OxyR mediated compensatory expression between \( \textit{ahpC} \) and \( \textit{katA} \) and the significance of \( \textit{ahpC} \) in protection from hydrogen peroxide in \( \textit{Xanthomonas campestris} \)

Nisanart Charoenlap \(^b\), Warawan Eiamphungporn \(^b\), Nopmanee Chauvatcharin \(^{b,1}\), Supa Utamapongchais \(^a\), Paiboon Vattanavibo \(^{a,*}\), Skorn Mongkolsuk \(^{a,b}\)

\(^a\) Laboratory of Biotechnology, Chulabhorn Research Institute, Lak Si, Bangkok 10210, Thailand
\(^b\) Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Received 7 January 2005; received in revised form 15 April 2005; accepted 1 June 2005

First published online 20 June 2005

Edited by C.W. Penn

Abstract

\( \textit{katA} \) and \( \textit{ahpC} \), encoding monofunctional catalase and alkyl hydroperoxide reductase, respectively, play important protective roles against peroxide toxicity in \( \textit{Xanthomonas campestris pv. phaseoli} \) (Xp). The expression of both \( \textit{katA} \) and \( \textit{ahpC} \) is controlled by the global peroxide sensor and transcriptional activator, OxyR. In Xp, these two genes have compensatory expression patterns. Inactivation of \( \textit{katA} \) leads to an increase in the level of AhpC and a concomitant increase in resistance to tert-butyl hydroperoxide (tBOOH). High-level expression of \( \textit{katA} \) from an expression vector in Xp also lowered the level of \( \textit{ahpC} \) expression. The compensatory regulation of \( \textit{katA} \) and \( \textit{ahpC} \) was mediated by OxyR, since the compensatory response was not observed in an \( \textit{oxyR} \) mutant background. \( \textit{ahpC} \) and \( \textit{katA} \) play important but unequal roles in protecting Xp from H\(_2\)O\(_2\) toxicity. These observations, taken together with a previous observation that an \( \textit{ahpC} \) mutant expresses high levels of KatA and is hyper-resistant to H\(_2\)O\(_2\), suggest the possibility that inactivation of either gene leads to accumulation of intracellular H\(_2\)O\(_2\). This in turn oxidizes reduced OxyR and converts the regulator to the oxidized form that then activates expression of genes in the OxyR regulon.

© 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: \( \textit{ahpC} \); Catalase; H\(_2\)O\(_2\); \( \textit{katA} \); OxyR; \( \textit{Xanthomonas} \)

1. Introduction

\( \textit{Xanthomonas campestris} \) is an important bacterial plant pathogen that causes disease in many economically important crops. During infection, a key component of the initial plant defense response against the invading microorganisms is the rapid production and accumulation of reactive oxygen species (ROS), primarily hydrogen peroxide (H\(_2\)O\(_2\)) and superoxide anions [1]. Elevated ROS are highly toxic and can cause detrimental effects to living cells through their ability to cause lipid peroxidation, protein modification and DNA damage [2]. In order to survive oxidative stress, bacteria have evolved a variety of enzymes capable of detoxifying ROS and repairing damage generated by them.

\( \textit{X. campestris} \) produces at least one isozyme of manganese superoxide dismutase and two isozymes of

0378-1097/$22.00 © 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.
monofunctional catalase, namely KatA (formerly Kat1) and KatE, to cope with superoxide anions and H₂O₂, respectively [3–5]. KatA is the major catalase produced throughout all phases of growth and its expression is inducible by exposure to oxidants in an OxyR (a peroxide sensor and transcription regulator) dependent manner. KatE is only detected as cells enter the stationary phase of growth or during starvation conditions [4]. The bacterium also produces alkyl hydroperoxide reductase (AhpCF) and an organic hydroperoxide resistance enzyme (Ohr) that function synergistically to catalyze the conversion of organic peroxides to alcohols [6]. These genes are differentially regulated. Expression of ahpC is induced by exposure to oxidants in an OxyR dependent manner [7,8] while ohr expression is strongly induced only by organic hydroperoxide through the action of OhrR, a transcription repressor and sensor of organic hydroperoxide [9].

In several bacteria, the response to peroxides is complicated and essential for their survival under oxidative stress. An increase in catalase levels upon inactivation of ahpC has been reported in both Gram-negative and Gram-positive bacteria including Pseudomonas aeruginosa, X. campestris and Bacillus subtilis [10–12]. These compensatory alterations in gene expression are presumably mediated by transcriptional regulators that sense changes in the level of peroxides, either H₂O₂ or organic hydroperoxides. However, observations in catalase-peroxidase defective strains of Mycobacterium tuberculosis and Burkholderia pseudomallei revealed an enhanced ahpC gene transcription to compensate for the loss of catalase-peroxidase activity [13–15]. Here, we report the characterization of the compensatory expression of ahpC and katA in X. campestris pv. phaseoli and show that this process requires OxyR.

2. Materials and methods

2.1. Growth conditions and media

All Xanthomonas strains were grown aerobically at 28 °C in Silva-Buddenhagen (SB) medium containing the appropriate antibiotics [16]. All Escherichia coli strains were grown aerobically at 37 °C in Luria–Bertani (LB) medium.

2.2. Nucleic acid manipulations

All nucleic acid manipulations were performed according to standard molecular biology techniques [17] or to manufacturers’ recommendations. The labeling of the DNA probes with [α-³²P]-dCTP was performed using a DNA labeling bead (Amersham Pharmacia Biotech). Southern and Northern Blot analyses were performed as described [16].

2.3. Catalase activity gel staining and assay

Cell lysate preparation and catalase activity gel staining were performed as previously described [4]. Bacterial cells were lysed in 50 mM sodium phosphate buffer pH 7.0 by brief sonication followed by centrifugation at 10,000g for 10 min and the supernatants were used for catalase activity gels. Catalase isozymes were separated on native PAGE gels and visualized as previously described [4]. Catalase activity appeared as colourless bands against a dark brown background. The catalase activity assay was carried out spectrophotometrically as previously described [19]. One unit of catalase was defined as the amount of enzyme required to decompose 1.0 μmol of H₂O₂ at 25 °C at pH 7.0.

2.4. Construction of ahpC1 katA mutant

A X. campestris pv. phaseoli ahpC1 katA double mutant was constructed by transferring the katA mutant locus of strain Xp20 [19] into strain Xp ahpC1 [11] by electroporation using Xp20 chromosomal DNA. The Kan’ and Amp’, ahpC katA mutant, Xp29, was selected and confirmed by Southern blot hybridization (data not shown).

2.5. Determination of oxidant resistance

Analysis of the killing effects of various reagents on Xanthomonas strains was performed using inhibition zone assays as described by Mongkolsuk et al. [18]. Briefly, overnight cultures were subcultured as a 5% inoculum into fresh SB broth. One ml of exponential phase cells (4 h culture, OD₆₀₀ nm of 0.5) was mixed with 10 ml of molten top agar (SB containing 0.7% agar) held at 50 °C, and overlaid onto SB plates (14 cm-diameter Petri-dishes containing 40 ml of SB agar). The plates were left at room temperature for 15 min to let the top agar solidify. Sterile 6-mm-diameter paper discs soak with 5 μl of either 0.3 M H₂O₂ or 1.0 M tert-butyl hydroperoxide (tBOOH), were placed on the surface of the cell lawn. The diameter of inhibition zones was measured after 24 h of incubation at 28 °C.

3. Results and discussion

3.1. Alterations in the phenotype and gene expression patterns of a katA mutant

katA encoding a house-keeping clade II monofunctional catalase from X. campestris pv. phaseoli (Xp) has been cloned and characterized [3]. The katA mutant, Xp20, is highly sensitive to H₂O₂ during exponential and stationary phase growth [19]. In addition, it has been shown that inactivation of ahpC, encoding the major
peroxide metabolizing enzyme, alkyl hydroperoxide reductase, results in pleiotropic effects on the peroxide stress response [11]. Thus, the resistance level of Xp20 to the organic hydroperoxide, tert-buty1 hydroperoxide (tBOOH) was determined by growth inhibition zone assay. Surprisingly, Xp20 was more resistant to tBOOH (1.0 M) than the wild type strain (Xp) and displayed a zone of growth inhibition of 25 mm as compared to a zone of 29 mm for Xp (Fig. 1). In Xanthomonas, there are two major organic hydroperoxide detoxification systems, consisting of the organic hydroperoxidase, Ohr (organic hydroperoxide resistance), and the alkyl hydroperoxide reductase, AhpC, that act synergistically to protect cells from organic hydroperoxide toxicity [6,16,18]. In order to further investigate the mechanism responsible for the phenotype, the expression levels of these genes were determined in Xp20 and Xp using Northern blot hybridization. The results in Fig. 2(a) and (b) clearly illustrated that the level of ahpc transcripts in Xp20 was approximately 3-fold higher than the level in strain Xp, as determined by densitometry, while the level of ohr was not significantly different. The results suggested that the increased level of ahpc expression was responsible for the enhanced resistance against tBOOH killing phenotype of Xp20 (Fig. 1). The data also suggested that inactivation of katA, a major catalase, was likely to be responsible for the elevated ahpc expression. This assumption was supported by the observation that expression of KatA, from pKatA (a broad-host-range plasmid containing full-length katA in pBBR1MCS-3 [20]), in Xp20 caused a reduction in ahpc mRNA to a level that was close to that of the parental wild type strain (Fig. 2(a)). Analysis of the compensatory link between KatA and AhpC expression was extended by the determination of the effect of high-level katA expression from pKatA on the level of ahpc transcripts in the wild type strain Xp. The results in Fig. 2(a) clearly showed that Xp/pKatA produced a lower level of ahpc mRNA relative to Xp harbouring the vector control.

The previous finding that inactivation of X. campestris ahpc leads to an increase in KatA levels [11] suggested a two-way compensatory link between katA and ahpc expression. Thus, the effect of high-level expression of ahpcF on the level of KatA was investigated using strain Xp harbouring the AhpCF expression plasmid pAhpCF (pBBR1MSC-3 containing ahpcF).
Catalase activity was monitored in exponential phase cells using total catalase activity assays and catalase activity stained gels that could differentiate between different catalase isozymes. High-level expression of ahpCF in strain Xp/pAhpCF resulted in a 3.5-fold reduction (i.e. from 5.3 U/mg protein in Xp harbouring the vector control to 1.5 U/mg protein in Xp/pAhpCF) in total catalase activity (Fig. 3) that was due to a decrease in KatA levels (data not shown) (Note: In Xp, KatA is the only isozyme that is detectable by catalase activity stained gels during the exponential phase of growth [4]). The changes in the ability to survive peroxide stresses in these strains were also tested. The results in Fig. 1 revealed that Xp containing pAhpCF was more resistant to tBOOH, but significantly more sensitive to H2O2 than was Xp containing the plasmid vector. This suggested that the reduction in catalase was responsible for the H2O2 sensitive phenotype and that the high level of AhpCF in the strain could not fully compensate for the loss of catalase.

In X. campestris, katA and ahpC are members of a regulon controlled by the peroxide-sensing transcriptional regulator, OxyR [7,19]. OxyR senses the intracellular concentration of H2O2. When the concentration of H2O2 reaches a critical level, reduced OxyR is oxidized by H2O2 and can then activate transcription of genes in the regulon. It was possible that the compensatory expression of ahpC and katA was mediated by OxyR most likely in response to the accumulation or dissipation of intracellular H2O2 resulting from changes in the relative levels of expression of the peroxide metabolizing enzymes. The finding that an elevated level of KatA in the ahpCl mutant did not occur in the ahpCl oxyR double mutant suggests the involvement of OxyR in this compensatory process (Fig. 3 and [11]). The role of OxyR in the compensatory increase in ahpC expression in a katA mutant was also investigated. The direct investigation of the role of OxyR in the compensatory process was complicated by the fact that inactivation of the oxyR alone leads to increased basal expression of AhpC. This is thought to be due to repression of ahpC expression by reduced OxyR in the absence of H2O2 [21]. Therefore, an alternative approach, that involved testing the effect of high-level expression of katA on the level of ahpC expression in a wild type (Xp/pKatA) and an oxyR (XpoxyR/pKatA, [21]) background, was performed. In Xp/pKatA (Fig. 2(a)), high-level expression of katA led to a 3-fold reduction in the level of ahpC mRNA. This response was abolished in an Xp oxyR/pKatA (Fig. 2(a)) indicating that the compensatory expression of katA and ahpC is mediated by OxyR.

We postulate that the high catalase level in Xp/pKatA makes it more efficient at the removal of endogenously generated H2O2 than the wild-type control strain harbouring the vector alone. Thus, the OxyR pool remains reduced thereby repressing the expression of ahpC [7].

3.2. The compensatory increase in KatA in an ahpC mutant is not due to organic hydroperoxide accumulation

While the data clearly indicated that the compensatory expression of ahpC and katA was mediated by OxyR (Figs. 2 and 3), the nature of the signal responsible for the compensatory regulation remained unknown. Previous studies have shown that X. campestris pv. phaseoli OxyR could be activated by both H2O2 and organic hydroperoxides [16]. Thus, the compensatory expression of ahpC and katA could be due to increased levels of endogenous organic hydroperoxides and/or H2O2 that subsequently oxidized OxyR and activated expression of genes in the regulon. Since ahpC can metabolize and confer resistance to both H2O2 and organic hydroperoxide, it was possible that inactivation of the gene could lead to the intracellular accumulation of either H2O2 and/or organic hydroperoxides [11]. In addition to AhpC, X. campestris pv. phaseoli produces Ohr, a second organic hydroperoxide-specific hydroperoxidase [6]. The expression of ohr is regulated by OhrR, an organic hydroperoxide sensor and transcriptional repressor that specifically senses organic hydroperoxides and not other oxidants [9]. In order to distinguish between H2O2 and organic hydroperoxides as the signal molecule responsible for the compensatory response, the levels of ohr transcripts were measured in Xp wild type, Xp20 and ahpCl strains using Northern blot hybridization analysis (Fig. 2(b)). It was reasoned that the ohr–ohrR system should be a valid proxy for the

![Fig. 3. Total catalase activity in X. campestris pv. phaseoli strains. Crude lysates prepared from exponential phase cultures were monitored for total catalase activity. The values presented are the means and standard deviations of three replicates. Xp (wild type harbouring pBBR1MCS-3 vector control), ahpC1(Xp ahpCl), ahpCl oxyR (Xp ahpCl oxyR), Xp20 (katA mutant harbouring pBBR1MCS-3), Xp/pAhpCF (Xp harbouring pAhpCF); oxyR (Xp oxyR); oxyR/pAhpCF (Xp oxyR harbouring pAhpCF).](https://academic.oup.com/femsle/article-abstract/249/1/73/754762)
indirect monitoring of the intracellular accumulation of organic hydroperoxides since increased levels of organic hydroperoxide would be accompanied by increased expression of ohr. The results in Fig. 2(b) showed that both the ahpC1 mutant and the wild type strain Xp accumulated similar levels of ohr transcripts, indicating that the concentration of intracellular organic hydroperoxide was not increased in strain ahpC1. Since OhrR is more sensitive to the presence of organic hydroperoxides, especially to fatty acid hydroperoxides, than OxyR (unpublished data), organic hydroperoxides are unlikely to be responsible for the observed activation of katA by OxyR in the ahpC1 mutant.

3.3. Evaluation of the role of AhpC in \( \text{H}_2\text{O}_2 \) protection

The availability of mutants in both a regulatory gene (oxyR) and genes for metabolizing enzymes (ahpC and katA) allowed their roles in the protection against \( \text{H}_2\text{O}_2 \) to be evaluated. A katA mutant (Xp20) was highly sensitive to \( \text{H}_2\text{O}_2 \) and yet it was still less susceptible to \( \text{O}_2 \) (see Section 2). The results in Fig. 1 clearly illustrated the \( \text{H}_2\text{O}_2 \) to be evaluated. A katA together, we speculated that activation of OxyR in the transcription of endogenously produced \( \text{H}_2\text{O}_2 \). The same mechanism was also responsible for the compensatory increase in \( \text{ahpC} \) expression in the \( \text{katA} \) mutant.

Acknowledgements

The authors thank Dr. J.M. Dubbs for a critical review of the manuscript. The research was supported by a Research Team Strengthening Grant from the National Center for Genetic Engineering and Biotechnology (BIOTEC), a Senior Research Scholar Grant RTA4580010 from the Thailand Research Fund (TRF), and a grant from the STM through the Higher Education Development Project of the Ministry of University Affairs, to S. Mongkolsuk. N. Charoenlap was supported by a Royal Golden Jubilee Scholarship (PHD/0212/2545) from the TRF.

References


