Cloning and characterization of genes induced by hydrogen peroxide in *Bacillus subtilis*

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(Received 10 February 1992; revised 19 June 1992; accepted 30 June 1992)

Transcriptional fusions of *Bacillus subtilis* DNA to the *lacZ* gene were screened for induction, initially by ethanol and then by hydrogen peroxide (H_2O_2). Two fusions were identified which were induced late following treatment with sublethal concentrations of H_2O_2 (100 μM). The *oxy-1* promoter was induced 4–5-fold and mapped to 11° while the *oxy-2* promoter was induced 20-fold and mapped close to the right of the defective prophage PBSX, at about 120°. The *oxy-2* fusion was induced by mitomycin C as well as H_2O_2 , which correlated with the induction of PBSX by these agents. This was probably not a transcriptional induction, but rather a consequence of the induction of PBSX replication extending into adjacent regions of the chromosome.

Introduction

Many studies have reported the adaption of bacteria to a variety of environmental stresses. Bacteria have been shown to adapt to high levels of stress as a result of pretreatments that provoke lesser stress. Such protective inductions can be seen in *Bacillus subtilis* in response to oxidative stress. Pretreatment of *B. subtilis* with hydrogen peroxide (H_2O_2 ; 100 μM) protects the cells against the otherwise lethal effects of a high concentration (10 mM) of the oxidant (Murphy *et al.*, 1987; Dowds *et al.*, 1987). The pretreatment leads to an induced synthesis of a number of proteins which may play a protective role against H_2O_2 .

A regulatory protein OxyR, that has been identified in *Salmonella typhimurium* and *Escherichia coli*, induces, in its oxidized form, at least nine genes involved in stress tolerance (Christman *et al.*, 1985; Storz *et al.*, 1990). Among these are the genes encoding glutathione reductase, catalase and an alkyl hydroperoxide reductase. Mutants with deletions of the *oxyR* gene, which cannot induce the nine proteins, are sensitive to H_2O_2 , alkyl hydroperoxide and some redox-cycling agents. Furthermore, a dominant mutation in this gene causes resistance to H_2O_2 , as well as overexpression of the nine proteins.

Isolation of DNA repair mutants in *E. coli* has led to some understanding of events following H_2O_2 treatment. Among the gene products that are needed by the cell after challenge with H_2O_2 are exonuclease III and endonuclease IV (Dempfle *et al.*, 1986), which restore

normal 3' termini at single strand breaks generated by H_2O_2 , thus facilitating DNA repair. In addition mutants defective in DNA polymerase I (Ananthaswamy & Eisenstark, 1977), DNA polymerase III (Hagansee *et al.*, 1987) and *recA* (Carlsson & Carpenter, 1980) are extremely sensitive to H_2O_2 . The *recA* gene acts as a positive regulator of the SOS operon, which is induced following DNA damage in the cell. A similar repair response, SOB, has been identified in *B. subtilis*, and is regulated by *recA*⁺ (previously called *recE*⁺ and homologous to *recA* of *E. coli*). A strain carrying the *recE4* mutation is sensitive to low concentrations of H_2O_2 (Bol & Yasbin, 1990).

Catalase also plays a role in the protection of bacteria against H_2O_2 toxicity. An *E. coli* strain carrying the *katG*⁺ plasmid, and consequently containing significantly more catalase, was found to be far more resistant to H_2O_2 than a non-plasmid-bearing strain (Eisenstark & Perrot, 1987). However, these authors also reported that *recA*, *polA* and *oxyΔ3* mutants all had wild-type levels of catalase activity, but much lower survival in response to H_2O_2 than a *kat* mutant with no catalase activity. Thus catalase appears to play a minor role compared with repair enzymes or the range of products regulated by *oxyR* in terms of protecting cells against H_2O_2 . High levels of catalase and superoxide dismutase were found in *B. subtilis*. Whereas it was originally reported that these two enzymes were not induced by H_2O_2 (Murphy *et al.*, 1987), a 5–7-fold induction of catalase has been observed recently (J. F. O'Rourke, O. M. Hartford & B. C. A. Dowds, unpublished).

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In addition to inducing protective genes, H₂O₂ has also been reported to induce the release of phage particles from *B. subtilis* (Stickler *et al.*, 1965).

Genes induced by H₂O₂ have not yet been systematically cloned. Kogoma *et al.* (1988) identified gene fusions in *E. coli* that were induced by superoxide-radical-generating drugs. However, these genes were only gradually induced by these agents, and were not induced by H₂O₂. Damage-inducible (*din*) genes were isolated from *B. subtilis* following treatment with mitomycin C. Some of these genes were also induced by concentrations of H₂O₂ (3 mM) that were high enough to decrease the survival rate of the cultures (Bol & Yasbin, 1990). Here we describe the approach we took towards cloning genes of *B. subtilis* induced by low, protective concentrations of H₂O₂, and the effects of this oxidant on induction of prophages resident in wild-type strains.

Methods

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

Growth of *B. subtilis*. *B. subtilis* JH642 and YB886 were inoculated from fresh plate cultures and grown in Schaeffer's medium (SM) (Schaeffer *et al.*, 1965) for 3 h. A 1 ml volume of culture was diluted into 50 ml SM or minimal medium [Spizizen salts (Anagnostopoulos & Spizizen, 1961) supplemented with glucose (0.5%), L-tryptophan (0.0025%) and either L-phenylalanine (0.0035%) (JH642) or L-methionine (0.004%) (YB886)] and allowed to grow to early exponential phase. Cultures were treated with different concentrations

(100 µM–10 mM) of H₂O₂ at OD₅₅₀ = 0.1. Optical density readings (OD₅₅₀) of the culture were noted at different stages of the growth cycle.

Induction of protection against oxidative stress. *B. subtilis* JH642 was grown to OD₅₅₀ = 0.15 in Luria both (LB) or minimal medium. Aliquots (1 ml) were placed in six test-tubes and treated as shown in Table 2. Treatment times were 30 min for ethanol and 15 min for H₂O₂. Samples subjected to two treatments were treated sequentially unless stated otherwise.

Screening the bank of gene fusions for induction of β-galactosidase expression by ethanol. The bank of gene fusions was that assembled by K. Woodson and others (unpublished). (1) Chloramphenicol-resistant *B. subtilis* transformants were plated on: (a) LB plates containing chloramphenicol (5 µg ml⁻¹), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 µg ml⁻¹) and ethanol (4%, v/v), and (b) LB plates containing chloramphenicol and X-Gal.

(2) Chloramphenicol-resistant transformants that were positive on plates were screened in liquid culture for expression of β-galactosidase in the presence and absence of 100 µM-H₂O₂ or 4% ethanol. Individual transformants were grown at 37 °C to OD₅₅₀ = 0.1. Samples (1 ml) of cells were taken at 20 min intervals after addition of H₂O₂, and were centrifuged at 4 °C for 5 min. The supernatant was then removed by suction and the pellets were frozen at -20 °C. β-Galactosidase levels in the pellets were measured according to Ferrari *et al.* (1986). One unit of β-galactosidase is defined as the amount of enzyme activity hydrolysing 1 nmol *o*-nitrophenyl galactopyranoside min⁻¹ at 28 °C.

Cloning of gene fusions in *E. coli*. High molecular mass DNA was isolated from the fusion strains of *B. subtilis* and digested with a restriction enzyme that cleaved pJM783 just once (in the multiple cloning site in the case of *EcoRI* or in the *lacZ* gene in the case of *Clal*). The digested DNA was then extracted with phenol and precipitated with ethanol before circularizing the digested fragments by ligating at DNA concentrations of 0.1–1 ng µl⁻¹ at 15 °C for 16 h. The circularized

Table 1. *Bacterial strains and plasmids*

Strain/plasmid	Relevant genotype	Source or reference
<i>B. subtilis</i> *		
JH642	<i>trpC2 pheA1</i>	BGSC
YB886	<i>trpC2 metB5 xin-1 SPB</i> ⁻	Yasbin <i>et al.</i> (1980)
SO113	<i>trpC2 amy-3</i>	Ortlepp <i>et al.</i> (1983)
L8508	<i>xhi-1479 lyt-2</i>	D. Karamata, Institut de génétique et de biologie microbiennes, Lausanne, Switzerland.
QB944 (1A3)	<i>cysA14 purA26 trpC2</i>	} BGSC
QB934 (1A5)	<i>glyB133 metC3 tre-12 trpC2</i>	
QB935 (1A8)	<i>aroD120 lys-1 trpC2</i>	
QB917 (1A10)	<i>hisA1 thr-5 trpC2</i>	
QB879 (1A125)	<i>argF4 metA29 thiB4</i>	
VUB79 (1A158)	<i>metA4</i>	
<i>E. coli</i>		
5K	<i>hsdR_k⁻ thi thr leu tonA supE</i>	Hubacek & Glover (1970)
Plasmid		
pJM783†	Amp ^r <i>oriC</i> (<i>E. coli</i>) Cm ^r , but no <i>ori</i> for <i>B. subtilis</i> , promoterless <i>lacZ</i>	J. A. Hoch, Research Institute of the Scripps Clinic, La Jolla, CA, USA.
pWD31	Clones of PBSX	Wood <i>et al.</i> (1990a)
pWD35		
pWD37		
pWD38		
pWD316		

* Numbers in parentheses are BGSC (Bacillus Genetic Stock Center, Ohio State University, Columbus, OH, USA) numbers.

† This plasmid was used for the construction of the bank of gene fusions.

chromosomal DNA was then transformed into *E. coli* 5K and ampicillin-resistant transformants were selected.

Genetic mapping. Mapping of the gene fusions was performed by PBS-1 transduction using the *B. subtilis* mapping kit strains (Dedonder *et al.*, 1977) and *metA* strains (BGSC) as recipients and the fusion strains as donors, and selecting for chloramphenicol resistance. The transduction method described in O'Kane *et al.* (1985) was used with the following variations. When preparing lysates, bacteria were removed from the lysate supernatant by filtration (0.45 µm Acrodiscs from Gelman Sciences) instead of being killed by chloroform treatment. Transductions were not performed on overnight cultures of the recipient strain. Rather, the cultures were grown in LB until they were in a state of maximum motility (usually at a culture density of OD₅₅₀ = 0.6–0.8). In addition, the recipient strain was incubated with the transducing lysate for 15 min instead of 20 min.

Other techniques. DNA preparation, restriction enzyme digestion, transformation of *E. coli*, Southern blotting, hybridization and DNA sequencing were as described in Sambrook *et al.* (1989).

Results

Induction of protection against H₂O₂ by pretreatment with ethanol

Pretreatment of *B. subtilis* JH642 with 50–100 µM-H₂O₂ for 10 min protects cells against the lethal effects of 10 mM-H₂O₂ (Murphy *et al.*, 1987; Dowds & Hoch, 1991). Synthesis of at least six proteins is induced within 5 min of the 50 µM pretreatment. Pretreatment of cultures with ethanol (4%, v/v) for 30 min resulted in an approximately 100-fold increase in survival in the presence of 10 mM-H₂O₂ (Table 2). Ethanol at micromolar concentrations acts as a scavenger of hydroxyl radicals generated by breakdown of H₂O₂ (Imlay & Linn, 1988). Pre-induction of cultures with concentrations of ethanol (0.001%) that scavenge radicals did not protect against lethal levels of H₂O₂ (data not shown). Cells treated with ethanol (4%) and H₂O₂ (10 mM) at the same time showed a survival rate similar to those treated only with 10 mM-H₂O₂ (data not shown). Thus ethanol must be added before H₂O₂ in order to exert the protective effect. Cultures pretreated with chloramphenicol (100 µg ml⁻¹) and ethanol (4%) showed little resistance to H₂O₂ (data not shown). Protein synthesis appears, therefore, to be essential for the observed protection by ethanol.

Identification of gene fusions induced by ethanol

Cloning of the oxidative stress genes relied on the observation that cultures pretreated with ethanol are protected against the lethal effects of H₂O₂. Thus ethanol seems to induce the genes that protect the cells against H₂O₂. Ethanol is more stable than H₂O₂ on agar plates so ethanol was used to identify the oxidative stress

Table 2. *Survival of B. subtilis* JH642 after ethanol and H₂O₂ treatments

Samples subjected to two treatments were treated sequentially. The values marked *a* and *b* were obtained in two different experiments. For reproducibility of this type of data see Murphy *et al.* (1987) and Dowds & Hoch (1991).

Treatment	Percentage survival
Untreated	100 ^{ab}
4% Ethanol	95 ^a
100 µM-H ₂ O ₂	100 ^b
10 mM-H ₂ O ₂	0.15 ^a , 0.05 ^b
4% Ethanol, then 10 mM-H ₂ O ₂	15 ^a
100 µM, then 10 mM-H ₂ O ₂	1.8 ^b

genes. A bank of transcriptional fusions to the *lacZ* indicator gene was constructed in the integrating plasmid pJM783 (Perego & Hoch, 1988) by ligating a partial *Sau*3A digest of *B. subtilis* chromosomal DNA to *Bam*HI-cut pJM783 DNA. The *B. subtilis* chromosomal DNA fragments were thus inserted at the 5' end of the promoterless *lacZ* gene on the plasmid, placing the *lacZ* gene under the control of a promoter in the insertion. pJM783 also contains a chloramphenicol-resistance gene which functions in *B. subtilis*, a gene which confers ampicillin resistance to *E. coli* and an origin of replication for *E. coli* but not for *B. subtilis*. Thus after transformation into *B. subtilis* JH642, the recombinant plasmid must integrate in order to be maintained.

Transformants were grown on medium containing chloramphenicol to select for integrants, ethanol to induce the gene fusions and X-gal, the chromogenic substrate of β-galactosidase. Blue colonies (i.e. those expressing β-galactosidase) were then streaked on X-Gal plates in the presence and absence of ethanol. Out of a total of 12000 chloramphenicol-resistant colonies, 108 were blue in the presence of ethanol and white in its absence on agar plates. Each of these colonies was tested in liquid medium with additions of inducing non-lethal levels of H₂O₂ (100 µM). Three of the 108 ethanol-induced colonies were found to be induced by H₂O₂ in liquid culture. Two of these were later found to be different isolates of the same gene (*oxy-2*). Thus a total of two different gene fusions was detected by this method.

Induction with H₂O₂

β-Galactosidase activity increased significantly in two gene fusions when cells were incubated in the presence of 100 µM-H₂O₂. These fusions showed different patterns of β-galactosidase activity. The *oxy-1* fusion was induced 4–5-fold (Fig. 1*a*) and showed a continuous increase in β-galactosidase activity throughout the growth cycle, with induction being first observed about 75 min after

Fig. 1. Growth (OD₅₅₀; open symbols) and β -galactosidase specific activity (filled symbols) of *B. subtilis* strains JH642::oxy-1 (a) and JH642::oxy-2 (b) in the absence (Δ , $\blacktriangle$) or presence ($\circ$, $\bullet$) of H₂O₂ (100 μ M, added at time zero).

addition of H₂O₂. The second pattern of β -galactosidase expression, seen for the oxy-2 fusion (Fig. 1b), showed a sharp increase followed by a decline in specific activity peaking at 80 min following treatment with H₂O₂. The oxy-2 fusion was maximally induced 20-fold by 100 μ M-H₂O₂.

Effect of H₂O₂ on the growth of *B. subtilis*

Peak induction of the oxy-2 clone occurred shortly after the time at which low concentrations of H₂O₂ began to affect the growth of cultures of *B. subtilis* strain JH642 (Fig. 2a). About 60 min after addition of 100 μ M-H₂O₂ the culture density declined before growth resumed, 20–40 min later. The decrease in the culture density did not occur when catalase was added simultaneously with H₂O₂, but did occur when catalase was added at the actual time of the decrease. This suggests that killing of the cells and induction of β -galactosidase in the clones is mediated not by H₂O₂ but by a product of the action of H₂O₂ on the cells, produced after about 60 min.

Most strains of *B. subtilis* 168 (including JH642) contain two defective prophages: SP β and PBSX (Warner *et al.*, 1977; Seaman *et al.*, 1964). It has been shown that H₂O₂ (440 μ M) induces phages in *B. subtilis* (Stickler *et al.*, 1965). Strain YB886, which has been cured of SP β and rendered non-inducible for PBSX (Yasbin *et al.*, 1980), was tested for its response to H₂O₂.

This strain did not display the dip in the growth curve characteristic of strain JH642 when treated with H₂O₂ (Fig. 2b). This suggests that one or both of the two prophages is being induced by H₂O₂ in *B. subtilis* JH642. When PBSX was disrupted in JH642 with an integrating plasmid containing a fragment from the centre of the late operon (pWD37 from Wood *et al.*, 1990a), the dip in the growth curve following treatment with H₂O₂ (250 μ M) was not observed (Fig. 3a). This implies that the dip observed in JH642 (PBSX intact) after treatment with low concentrations of H₂O₂ is caused by PBSX and not by other prophages such as SP β . However, another phage (possibly SP β) may be induced by high concentrations of H₂O₂ since a 1 mM treatment did cause a dip in the growth curve of JH642::pWD37 (Fig. 3b). The kinetics of cell lysis were different in this case, lysis peaking at 130 min instead of the 80 min observed for PBSX.

Induction with mitomycin C

The correlation between induction of phages and induction of the gene fusions by H₂O₂ suggested that the gene fusions might contain phage genes. We tested this further by assessing their induction by mitomycin C, which has been reported to induce phages PBSX and SP β (Warner *et al.*, 1977) as well as the SOB response (O'Kane *et al.*, 1986). Mitomycin C (0.1, 1 and 3 μ g ml⁻¹) was added to an early exponential phase culture of JH642 and was found to cause a decline in the density of the culture, starting at about 80 min. The density of the culture stabilized at about 110 min but growth was not resumed (data not shown). This dip was also found during growth of JH642::pWD37 in the presence of mitomycin C (data not shown), thus suggesting that mitomycin C induces other phages in addition to PBSX. β -Galactosidase expression was induced by mitomycin C in oxy-2 (Fig. 4) but not in the oxy-1 clone (data not shown), suggesting that the gene present in oxy-2 might be a phage gene while the oxy-1 gene is not.

Isolation of gene fusions

In order to isolate the H₂O₂-inducible promoters, DNA was prepared from the fusions strains of *B. subtilis*. The chromosomal DNA was cleaved with *Eco*RI (which cuts pJM783 in the multiple cloning site) and the fragments were then circularized by ligation at low DNA concentration and transformed into *E. coli*, where pJM783 can replicate. Plasmid DNA was prepared from ampicillin-resistant transformants and was treated with *Sal*I and *Eco*RI (which cut the insert from pJM783). This treatment revealed a 2.3 kb insert for the oxy-2 clone,

Fig. 2

Fig. 2. Effect of H₂O₂ concentration on the growth (OD₅₅₀) of *B. subtilis* strains JH642 (a) and YB886 (b). H₂O₂ was added to cultures in early exponential phase at the following concentrations: Δ , zero; $\circ$, 50 μ M for (a) 200 μ M for (b); $\blacktriangle$, 100 μ M for (a), 500 μ M for (b); $\square$, 200 μ M for (a), 1 mM for (b); $\bullet$, 10 mM for (a) and (b).

Fig. 3

Fig. 3. Effects of H₂O₂ on the growth of *B. subtilis* strain JH642::pWD37. (a) Comparison with the control strain JH642. Δ , $\blacktriangle$, JH642; $\circ$, $\bullet$, JH642::pWD37. Open symbols, absence of H₂O₂; filled symbols, 250 μ M-H₂O₂. (b) Effect of H₂O₂ concentration on strain JH642::pWD37. Δ , zero; $\blacktriangle$, 500 μ M; $\bullet$, 1 mM H₂O₂ was added at time zero.

Fig. 4. Growth (OD₅₅₀; Δ , $\blacktriangle$) and β -galactosidase activity ($\circ$, $\bullet$) of *B. subtilis* strain JH642::oxy-2 in the absence (open symbols) or presence (filled symbols) of mitomycin C (0.1 μ g ml⁻¹; added at time zero).

whereas the insert of *oxy-1* was apparently deleted during cloning. Chromosomal DNA from JH642::oxy-1 was then digested with *Cla*I (which cuts pJM783 once, within the *lacZ* gene), and the fragments were ligated and transformed into *E. coli* 5K. By gel analysis, it was shown that the insert size in the *oxy-1* clone was approximately 1.7 kb. Plasmid DNA was prepared on a

large scale from the positive clones and analysed with various restriction enzymes. The restriction maps differ for the two clones (data not shown), implying that the clones represent two different genes. A second isolate of the gene in the *oxy-2* clone was also found, as determined by restriction mapping, partial sequencing and induction characteristics (data not shown).

Induction of *oxy-2* in YB886

In order to further investigate whether the *oxy-2* gene was derived from PBSX, the clone was transformed into YB886 and its induction was assessed in this strain. After transformation of the *oxy-2* clone into YB886, chloramphenicol-resistant colonies were obtained. Thus we can conclude that the *B. subtilis* gene in *oxy-2* is not located in SP β , which is deleted in YB886 and is therefore not available for recombination with an SP β gene fusion. The gene fusion was not induced by H₂O₂ in YB886 as expected if it is part of one of the PBSX operons, since the prophage is uninducible in this strain. This result appeared to confirm that the *oxy-2* gene is part of PBSX. However, it was also noted that the level of uninduced synthesis of β -galactosidase is zero in this strain compared to an appreciable level of uninduced synthesis

Table 3. Mapping of *oxy-1*
The donor strain was JH642::*oxy-1*.

Recipient strain (BGSC no.)	Marker	Percentage cotransduction with Cm ^r
1A3	<i>purA26</i>	19, 21
	<i>cysA14</i>	98, 100
1A5	<i>glyB133</i>	0
	<i>metC5</i>	0
1A8	<i>lys-1</i>	0
	<i>aroD120</i>	0
1A10	<i>thr-5</i>	0
	<i>hisA1</i>	0

in JH642 (Fig. 1b). It was similarly noted that the level of uninduced and induced synthesis was zero in another wild-type strain, SO113, and in a strain, L8508, in which PBSX is heat-inducible. This suggested that possibly the clone had integrated into these strains at a different site from the one at which the fusion had been located in JH642. Thus the position of the inserted plasmid was determined in these strains and was found to be at approximately the same location in all three [frequency of cotransduction with *metC* = 52% (JH642), 45% (YB886) and 55% (SO113)]. The difference in the level of uninduced synthesis of β -galactosidase might possibly be caused by the promoter for this particular gene being absent in YB886, SO113 and L8508.

Hybridization to PBSX region

pWD316, 38, 37, 35, 31 and a possible repressor fragment (Wood *et al.*, 1990a, b) are clones of fragments of PBSX. Clones pWD38, 37, 35 and 31 span most of the late operon of PBSX, while the other two clones are probably derived from the early operon. These clones were Southern blotted and hybridized to the *oxy-2* probe. *oxy-2* failed to hybridize to any of these fragments. Thus if the *oxy-2* gene is part of PBSX, it is probably located within a part of the phage genome that has not yet been cloned.

Genetic mapping

The *oxy-1* gene mapped very close to *cysA14* at 11°. It displayed 99% and 20% cotransduction with the *cysA14* and *purA26* markers respectively (Table 3).

The gene fused to *lacZ* in *oxy-2* was mapped relative to *metC* and *metA* and compared with similar mapping data from clones representing the late operon of PBSX. The data (Table 4) show that *oxy-2* maps closer to *metC* than to *metA*. It cannot be located between these two genes, i.e. in the PBSX genome, since the PBSX marker pWD37 (Wood *et al.*, 1990a) is closely linked to both

Table 4. Mapping of *oxy-2* and pWD37

Recipient strain (BGSC no.)	Marker	Percentage cotransduction with Cm ^r
(a) <i>oxy-2</i> (donor strain JH642:: <i>oxy-2</i>)		
1A3	<i>purA26</i>	0
	<i>cysA14</i>	0
1A5	<i>metC5</i>	55, 46, 54, 54
	<i>glyB133</i>	0
1A8	<i>lys-1</i>	0
	<i>aroD120</i>	0
1A125	<i>metA29</i>	3-6
1A158	<i>metA29</i>	3-0
(b) pWD37 (donor strain JH642::pWD37)		
1A5	<i>metC5</i>	74
1A125	<i>metA29</i>	49
1A158	<i>metA29</i>	51

metC and *metA* (Table 4). This implies that the *oxy-2* gene maps to the right of the *metC* gene and it is therefore close to, but not part of the PBSX genome. It is at a similar distance to the right of *metC* as pWD37 is to the right of *metA*, i.e. it maps at about 120°.

Discussion

In this paper, we describe the cloning of two genes induced by H₂O₂. The two fusions display different induction kinetics with respect to the time of H₂O₂ addition. *oxy-1* clone induction begins about 75 min after H₂O₂ addition and gene fusion expression continues to rise up to 180 min post addition of the oxidant. Induction of the *oxy-2* fusion occurs immediately, peaks at 80 min and then declines.

Genetic mapping places the *oxy-1* fusion very close to the *cysA* gene (99% cotransduction) at about 11° on the *B. subtilis* chromosome. The gene in the vicinity that appears most relevant is the *recP* gene which maps at 10-5°. Mutations in this gene render the cell sensitive to mitomycin C, suggesting that the gene may be involved in repairing DNA damage caused by this agent. However, we found that *oxy-1* is not induced by mitomycin C, suggesting that this fusion is unlikely to be *recP*. A partial sequence of *oxy-1* (300 nucleotides) did not display homology to any sequences in the GenBank data base (data not shown).

The *oxy-2* gene displays many of the features expected of a gene in the PBSX prophage genome. We found that this defective prophage is induced by H₂O₂ as is the *oxy-2* clone. In addition, the clone was induced by mitomycin C and it has been reported that PBSX is also induced by this agent (Warner *et al.*, 1977). However, we found that *oxy-2* did not hybridize to any of the clones that span the late operon of PBSX and that a 170

nucleotide partial sequence of *oxy-2* was not homologous to any sequence in the GenBank database. Mapping of the *oxy-2* fusion located it to the right of PBSX and *metC* (118°). It has been reported that the *metC* marker is present in multiple copies in cells induced for PBSX replication, suggesting that replication of PBSX extends into adjacent regions of the chromosome (Thurm & Garro, 1975). Thus it is probable that the observed induction of *oxy-2* by H₂O₂ and mitomycin C is actually induction of replication rather than transcription, and that it occurs as a secondary effect following induction of PBSX replication by these agents.

A second isolate of the *oxy-2* gene fusion was obtained during the initial screening. It was shown to be the same as *oxy-2* based on the kinetics of induction, restriction mapping and partial sequencing (data not shown). The fact that we obtained two isolates of one gene suggests that we identified most of the genes which are induced by H₂O₂ and which can be identified by means of gene fusion technology. Using these methods, we were unable to clone the genes which are induced immediately following addition of low concentrations of H₂O₂, and which are probably involved in protecting the cells against high concentrations of the oxidant. The gene fusion approach is probably insufficiently sensitive to find genes which are induced transiently (10–35 min in this case; Murphy *et al.*, 1987). We have now isolated a H₂O₂-resistant mutant which constitutively accumulates high levels of the proteins induced immediately following addition of H₂O₂. We have obtained N-terminal sequence information on these proteins which has allowed us to design probes to clone the 'protective' genes (O. M. Hartford & B. C. A. Dowds, unpublished).

We thank Ms Sheila Morgan for isolating the *oxy-2* clone, Dr Heather Wood for the PBSX clones and Dr Anne Moir for advice on transduction. The work was supported by Eolas, the Irish Science and Technology Agency.

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