J Ind Microbiol Biotechnol (2005) 32: 687–690
DOI 10.1007/s10295-005-0036-5

ENVIRONMENTAL BIOTECHNOLOGY

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Chemical modulation of physiological adaptation and cross-protective responses against oxidative stress in soil bacterium and phytopathogen, *Xanthomonas*

Received: 25 August 2004 / Accepted: 8 August 2005 / Published online: 6 October 2005

Abstract Soil bacteria need to adapt quickly to changes in the environmental conditions. Physiological adaptation plays an important role in microbial survival, especially under stressful conditions. Here the abilities of chemicals and pesticides to modulate physiological adaptive and cross-protective responses, that make the bacteria more resistant to oxidative stress, are examined in the soil bacterium and phytopathogen, *Xanthomonas*. The genetic basis for the observed stress resistance, as well as the regulatory mechanisms controlling gene expression during the process, has begun to be elucidated.

Key words Physiological adaptive response · Oxidative stress · Gene regulation · OxyR

Introduction

Bacteria, like other living organisms, alter their physiological processes in a manner dictated by outside environmental factors. These adaptations, in response to different stimuli, are often preceded by alterations in the pattern of gene expression resulting in increased survivability under unfavorable conditions. For the purpose of this review, physiological adaptation is defined as the ability of a chemical or a stress to induce protection against a subsequent exposure to the same chemical or stress. By contrast, physiological cross protection occurs when exposure to a chemical or a stress subsequently induces protection to unrelated chemicals or stresses. Here, we examine the ability of low-level exposure to various chemical inducers to stimulate adaptations that result in increased resistance to hyper-stress conditions, such as exposure to oxidants in the soil bacterium and phytopathogen, *Xanthomonas*.

By definition, an oxidative stress condition arises from an excess of reactive oxygen species (ROS), including superoxide anions, H_2O_2, and organic hydroperoxides, which are highly toxic to biological macromolecules. Bacteria have evolved both enzymatic and non-enzymatic mechanisms to remove these ROS [3]. Questions remain as to where and when *Xanthomonas* would encounter ROS and oxidative stress. Aerobic respiration generates large quantities of ROS. Also, it has been shown that plants increase the production and accumulation of ROS as a part of the active defense response to microbial infection [5]. Thus, during interactions with plants, *Xanthomonas* must overcome toxic ROS in order to proliferate and multiply. As a soil bacterium, *Xanthomonas* is also exposed to environmental pollutants, some of them are strong oxidants. Widely used herbicides such as paraquat, as well as some metal pollutants, are potent generators of ROS, in vivo.

The simplest bacterial adaptation to stress probably occurs during the stationary phase of growth. During stationary phase, cells become starved for nutrients. Many gram-negative bacteria enter into a physiological state during stationary phase that allows them to remain viable for a long period of time. Interestingly, these stationary phase cells also become hyper-resistant to many types of stresses including oxidative stress. In some bacteria, general stress resistance is mediated, at least in part, by the product of the stationary phase sigma factor gene, *rpoS* [4]. In *Xanthomonas*, we have observed that stationary phase cells are hyper-resistant to H_2O_2, organic peroxides and superoxide generators, but not to either pH or heat stress [12]. Once stationary phase induced stress resistance is established, de novo protein synthesis is no longer required. At present, the regulatory mechanism(s) controlling the establishment of this general stress resistance phenotype in *Xanthomonas* is

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unknown, but the response appears to be independent of nutrient levels or the presence of cell derived metabolites in the stationary phase medium [12]. Some of the characteristics of the stationary phase induced oxidative stress resistance response in *Xanthomonas* are unlike that observed in other bacteria suggesting that a wide range of mechanisms may be employed by different bacteria to protect themselves from stresses during stationary phase.

The inducible adaptive response

Actively growing bacteria are generally more susceptible to stresses. Most bacteria have inducible stress protective systems to deal with a variety of environmental assaults. One of the most studied of these systems is the oxidative stress defense system. In *Xanthomonas*, as well as in other bacteria, exposure to low concentrations of the oxidative stress inducer, H_2O_2, induces high-level resistance to a subsequent challenge with lethal concentrations of H_2O_2 (Fig. 1) [8]. This inducible physiological adaptive response is not unique to H_2O_2. In several bacteria, other oxidants have been shown to be capable of inducing an adaptive protective response [2]. In *Xanthomonas*, as in many gram-negative bacteria, OxyR is one of the major peroxide sensing transcription regulators involved in controlling the oxidative stress response. Normally, the OxyR dimer exists in a reduced form that represses expression of genes in its regulon. Upon exposure to peroxides, the reactive sensing cysteine residue, C199 of OxyR becomes oxidized resulting in the subsequent formation of an intermolecular disulphide bond [13]. Oxidized OxyR is then able to interact with RNA polymerase and activate transcription of the genes in its regulon [11]. The role of OxyR in the *Xanthomonas* adaptive response to oxidative stress has been investigated using an *oxyR* mutant [8]. The results show that the regulation of this stress response is complex. Inactivation of *oxyR* renders cells highly susceptible to all oxidants confirming the importance of this global regulator in the oxidative stress response. H_2O_2 induced adaptive protection against H_2O_2 killing is clearly dependent on a functional *oxyR* as shown by the loss of the response in the *oxyR* mutant (Fig. 1). *oxyR* is also a regulator of genes involved in peroxide metabolism [11]. Thus, changes in the levels of enzymes such as catalase (involved in H_2O_2 degradation) and alkyl hydroperoxide reductase (involved in organic peroxide and H_2O_2 degradation) in response to various inducers have also been investigated. As expected, the expression of catalase (KatA) and alkyl hydroperoxide reductase (AhpC) is highly induced (at least five- to tenfold over uninduced levels) by pre-treatments with low levels of peroxides in an OxyR-dependent manner (Fig. 2). During exponential phase, the relative levels of these enzymes show a direct correlation with the level of resistance to H_2O_2. Thus, the H_2O_2 induced adaptive response is most likely a result of the increased expression of *katA* and *ahpC*. Ohr, a thiol peroxidase first discovered in *Xanthomonas* [9], belongs to a new class of genes involved in organic peroxide metabolism. The enzyme metabolizes organic peroxide much more efficiently than H_2O_2 [1]. *ohr* is regulated by OhrR, an organic peroxide sensing

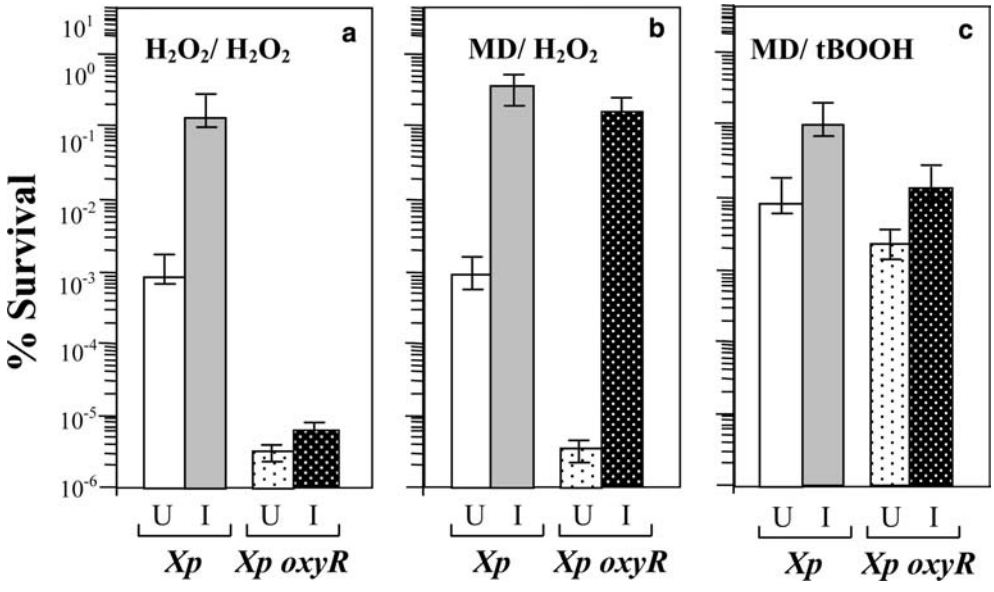

Fig. 1 Inducible adaptive and cross-protection responses to peroxide killing in *Xanthomonas*. *X. campestris* pv. *phaseoli* (*Xp*) and an *oxyR* mutant (*Xp oxyR*) were grown in SB medium [8]. Each culture was induced with a nonlethal concentration (30 μM) of either H_2O_2 or menadione (MD) for 30 min prior to exposure to a lethal concentration (100 μM) of either H_2O_2, menadione (MD) or *tert*-butyl hydroperoxide (tBOOH) for 30 min. Surviving cells were counted after 24 h incubation. The percent survival was calculated as the colony forming units (cfu) obtained after treatment divided by the cfu obtained from an untreated culture. Uninduced (U) cultures (i.e. those that received no pretreatment) were used as controls

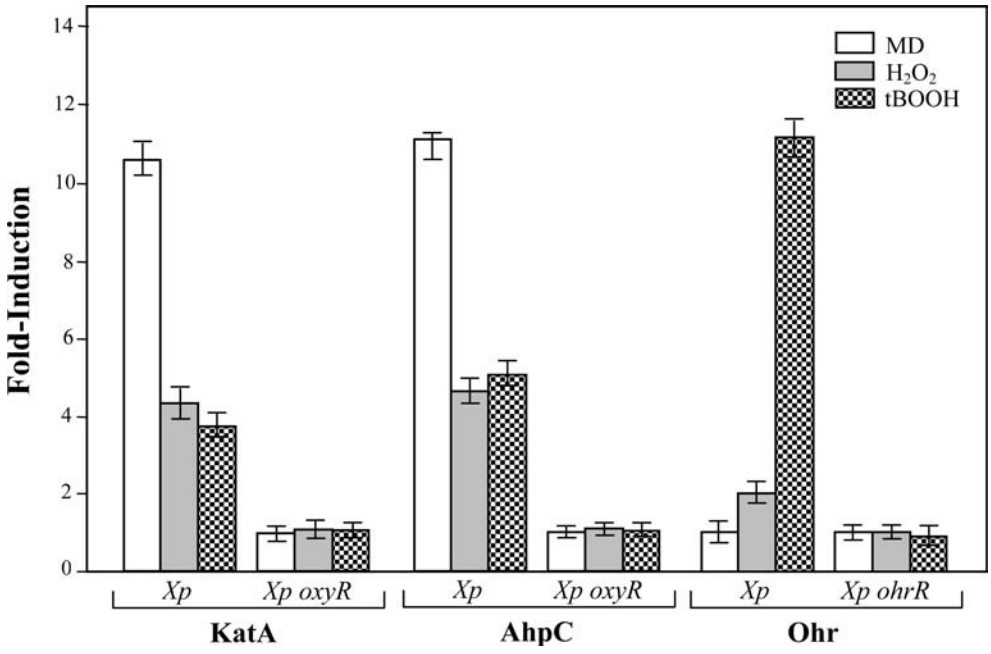

Fig. 2 Induction of *Xanthomonas* peroxide metabolizing enzymes following treatment with oxidants. Catalase (KatA) activity was assayed as previously described [8]. AhpC and Ohr protein levels were measured by western blot using antibodies specific to each protein as described previously [9]. The fold-induction is defined as the induced level divided by the uninduced level. *Xp* and *Xp oxyR* were grown in SB medium until exponential phase. Induced cultures received 100 μM of a given oxidant. Uninduced cultures received no oxidant treatment

transcription repressor [10]. OhrR is efficiently oxidized by organic peroxide but, only poorly by H_2O_2 [6]. Inactivation of *ohr* resulted in an increased sensitivity to organic peroxide, but not to other oxidants. Preliminary data suggest that the *ohrR–ohr* system may have a role in adaptation to exposure to complex organic peroxides. While it is clear that peroxides elicit a strong adaptive response in *Xanthomonas*, it is interesting that no physiological adaptive response to superoxide generators has been observed in this organism [7].

The inducible cross-protective response

Superoxide generators such as paraquat and menadione are strong inducers of cross-protective responses to H_2O_2 and organic peroxide killing (Fig. 1). The genetic basis of the regulation of the induced cross-protective response is also complex. Unlike the H_2O_2 adaptive response, the superoxide generator (menadione) induced cross protection against H_2O_2 and organic peroxide is OxyR independent (Fig. 1) [8]. Interestingly, the superoxide generator, menadione is the most potent inducer of both catalase and *ahpC* expression and can increase enzyme expression by up to tenfold (Fig. 2). This induction mechanism is dependent on the oxidation of OxyR, possibly by H_2O_2 generated through the dismutation of superoxide anions. By contrast, menadione treatment has no effect on *ohr* expression. Nonetheless, in the absence of OxyR, menadione could still induce high levels of resistance to H_2O_2 and organic peroxides. Thus, menadione induced physiological cross protection against H_2O_2 and organic peroxide involved activation of genes other than catalase, *ahpC* and *ohr* that could efficiently metabolize or repair peroxides induced cellular damage. Current work involves the identification of novel menadione regulated genes that have the ability to confer high levels of peroxide resistance. The regulators responsible for the cross-protection responses have not been identified. Inducible cross-protective responses to different types of peroxides have been observed in *Xanthomonas*. It is known that *oxyR* is involved in the regulation of these cross-protective responses since an organic peroxide such as *tert*-butyl hydroperoxide is able to induce protection against H_2O_2 killing in an *oxyR*-dependent manner [8]. In addition to being an H_2O_2 sensor, OxyR has also been shown to be able to sense organic peroxides. The protein could be oxidized and activated in vivo by organic peroxide leading to the activation of *ahpC* and *katA* expression. Up regulation of genes involved in peroxide metabolism is responsible for the cross-protective responses that are induced by organic peroxide. Although, organic peroxide is a strong inducer of *ohr*, it is unlikely that the thiol peroxidase enzyme is also involved in H_2O_2 protection due to the enzyme's low substrate affinity for H_2O_2 [1]. However, H_2O_2 only induces low-level cross protection to organic peroxide killing. H_2O_2 activation of OxyR and the resulting increased *ahpC* expression levels is not sufficient to confer high-level cross protection to organic peroxide in *Xanthomonas*. This is consistent with the observation that the *ohrR–ohr* system has the major role in protecting *Xanthomonas* from organic peroxide toxicity but is only weakly induced by H_2O_2 [6].

Conclusion

Exposure of *Xanthomonas* to pollutants, that have the ability to generate strong oxidants such as the herbicide paraquat and metals, could stimulate both adaptive and cross-protective physiological responses to oxidative

stress in the bacteria. These responses are, in part, mediated by the oxidant-dependent activation of the global peroxide sensing transcription regulators OxyR, OhrR that in turn, activate/derepress genes in their respective regulons. Since increased production and accumulation of ROS are an important part of the plant active defense response to microbial invasion, environmental conditions that render bacterial phytopathogens more resistant to these ROS could alter both the frequency and severity of crop disease. Therefore, exposure to environmental pollutants could affect crop yields in ways that are not immediately apparent.

Acknowledgments Research work is supported by grants from the BIOTEC (Research Team Strengthening), the Thailand Research Fund (RTA4580010), and the ESTM through the Higher Education Development Project of the Ministry of Education.

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