Manganous ions suppress photosynthesis gene expression in *Rhodobacter sphaeroides*

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The effect of manganous ions [Mn(II)] and ferrous ions [Fe(II)] on expression of photosynthesis genes in *Rhodobacter sphaeroides* was investigated. The presence of Mn(II) during phototrophic (anaerobic) and chemotrophic (aerobic) growth of *R. sphaeroides* caused a decrease in the amount of bacteriochlorophyll and carotenoid pigments which were synthesized and this was associated mainly with a decrease in the level of light-harvesting complex II. Mn(II) was shown to cause a decrease in expression of the *puc* operon, which encodes the polypeptides of light-harvesting complex II. Expression of the *puc* operon is controlled by the central repressor of photosynthesis gene expression, PpsR. In a *ppsR* mutant there was no effect of Mn(II) on photosynthesis gene expression. It is concluded that Mn(II) may act as a co-repressor in the action of PpsR or act via an as yet uncharacterized protein that interacts with PpsR. In contrast to the effects of Mn(II), Fe(II) was required for high levels of photosynthesis gene expression. This requirement for Fe(II) was shown to be related to the regulation of *hemA*, a gene under the control of the transcriptional regulator, FnrL. Mn(II) did not affect FnrL-dependent gene expression.

Keywords: photosynthesis, gene expression, *Rhodobacter sphaeroides*, repressor PpsR, manganous ion

INTRODUCTION

Most species of purple non-sulfur phototrophic bacteria are facultative aerobes. They are able to grow chemoheterotrophically using oxygen as a terminal electron acceptor, and under anaerobic conditions in the light they can catalyse cyclic photosynthetic electron transfer during phototrophic growth (McEwan, 1994). Two environmental signals control the synthesis of the photosynthetic apparatus: oxygen represses its formation while under anaerobic conditions the size of the photosynthetic apparatus is inversely related to the incident light intensity (Aagaard & Sistrom, 1972). The photosynthetic apparatus of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* contains three distinct pigment–protein complexes involved in the transduction of light energy into electrochemical energy (Kiley & Kaplan, 1988). Two types of light-harvesting (LH) complex, LHI (or B875) and LHII (or B800–850), are identified by the distinctive absorbance maxima of their

bacteriochlorophyll *a* (Bch) pigments. The core of the photosynthetic unit consists of a reaction centre (RC) surrounded by 10–15 LHI. The peripheral LHII complex surrounds the LHI/RC core and its level is more variable with light intensity.

A number of the regulators of photosynthesis (PS) gene expression have now been identified, although their mechanism of oxygen and/or light sensing is still unclear. The oxygen-responsive transcriptional regulator FnrL has been identified as an activator of PS gene expression in *R. sphaeroides* (Zeilstra-Ryalls & Kaplan, 1995) while the sensor histidine kinase–response regulator system PrrB–PrrA is also essential for activation of PS gene expression in response to a decrease in oxygen tension (Eraso & Kaplan, 1994, 1995). Another key regulatory gene has been identified within the PS gene cluster. *ppsR* (formerly known as *crtI*) encodes a repressor which shuts down PS gene expression in response to the presence of oxygen (Penfold & Pemberton 1991, 1994). A *ppsR* mutant of *R. sphaeroides* was shown to synthesize high levels of LH complexes under aerobic conditions and this led to the view that PpsR must be a repressor of the transcription

Abbreviations: Bch, bacteriochlorophyll *a*; LHI, LHII, light-harvesting complex I, II; PS, photosynthesis.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>R. sphaeroides</i>		
RS630	Wild-type	Pemberton & Bowen (1981)
PPSR	RS630 (<i>ppsR</i>), Kn^r	Penfold & Pemberton (1994)
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15, <i>hsdR17</i> ($r_K^- m_K^+$) <i>recA1</i> λ^-	Bethesda Research Labs
S17-1	MM294 with RP4-2 (<i>tet::Mu</i> , <i>neo::Tn7</i>) integrated into the chromosome, Tra^+ Sm^r Tp^r	Simon <i>et al.</i> (1983)
Plasmids		
pCF200	<i>puc</i> (URS+DRS):: <i>lacZYA'</i> , IncQ	Lee & Kaplan (1992)
pUI1063	<i>hemA::lacZYA'</i> , IncQ	Zeilstra-Ryalls & Kaplan (1995)
pUI1830	<i>pufB::lacZYA'</i> , IncQ	Gomelsky & Kaplan (1995a)

of PS genes. It has been confirmed that PpsR represses the expression of a number of *crt* and *bch* genes (Penfold & Pemberton, 1994; Gomelsky & Kaplan, 1995a) and a consensus DNA sequence for the binding of PpsR has been identified (Gomelsky & Kaplan, 1995a). Similar results for the PpsR homologue in *R. capsulatus*, still known as CrtJ, have been obtained by Bauer and co-workers and the ability of this protein to bind to its DNA target has been confirmed (Ponnampalam *et al.*, 1995; Ponnampalam & Bauer, 1997). PpsR can repress the expression of *R. sphaeroides bch* and *crt* genes in the heterologous hosts *Paracoccus denitrificans* and *Pseudomonas stutzeri* (Penfold & Pemberton, 1994; Gomelsky & Kaplan, 1995a), and this has led to the suggestion that PpsR may sense oxygen or an oxygen-induced metabolite directly.

Changes in metal concentration have been shown to affect the levels of pigments in *R. sphaeroides*. Lascelles and coworkers have extensively studied the affects of iron on pigment levels, porphyrin excretion and 5-aminolaevulinic acid synthase activity (Burnham & Lascelles, 1963; Lascelles, 1959). Iron-dependent regulators of gene expression have been identified in a variety of organisms; examples include the Fur protein (Hickey & Ciancotto, 1994; Staggs & Perry, 1992; Ernst *et al.*, 1978) and the diphtheria toxin repressor (DtxR) of *Corynebacterium diphtheriae* (Boyd *et al.*, 1990). In addition to proteins which may reversibly bind iron, there are other regulatory proteins which contain iron-sulfur clusters (Roualt & Klausner, 1996). Perhaps the best-known example of this class of proteins is the transcriptional regulator Fnr, which, in *Escherichia coli*, has been shown to be regulated by iron availability (Trageser & Uden, 1989). The presence of FnrL in *R. sphaeroides* (Zeilstra-Ryalls & Kaplan, 1995) suggests that the iron-dependent stimulation of PS gene expression may involve this protein. The possibility that other metal ions have a role in the control in PS gene expression has not been explored in recent years. In this

paper we describe experiments to determine whether Mn(II) ions affected PS gene expression.

METHODS

Bacterial strains, plasmids, media and growth conditions.

The bacterial strains and plasmids used are listed in Table 1. RCV medium was routinely used for the cultivation of *R. sphaeroides* strains (Weaver *et al.*, 1975). For 'Mn-free' RCV, MnSO_4 was omitted and Mn(II) added from a sterile stock MnSO_4 solution to the required concentration. This medium contained $24 \mu\text{g FeSO}_4 \cdot 7\text{H}_2\text{O ml}^{-1}$. 'Fe-free' medium was prepared by omitting $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ from the RCV medium, and Fe was added to the appropriate concentration from a freshly prepared filter-sterilized solution of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. This medium contained $1.6 \mu\text{g MnSO}_4 \text{ ml}^{-1}$. Ni(II) was added to RCV from a sterile stock solution of NiCl_2 . All *E. coli* strains were grown on LB medium (Sambrook *et al.*, 1989).

E. coli strains were grown at 37°C in 25 ml liquid in 250 ml flasks. *R. sphaeroides* strains were inoculated as previously described (Horne *et al.*, 1996) in completely filled 30 ml McCartney bottles and incubated at a light intensity of approximately 200 W m^{-2} calculated by the method of Clayton (1970). Antibiotics were added to the following final concentrations: tetracycline $10 \mu\text{g ml}^{-1}$ (*E. coli*), spectinomycin $25 \mu\text{g ml}^{-1}$ (*E. coli* and *R. sphaeroides*), and kanamycin $25 \mu\text{g ml}^{-1}$ (*R. sphaeroides*).

Spectroscopic, protein and enzyme analyses. Membranes for analysis of LH complexes were prepared from *R. sphaeroides* cell cultures as described previously (Horne *et al.*, 1996). The level of Bch in the LH complexes was determined by the method of Meinhardt *et al.* (1984). The LH concentration was calculated assuming 2 mol Bch per mol LHI and 3 mol Bch per mol LHII. β -Galactosidase assays were performed according to the method of Clark & Switzer (1977). Protein was determined using the bicinchoninic acid reagent with bovine serum albumin as a standard (Smith *et al.*, 1985). All data are the means of at least two independent analyses and errors did not exceed 15%.

Genetic manipulations. *E. coli* S17-1 (Simon *et al.*, 1983) was used to transfer plasmids into *R. sphaeroides* strains. The

mating method of Bonnett *et al.* (1995) was used for conjugal transfer. Molecular biological enzymes and reagents were used according to the specifications of the manufacturers.

RESULTS

Effect of Mn(II) on the level of LH complexes in *R. sphaeroides* RS630

Kassner & Kamen (1968) observed that the level of Bch pigment in phototrophically grown *R. sphaeroides* was lowered when Mn(II) ions were supplied in the growth medium. This result was confirmed in our experiments and a more detailed analysis was performed. Fig. 1 shows the spectra of membranes from *R. sphaeroides* grown phototrophically at 200 W m^{-2} in the presence of Mn(II) (at a final concentration of $100 \mu\text{g MnSO}_4 \text{ ml}^{-1}$) and in the absence of added Mn(II). Absorption due to Bch in the LHII complex can be seen as a peak at 800 nm and at 850 nm while the Bch in LHI can be seen as a shoulder on the absorption peak at 850 nm. The presence of Mn(II) in the growth medium caused a decrease in the level of Bch pigments in LHII and LHI under phototrophic (anaerobic) conditions (Fig. 1). The absorption peaks between 450 nm and 550 nm correspond to Crt pigments. The presence of Mn(II) in the growth medium also caused a lowering in the level of Crt pigments (Fig. 1). This is taken to indicate that Mn(II) ions were affecting the level of LH complexes and not their composition.

Table 2 shows the level of LH complexes in membranes from *R. sphaeroides* grown phototrophically and aerobically as a function of the Mn(II) concentration in the growth medium. In phototrophically grown cells the presence of $100 \mu\text{g MnSO}_4 \text{ ml}^{-1}$ caused the level of LHI to be 20% lower than its level in cells grown without added Mn(II). The decrease in LHII caused by the

Table 2. Level of light-harvesting complexes in membranes from *R. sphaeroides* RS630 grown at various Mn(II) ion concentrations

Growth conditions*	LH level [nmol (mg protein) ⁻¹]			
	MnSO ₄ concn ($\mu\text{g ml}^{-1}$):			
	0	1	10	100
PS				
LHI	15.0	14.2	13.3	12.0
LHII	23.8	18.3	17.4	14.7
O ₂				
LHI	1.65	1.35	1.0	0.97
LHII	0.83	0.62	0.4	0.36

*PS, phototrophic conditions; O₂, chemotrophic conditions.

presence of Mn(II) was even greater (38%) (Table 2). Mn(II)-dependent suppression of LH levels was also observed in cells grown at lower light intensity (16 W m^{-2}) (data not shown). During chemoheterotrophic aerobic growth of *R. sphaeroides* there is strong repression of PS gene expression but the presence of Mn(II) also caused a decrease in the level of LH complexes under these conditions (Table 2). This showed that the effect of Mn(II) ions was not restricted to phototrophic conditions.

Comparison of the effect of Mn(II), Fe(II) and Ni(II) ions on LH levels in *R. sphaeroides*

Since Mn(II) affected the level of LH complexes in *R. sphaeroides*, the effect of other divalent cations from the first transition series was examined. Fe(II) was chosen because in some proteins it has been shown that Mn(II) can substitute for Fe(II) (Hantke, 1987; Trageser & Uden, 1989). The effect of Fe(II) concentration on the level of LH complexes in *R. sphaeroides* grown phototrophically is shown in Fig. 2. There was an increase in the level of both LHI and LHII as the Fe(II) concentration in the growth medium increased. In this medium, Mn(II) was at a constant concentration of $1.6 \mu\text{g MnSO}_4 \text{ ml}^{-1}$. The effect of Fe(II) concentration on the level of LH complexes was opposite to the effect of Mn(II). Fig. 2 also shows that low levels of Mn(II) exert a dramatic effect on the level of LH complexes. The growth medium used for the titration with Mn(II) ions contained $24 \mu\text{g FeSO}_4 \cdot 7\text{H}_2\text{O ml}^{-1}$ and this suggested that Mn(II) exerted its effect on the formation of LH complexes independently of Fe(II). The level of LH complexes did not change significantly as a function of Ni(II) concentration except at high concentrations ($100 \mu\text{g ml}^{-1}$) of this ion (data not shown). This can be attributed to toxic effects of Ni(II) because it caused the growth rate of *R. sphaeroides* to decrease. In contrast, Mn(II) at $100 \mu\text{g ml}^{-1}$ in the medium had no effect on the growth rate of *R. sphaeroides* under the experimental conditions used.

Fig. 1. Absorption spectra of membranes of *R. sphaeroides* strain RS630 grown phototrophically in the absence (solid line) and presence (dashed line) of $100 \mu\text{g MnSO}_4 \text{ ml}^{-1}$ in RCV medium. Both samples contained $195 \mu\text{g}$ protein.

Fig. 2. Effect of Mn(II) (●) and Fe(II) (■) concentration on the Bch content of membranes from *R. sphaeroides* strain RS630 grown phototrophically in RCV medium. The concentrations plotted are those of the respective sulfate.

Effect of Mn(II) and Fe(II) ions on PS gene expression

FnrL has been identified as a key regulator of PS gene expression in *R. sphaeroides* by virtue of its role as an activator of the expression of the *hemA* gene (Zeilstra-Ryalls & Kaplan, 1995). The *hemA* gene encodes an isozyme of 5-aminolaevulinic acid synthase, the first step in Bch synthesis. Fnr is known to contain an [Fe-S] cluster (Lazazzara *et al.*, 1996) and Fe(II) ions have been shown to affect Fnr-dependent gene expression in *E. coli* (Niehaus *et al.*, 1991). Mn(II) has also been suggested to be capable of affecting the Fnr-dependent regulation of gene expression (Trageser & Uden, 1989). Therefore we examined the effect of Mn(II) and Fe(II) ions on the expression of an *hemA-lacZ* transcriptional fusion. Table 3 shows the β -galactosidase activity, corresponding to the degree of expression from the *hemA* promoter, in cell-free extracts from *R. sphaeroides* grown phototrophically. The expression of *hemA* did not appear to change as a function of Mn(II) concentration but, as expected for an Fnr-regulated gene, *hemA* expression increased with the concentration of Fe(II) ions in the

medium. These data supported the view that the effect of Mn(II) ions on PS gene expression is not exerted via FnrL.

Regulation of the formation of the photosynthetic apparatus occurs at the level of transcription as well as at the post-transcriptional and post-translational level (Kiley & Kaplan, 1988). To determine whether the effect of Mn(II) ions on LH complex formation was mediated at the level of transcription, the expression of *puf-lacZ* and *puc-lacZ* gene fusions was examined in *R. sphaeroides* RS630 grown phototrophically in the presence and absence of Mn(II). The *puf* operon encodes the structural polypeptides of LHI and two polypeptides of the photochemical reaction centre, and the *puc* operon includes genes encoding the structural polypeptides of LHII (Kiley & Kaplan, 1988). The β -galactosidase activity in cell-free extracts from strains expressing these gene fusions is shown in Table 3. A 28% reduction in *puc* expression was observed in *R. sphaeroides* grown in the presence of 100 $\mu\text{g Mn(II) ml}^{-1}$ compared to cells grown in the absence of this ion. In contrast there was no significant effect of Mn(II) concentration on the level of *puf* expression (Table 3).

Effect of Mn(II) and Fe(II) concentration on LH complex levels and PS gene expression in a *ppsR* mutant

The *ppsR* gene encodes a repressor of the expression of the *puc* operon and a number of *bch* genes (Penfold & Pemberton, 1994; Gomelsky & Kaplan, 1995a). The effect of *ppsR* on PS gene expression is most obvious under aerobic conditions; the presence of *ppsR* in multi-copy causes suppression of LH complex formation (Penfold & Pemberton, 1991) whilst a *ppsR* mutant fails to repress PS gene expression in response to the presence of oxygen (Penfold & Pemberton, 1994). However, it has been shown that a *ppsR* mutant of *R. sphaeroides* synthesizes higher levels of LH complexes compared to wild-type cells under phototrophic conditions and this indicates that PpsR is active in the absence of oxygen (Gomelsky & Kaplan, 1997). The role of Mn(II) and Fe(II) in PpsR-dependent regulation of PS gene expression was investigated in *R. sphaeroides* grown

Table 3. β -Galactosidase activity in cell extracts of *R. sphaeroides* RS630 bearing *lacZ* transcriptional fusions grown phototrophically with various metal ions

<i>lacZ</i> fusion	β -Galactosidase activity [nmol ONPG hydrolysed min ⁻¹ (mg protein) ⁻¹]			
	MnSO ₄ concn ($\mu\text{g ml}^{-1}$)		FeSO ₄ concn ($\mu\text{g ml}^{-1}$)	
	0	100	0	100
<i>puc</i>	5300	3800	ND	ND
<i>puf</i>	5700	5200	ND	ND
<i>hemA</i>	1500	1500	500	1100

ND, Not determined.

Table 4. Level of LH complexes and *puc* expression in *R. sphaeroides* PPSR grown under phototrophic conditions with various concentrations of Fe(II) ions and Mn(II) ions

Metal ion concn ($\mu\text{g ml}^{-1}$ of sulfate)	LH complex level [nmol (mg protein) $^{-1}$]		β -Galactosidase activity [nmol ONPG hydrolysed min $^{-1}$ (mg protein) $^{-1}$]*
	LHI	LHII	
MnSO₄			
0	18.9	26.3	7800
100	18.2	24.6	7500
FeSO₄			
0	11.0	16.6	ND
100	16.3	26.3	ND

*Expression of a *puc-lacZ* fusion. ND, Not determined.

phototrophically. Table 4 shows that the level of LH complexes in the *ppsR* mutant *R. sphaeroides* PPSR was higher than that of *R. sphaeroides* RS630 under these conditions (compare Table 2). However, the level of LH complexes in the *ppsR* mutant did not appear to alter in response to increased Mn(II) concentration, in contrast to the Mn(II)-dependent suppression of LH complex formation observed with the wild-type. Table 4 also shows that the level of LH complexes increased with increasing Fe(II) concentration in PPSR as it did in RS630. Since *puc-lacZ* expression was affected by Mn(II) concentration in strain RS630 (Table 3) the effect of Mn(II) concentration on the activity of this gene fusion in the *ppsR* mutant was examined. The expression of *puc-lacZ* was independent of Mn(II) concentration in strain PPSR (Table 4). These data are consistent with the view that the effect of Fe(II) concentration on LH complex formation is independent of PpsR but suggest that Mn(II) might exert its effect via this repressor.

DISCUSSION

The role of transition metals, particularly iron, in the regulation of gene expression is well documented (Hennecke, 1990). PS gene expression is no exception and it is established that one activator of PS gene expression is the [Fe-S] protein FnrL (Zeilstra-Ryalls & Kaplan, 1995). An area of cell physiology where iron has particular importance is in the regulation of gene expression in response to oxygen. In the case of Fnr it has been suggested that regulation by oxygen may involve oxygen-dependent destruction of the [Fe-S] cluster (Beinert & Kiley, 1996). Iron can exist in the Fe(II) and Fe(III) states and this raises the possibility that changes in the redox state of iron might also be important in the regulation of metabolism and gene expression (Williams, 1982; Uden *et al.*, 1995). The requirement for iron in haemoproteins and [Fe-S] proteins means that iron-limitation often restricts bacterial cell growth and gene expression, and this is the case for PS gene expression. The effect of Fe(II) limitation on the level of LH complexes can be explained

as arising from a restriction in the synthesis of key [Fe-S] proteins. FnrL is a positive regulator of *hemA* and *puc* expression (Zeilstra-Ryalls & Kaplan, 1995) while BchX is an [Fe-S] protein which is involved in a key step in Bch synthesis (Burke *et al.*, 1993). Fnr-dependent gene expression in *E. coli* has been shown to be affected by changes in Fe(II) concentration (Niehaus *et al.*, 1991; Trageser & Uden, 1989) and the situation appears to be similar for the FnrL-dependent expression of *hemA* expression in *R. sphaeroides* (this work). Experiments with permeabilized *E. coli* cells have suggested that Mn(II) might affect Fnr (Trageser & Uden, 1989) but there is no indication that Mn(II) concentration affects the FnrL in *R. sphaeroides*. Mn(II) can substitute for Fe(II) as an activating ion for the Fur protein (Hantke, 1987). However, the concentrations of Mn(II) which are required are in a huge excess over the levels used in the experiments described in this paper and this leads us to conclude that the effect of Mn(II) on PS gene expression is not due to its ability to partially substitute for Fe(II).

The observation that Mn(II) had no effect on PS gene expression or LH complex levels in a *ppsR* mutant leads to the possibility that Mn(II) is a co-repressor of the PpsR repressor system of *R. sphaeroides*. The observation that Mn(II) affected the expression of the *puc* operon but not the *puf* operon is consistent with this view since only the former has a consensus binding sequence for PpsR (Gomelsky & Kaplan, 1995a). The lowering of *puf-lacZ* expression in response to an increase of Mn(II) concentration probably reflects the coupling of *puf* expression to Bch production; the expression of a number of operons encoding enzymes of Bch biosynthesis is repressed by PpsR (Gomelsky & Kaplan, 1995a, 1997). PpsR was first identified as a repressor of PS gene expression which operated under aerobic conditions (Penfold & Pemberton, 1991, 1994) but it now appears that PpsR can operate under anaerobic conditions (Gomelsky & Kaplan, 1997; this work). Recently, Ponnampalam & Bauer (1997) have presented evidence that the PpsR homologue in *R. capsulatus* is a redox sensor, although no mechanism of action was obvious. Inspection of the amino acid

sequence of PpsR reveals that it possesses only two cysteine residues and one of these (Cys424) lies in the DNA-binding motif (Penfold & Pemberton, 1994); it seems highly unlikely that PpsR would contain an [Fe-S] cluster as a sensory component. If Mn(II) were bound to PpsR, it would most likely involve amino acid residues with N and O side chains and not a cysteine residue. This is because sulfur donors would be overwhelmingly occupied by Fe(II), Cu(II) or Zn(II) as predicted by the Irving-Williams series of divalent cation-ligand stability constants (Williams, 1982). A possible role for Mn(II) in PpsR could be to stabilize the oxidized form of PpsR, thereby enhancing the repression of PS gene expression. However, there is no direct evidence for this model at present. Indeed, CrtJ, the PpsR homologue in *R. capsulatus*, was shown not to contain metals as part of the protein (Ponnampalam & Bauer, 1997). However, Mn(II) can often be lost from proteins during purification and so the question of whether PpsR contains metal ions should be approached with caution.

Since there is no direct evidence that Mn(II) is a component of PpsR, then it is also possible that Mn(II) influences the activity or expression of proteins which interact with PpsR. Genetic experiments have indicated that a protein AppA may positively regulate PS gene expression by antagonizing the action of the repressor PpsR (Gomelsky & Kaplan, 1997). Furthermore, it seems likely that the Prr system may interact with PpsR because overexpression of the *appA* gene can suppress the pigmentless phenotype of a PrrA mutant (Gomelsky & Kaplan, 1995b).

It has recently been shown in *Bacillus subtilis* that low levels of Mn(II) inhibit the production of MrgA, a Dps homologue involved in protection of DNA during oxidative stress (Chen *et al.*, 1993; Chen & Helmann, 1995). Further experiments have shown that Mn(II) suppresses the coordinate derepression of a number of genes involved in a peroxide stress response (Chen *et al.*, 1995). These data have been interpreted as indicating the existence of a peroxide stress gene repressor which binds Mn(II) and senses hydrogen peroxide. Although putative operator sites have been identified in a number of *B. subtilis* peroxide stress genes (Chen *et al.*, 1995) no *trans*-acting factor has yet been identified. Our results suggest that Mn(II)-dependent regulation of gene expression is not restricted to Gram-positive bacteria such as *Bacillus*.

ACKNOWLEDGEMENTS

I. M. H. was supported by an Australian Postgraduate Award. A. G. M and J. M. P thank the University of Queensland for an Enabling Research Grant. The authors thank Therese Del Dot for help with preliminary investigations.

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Received 5 February 1998; revised 5 May 1998; accepted 12 May 1998.