Preparation of spore coat proteins. Cultures were grown in Schaeffer sporulation medium (Schaeffer *et al.*, 1965) for 7 or 21 h after entry into stationary phase (*t*₇ and *t*₂₁, respectively). Spore-coat proteins were extracted by the SDS/DTT method of Donovan *et al.* (1987).

Protein sequencing. Early-exponential-phase (OD₅₅₀ 0.2) extracts were used for sequencing of all proteins except the 78 kDa protein, which was sequenced from a late-exponential-phase (OD₅₅₀ 0.8) extract. Samples were prepared for gels as described by Murphy *et al.* (1987), except that the dye was added after boiling and the early-exponential-phase samples were concentrated 10-fold on a Centricon 10 (Amicon) after clearing of the lysates. The 12.5% (w/v) SDS-PAGE gels were prepared and run as described by Murphy *et al.* (1987) with the following exceptions. Piperazine diacrylamide was used as a crosslinker (0.8% in a 30%, w/v, acrylamide stock), and the upper reservoir contained glutathione (0.05 mM) for a prerun at 3 mA for 2 h and sodium thioglycolate (0.1 mM) for the electrophoresis run after loading of the samples. After electrophoresis, the contents of the gel were blotted onto ProBlott membrane (Applied Biosystems) by the method of Matsudaira (1987), and the protein bands stained with amido black (0.1%) according to the instructions of Applied Biosystems. N-terminal sequencing was performed at the SERC Protein Sequencing Facility at the University of Aberdeen, Scotland, UK. The sequences were compared to protein and translated DNA sequences in the GenBank database using the program of the National Centre for Bioinformatics in Trinity College Dublin.

Electron microscopic analysis. YB886 and MA991 were grown in LB overnight, and the stationary-phase cultures were placed on carbon-coated grids, negatively stained with 1%, w/v, sodium silicotungstate for 2–3 min and examined with a transmission electron microscope (Hitachi H-7000, accelerating voltage 100 kV).

DNA manipulation. All of these methods were as described by Sambrook *et al.* (1989).

RESULTS

Isolation of H₂O₂-resistant mutant

An attempt was made to isolate a H₂O₂-resistant mutant by selecting mutagenized cells on LB medium containing 3.1 mM H₂O₂. No resistant colonies were found by this

Fig. 1. Effect of (a) 150 mM H₂O₂, (b) 1 mM cumene hydroperoxide and (c) 1 mM *t*-butyl hydroperoxide on the growth of YB886 (wild-type, ◇) and MA991 (mutant, ◆). Cultures were grown in MM+CAA and were treated at an OD₅₅₀ of 0.2 at *t* = 0 min.

means. Failure to isolate a mutant with this method probably stemmed from difficulty with the selection procedure. The instability of H₂O₂ in hot medium probably led to day-to-day variation in the concentration of H₂O₂ on the plates and therefore variation in the ability of the wild-type to grow on the medium. Thus an alternative selection method was used in which there was no possibility of the wild-type surviving.

A single colony of YB886 was subjected to continuous selection over 16 growth cycles in increasing concentrations of H₂O₂ from 200 µM to 74 mM. After the last cycle, cells were plated and six colonies were picked for measurement of their growth rates in the presence of H₂O₂ (50 mM). All six had the same growth rate, which was considerably higher than that of the wild-type. One of the six was used in further experiments and has been designated MA991. It was a rod-shaped bacterium which was auxotrophic for tryptophan and methionine, like the parent strain YB886.

Table 1. Percentage survival of *B. subtilis* after H₂O₂ treatment

With no treatment there was 100% survival. The values marked *a–h* were obtained in eight different experiments. H₂O₂ was added to cultures for 15 min starting at an OD₅₅₀ of 0.15.

[H ₂ O ₂] (mM)	Percentage survival	
	MA991 (mutant)	YB886 (wild-type)
1	100 ^a , 96 ^b	
10	11 ^c	0.9 ^c
50	10 ^c , 11 ^d	0.0003 ^c
100	11 ^d	
150	9 ^d	
200	8 ^d , 7 ^e	
350	0.4 ^f , 0.4 ^g , 0.2 ^h	< 0.00003 ^f
500	0.05 ^e	
1000	< 0.0001 ^e	

Growth rate of mutant in presence of peroxides

MA991 was grown in MM + CAA in the presence of 0, 50, 100, 150 and 350 mM H₂O₂. It grew well in the first four conditions with doubling times (*t*_d) of 33, 61, 73 and 85 min, respectively, whereas it failed to grow at the 350 mM concentration. In contrast to the mutant, a culture of the parent strain, YB886, lysed in the presence of 100–350 mM H₂O₂ but grew with *t*_d values of 35 and 200 min in MM + CAA with 0 and 50 mM H₂O₂, respectively (see Fig. 1a for the 150 mM concentration). The mutant also grew better than the wild-type in the presence of two other peroxides: cumene hydroperoxide and *t*-butyl hydroperoxide. The culture density of MA991 remained stationary in cumene hydroperoxide (1 mM) whereas YB886 lysed under these conditions (Fig. 1b). In the presence of *t*-butyl hydroperoxide (1 mM), MA991 had a *t*_d of 35 min compared to a *t*_d of 67 min for the wild-type (Fig. 1c).

Survival of MA991 after short-term H₂O₂ treatment

Table 1 shows the percentage survival of MA991 and YB886 after 15 min treatments with H₂O₂. The mutant displayed a similar response of 7–11% survival to treatments of 10–200 mM. The wild-type parent strain was more sensitive to 10 and 50 mM concentrations than was the mutant and, in addition, the wild-type was considerably more sensitive to the higher than the lower concentration.

Pretreatment of the wild-type strain with 100 µM of the oxidant partially protected it against the higher concentrations, but the mutant did not display any such inducible protection (Table 2). This protective concentration was

Table 2. Percentage survival of *B. subtilis* after pretreatment with low concentrations followed by treatment with high concentrations of H₂O₂

With no treatment there was 100 % survival. The values marked *a* and *b* were obtained in two different experiments. Cultures were treated at an OD₅₅₀ of 0.15 for 15 min. Samples subjected to two treatments were treated sequentially.

[H ₂ O ₂]	Percentage survival	
	MA991 (mutant)	YB886 (wild-type)
100 µM	100 ^b	100 ^a , 100 ^b
10 mM	11 ^b	0.2 ^a , 0.9 ^b
100 µM, 10 mM	10 ^b	8 ^a , 12 ^b
50 mM	10 ^b	0.0003 ^b
100 µM, 50 mM	9 ^b	0.002 ^b

Table 3. Percentage survival of mutant MA991 after pretreatment and treatment with a variety of H₂O₂ concentrations

With no treatment there was 100 % survival. The values marked *a–e* were obtained in five different experiments. Cultures were treated at an OD₅₅₀ of 0.15 for 15 min. Samples subjected to two treatments were treated sequentially.

Pretreatment [H ₂ O ₂]	Treatment [H ₂ O ₂]	Percentage survival
100 µM	100 µM	100 ^{a,b,c}
	400 mM	0.0008 ^a
	100 µM, 400 mM	0.0008 ^a
	500 mM	0.0001 ^a , 0.003 ^b , 0.05 ^c
	100 µM, 500 mM	0.0001 ^a , 0.008 ^b , 0.3 ^c
	1 M	< 0.0001 ^c
500 µM	100 µM, 1 M	< 0.0001 ^c
	500 µM	100 ^d
	200 mM	43 ^d
	500 µM, 200 mM	90 ^d
	300 mM	34 ^d
1 mM	500 µM, 300 mM	15 ^d
	1 mM	96 ^e
	350 mM	5 ^e
	1 mM, 350 mM	3 ^e
	400 mM	1 ^e
	1 mM, 400 mM	0.4 ^e
	450 mM	0.01 ^e
	1 mM, 450 mM	0.002 ^e

determined as the highest concentration which did not affect the viability of the wild-type strain, while the concentration of 10 mM was that which gave rise to survival of 0.1–1 % of the wild-type strain YB886. Thus these were not necessarily suitable concentrations for assessing the inducibility of the mutant. Therefore pro-

Fig. 2. Autoradiogram of SDS-PAGE gel (12.5%) of [³⁵S]methionine-labelled proteins from YB886 (wild-type) (lanes 1–4) and MA991 (mutant) (lanes 5–8). Cells grown to an OD₅₅₀ of 0.2 were pulse labelled for 5 min, as described by Murphy *et al.* (1987), in the absence of H₂O₂ treatment (lanes 1 and 5); 0–5 min (lanes 2 and 6) and 5–10 min (lanes 3 and 7) after 100 µM H₂O₂ treatment; and 0–5 min after 1 mM H₂O₂ treatment (lanes 4 and 8). The arrows beside lane 4 indicate the proteins induced by H₂O₂ in YB886, while the arrows beside lane 8 show the proteins whose rate of synthesis is greater in MA991 than in YB886 (after induction). The positions of molecular mass markers are indicated on the left.

TECTIVE and killing concentrations were established for MA991 to determine whether the mutant had an inducible response to H₂O₂. The effect of a range of concentrations on the mutant (Table 1) revealed that concentrations of 200–500 mM had to be used in order to effect survival of 0.05–8 %. A concentration of 1 mM had no effect on viability of MA991, while 10 mM killed 90 % of the cells. Thus concentrations of 1 mM down to the wild-type level of 100 µM were used to attempt to protect MA991 against killing concentrations of 200–1000 mM H₂O₂ (Table 3). None of the pairs of concentrations used suggested an inducible response in the mutant, implying that MA991 is constitutively resistant to H₂O₂.

Protein synthesis in wild-type and mutant with and without H₂O₂ treatment

Cells grown to mid-exponential-phase were pulse-labelled with [³⁵S]methionine for 5 min in the absence of H₂O₂ treatment and from 0–5 and 5–10 min after addition of inducing concentrations of H₂O₂ (100 µM and 1 mM) (Fig. 2). In the wild-type strain YB886, synthesis of four proteins was induced by H₂O₂ (compare lane 1 with lanes

Fig. 3. SDS-PAGE gel (12.5%) of cleared lysates of YB886 (wild-type) (lane 2) and MA991 (mutant) (lane 3) stained with Coomassie blue. Cultures were grown to an OD₅₅₀ of 0.8 and samples prepared as described by Murphy *et al.* (1987). Arrows indicate the position of proteins which accumulate to different extents in the two strains. Lane 1, molecular mass markers.

2–4, Fig. 2). These proteins of 16, 23, 54 and 59.5 kDa are four of the six proteins previously observed to be induced immediately following H₂O₂ treatment of another wild-type strain, JH642 (Murphy *et al.*, 1987). No proteins were induced in the mutant MA991 following H₂O₂ treatment (Fig. 2, lanes 5–8). However, a comparison of the mutant and the wild-type reveals that several proteins were synthesized at a much higher rate in the mutant. They include the four proteins induced in YB886 which were synthesized in the mutant at a rate even higher than the induced wild-type rate. In addition, proteins of 40 and 50 kDa were synthesized at a higher rate in the mutant (Fig. 2), and synthesis of a 39 kDa protein which was constitutive in the wild-type was not detected at all in the mutant.

Protein accumulation in wild-type and mutant

Lysates of the cell pellets of YB886 and MA991 were run on an SDS-PAGE gel and stained with Coomassie blue (Fig. 3). Proteins of 16, 23, 54, 59.5 and 113 kDa accumulated to a greater extent in the mutant than in the wild-type. In addition, the 40 kDa protein was repeatedly

Fig. 4. Coomassie-blue-stained SDS-PAGE gel (15%) of spore-coat proteins prepared from YB886 (wild-type) (lane 2) and MA991 (mutant) (lane 3) cultures grown to *t*₇. Arrows indicate proteins which accumulate to a different extent in the two strains. Lane 1, molecular mass markers.

observed to over-accumulate in the mutant compared to the wild-type, but this difference cannot be seen on the gel shown in Fig. 3. The 50 kDa protein which was synthesized at a higher rate in the mutant (Fig. 2) was not seen on the Coomassie blue-stained gels (Fig. 3), and is presumably a low-abundance protein. The 113 kDa protein which over-accumulated in the mutant (Fig. 3) was not clearly identifiable on the protein synthesis gels (Fig. 2). Proteins of 28, 39 and 78 kDa accumulated to a much smaller extent in the mutant. The difference in the accumulation of the 78 kDa protein was seen only in late-exponential-phase samples. All of the other differences were observed in both early- and late-exponential-phase samples.

Analysis of spore-coat proteins in MA991

MA991 appeared as brown colonies after 1 d growth on LB agar, unlike YB886, whose colonies are white in colour. The brown colour of MA991 intensified with time, suggesting that the colour might be associated with the formation of spores, whose brown pigment is a 65 kDa spore-coat protein encoded by the *cotA* gene (Donovan *et al.*, 1987). Thus, the spore-coat proteins of the mutant and wild-type were compared. The 65 kDa protein was a low-abundance protein under the conditions of growth and extraction used here, and there was no difference in the intensity of this band between the two strains (Fig. 4). However, two major spore-coat proteins accumulated to different extents in YB886 and MA991 at *t*₇, defined as 7 h after entry into stationary phase (Nicholson & Setlow, 1990). A 41 kDa protein under-accumulated in the mutant, while a thick band of

Fig. 6. Cells of (a) YB886 (wild-type) and (b) MA991 (mutant) and (c) flagella fragments of MA991 examined by transmission electron microscopy. Bars: (a) 1.43 μ m; (b) 588 nm; (c) 3.3 μ m.

from large-scale rearrangement or amplification of a region of the genome.

MA991 was highly resistant to short- and long-term exposure to H₂O₂, and was less sensitive than the wild-type to cumene hydroperoxide and *t*-butyl hydroperoxide. The resistance to H₂O₂ was constitutive and could not be further induced by treatment with low concentrations of H₂O₂. However, the mutant was no more resistant than the wild-type to cadmium chloride, mitomycin C, paraquat, plumbagin, *N*-ethylmaleimide or a 57 °C heat shock (data not shown). Protein synthesis and accumulation was examined in the two strains. Four proteins that were induced by H₂O₂ in the wild-type were constitutively synthesized at a high rate in the mutant (higher than in the induced wild-type). Three of these were identified as catalase and two subunits of alkyl hydroperoxide reductase, which degrade H₂O₂ and organic hydroperoxides (including cumene hydroperoxide and *t*-butyl hydroperoxide), respectively (Storz *et al.*, 1989). The quality of filamentation is another attribute of the mutant found in wild-type *B. subtilis* treated with H₂O₂ (3 mM) (Bol & Yasbin, 1990). The lack of attached flagella in MA991 probably derives directly from its filamentation, as these two characteristics have been associated in a variety of mutants studied (e.g. Helmann *et al.*, 1988).

In summary, MA991 is constitutively resistant to high concentrations of H₂O₂, it synthesizes all of the proteins induced by H₂O₂ in the wild-type and it grows as filaments of cells. Thus, MA991 has a collection of attributes that can be induced in the wild-type in response to H₂O₂ treatment. This is consistent with MA991 being affected in a gene controlling a peroxide-response regulon. Attempts were made to isolate this regulatory gene by trying to transform YB886 to H₂O₂ resistance with a bank of MA991 DNA on an integrating plasmid. These attempts were unsuccessful, and it is not clear whether MA991 possesses more than one mutation or whether the problem lay in the selection conditions used. Thus it is not clear whether the multiple traits of MA991 derive from a single or multiple mutations. However, it is probable that MA991 has been affected in at least one regulatory gene involved in induced resistance to H₂O₂.

We have previously shown that a *spo0A* mutant JH646, which is defective in responding to conditions which activate sporulation, is 100-fold more resistant to H₂O₂ than its wild-type parent, JH642 (Dowds *et al.*, 1987; Dowds & Hoch, 1991). However, some further resistance of this mutant can be induced by low concentrations of H₂O₂ (Dowds & Hoch, 1991). In addition, while only two of the proteins induced in the wild-type are also induced in the *spo0A* strain, most of the remaining proteins are not synthesized at a high level in the mutant. The only exception is the 20 kDa protein (the JH646 equivalent of the 23 kDa protein of MA991), which is constitutively synthesized in the *spo0A* mutant at approximately the same rate as in the induced wild-type (Dowds *et al.*, 1987). Thus, it appears that a gene for alkyl hydroperoxide reductase is expressed constitutively in both MA991 and in the *spo0A* mutant JH646. However, it is clear that MA991 and JH646 contain different

regulatory mutations, the former affecting the inducible phenotype and the latter affecting the basal, uninduced sensitivity to H_2O_2 .

ACKNOWLEDGEMENTS

We thank Bryan Dunbar for performing the protein sequencing, Colin Reid for help with the electron microscopy and Siobhan Byrne for performing the autolysin assays. This work was supported by Eolas, the Irish Science and Technology Agency.

REFERENCES

- Aebi, H. (1984). Catalase *in vitro*. *Methods Enzymol* **105**, 121–126.
- Bol, D. K. & Yasbin, R. E. (1990). Characterization of an inducible oxidative stress system in *Bacillus subtilis*. *J Bacteriol* **172**, 3503–3506.
- Bol, D. K. & Yasbin, R. E. (1991). The isolation, cloning and identification of a vegetative catalase gene from *Bacillus subtilis*. *Gene* **109**, 31–37.
- Christman, M. K., Morgan, R. W., Jacobson, F. S. & Ames, B. N. (1985). Positive control of a regulon for defences against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* **41**, 753–762.
- Darr, D., Zarilla, K. A. & Fridovich, I. (1987). A mimic of superoxide dismutase activity based upon desferrioxamine B and manganese (IV). *Arch Biochem Biophys* **258**, 351–355.
- DeLange, R. J., Chang, J. Y., Shaper, J. H. & Glazer, A. N. (1976). Amino acid sequence of flagellin of *Bacillus subtilis* 168. *J Biol Chem* **251**, 705–711.
- Demple, B. & Halbrook, J. (1983). Inducible repair of oxidative DNA damage in *Escherichia coli*. *Nature* **304**, 466–468.
- Donovan, W., Zheng, L., Sandman, K. & Losick, R. (1987). Genes encoding spore coat polypeptides from *Bacillus subtilis*. *J Mol Biol* **196**, 1–10.
- Dowds, B. C. A. & Hoch, J. A. (1991). Regulation of the oxidative stress response by the *hpr* gene in *Bacillus subtilis*. *J Gen Microbiol* **137**, 1121–1125.
- Dowds, B. C. A., Murphy, P., McConnell, D. & Devine, K. M. (1987). Relationship among oxidative stress, growth cycle, and sporulation in *Bacillus subtilis*. *J Bacteriol* **169**, 5771–5775.
- Farr, S. B. & Kogoma, T. (1991). Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol Rev* **55**, 561–585.
- Goldberg, I. & Mekalanos, J. J. (1986). Effect of a *recA* mutation on cholera toxin gene amplification and deletion events. *J Bacteriol* **165**, 723–731.
- Greenberg, J. T. & Demple, B. (1989). A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. *J Bacteriol* **171**, 3933–3939.
- Hartford, O. & Dowds, B. C. A. (1992). Cloning and characterization of genes induced by hydrogen peroxide in *Bacillus subtilis*. *J Gen Microbiol* **138**, 2061–2068.
- Helmann, J. D., Marquez, L. M. & Chamberlin, M. J. (1988). Cloning, sequencing, and disruption of the *Bacillus subtilis* σ^{28} gene. *J Bacteriol* **170**, 1568–1574.
- Hornemann, U., Otto, C. J., Hoffman, G. G. & Bertinsson, A. C. (1987). Spectinomycin resistance and associated DNA amplification in *Streptomyces achromogenes* subsp. *rubradiris*. *J Bacteriol* **169**, 2360–2366.
- Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**, 10035–10038.
- Murphy, P., Dowds, B. C. A., McConnell, D. J. & Devine, K. M. (1987). Oxidative stress and growth temperature in *Bacillus subtilis*. *J Bacteriol* **169**, 5766–5770.
- Nicholson, W. L. & Setlow, P. (1990). Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus*, pp. 391–450. Edited by C. R. Harwood & S. M. Cutting. Chichester: John Wiley.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sauret, G., Jouve, H. & Pelmont, J. (1979). Propriétés d'un mutant de *Proteus mirabilis* résistant au peroxyde d'hydrogène. *Canadian J Microbiol* **25**, 312–320.
- Schaeffer, P., Miller, T. & Aubert, J. (1965). Catabolic repression of bacterial sporulation. *Proc Natl Acad Sci USA* **54**, 701–711.
- Storz, G., Jacobson, F. S., Tartaglia, L. A., Morgan, R. W., Silveira, L. A. & Ames, B. N. (1989). An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of *ahp*. *J Bacteriol* **171**, 2049–2055.
- Storz, G., Tartaglia, L. A. & Ames, B. N. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation of oxidation. *Science* **248**, 189–194.
- Tartaglia, L. A., Storz, G., Brodsky, M. H., Lai, A. & Ames, B. N. (1990). Alkyl hydroperoxide reductase from *Salmonella typhimurium*. *J Biol Chem* **265**, 10535–10540.
- Trach, K., Burbulys, D., Spiegelman, G., Perego, M., Van Hoy, B., Strauch, M., Day, J. & Hoch, J. A. (1990). Phosphorylation of the Spo0A protein: a cumulative enviroinsensory activation mechanism. In *Genetics and Biotechnology of Bacilli*, vol. 3, pp. 357–365. Edited by M. M. Zukowski, A. T. Ganesan & J. A. Hoch. San Diego: Academic Press.
- Wu, J. & Weiss, B. (1991). Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. *J Bacteriol* **173**, 2864–2871.
- Yasbin, R. E., Fields, P. I. & Anderson, B. J. (1980). Properties of *Bacillus subtilis* 168 derivatives freed of their natural prophages. *Gene* **12**, 155–159.

Received 27 May 1993; revised 12 August 1993; accepted 19 August 1993.