EVALUATION OF THE ROLES OF OHR AND AHPC IN ORGANIC HYDROPEROXIDE DETOXIFICATION IN XANTHOMONAS CAMPESTRIS PV. PHASEOLI

CHANANAT KLOMSIRI

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Chananat Klomsiri

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ABSTRACT

Xanthomonas campestris pv. *phaseoli* (*Xp*) has two structurally and regulatory distinct genes namely alkyl hydroperoxide reductase (*ahpC*) and organic hydroperoxide resistance (*ohr*), which act synergistically to protect bacterial cell from organic hydroperoxide toxicity. A double mutant (*Xp ahpC-ohr*) was constructed to examine the physiological role of the genes. Inactivation of either *ohr* or *ahpC* resulted in increased sensitivity to killing with organic hydroperoxides. High expression of *ahpC* or *ohr* partially complements the hypersensitive phenotype of the double mutant. Functional analyses of Ohr and AhpC abilities to degrade *tert*-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CuOOH) and linoleic acid hydroperoxide (LOOH) revealed that both Ohr and AhpC could degrade all peroxides while the former was more efficient at degrading CuOOH and LOOH. Expression analyses of these genes in *ohr* and *ahpC* mutants showed no compensatory alteration between the levels of AhpC and Ohr. However, CuOOH induced *ahpC* expression was higher in the *ohr* mutant than in the parental strain, in contrast, the *ahpC* mutation has no effect on the level of *ohr* expression. Northern analyses showed that exposure of bacteria to sublethal concentrations of LOOH induced elevated expression of both *ahpC* and *ohr* by several folds. Ohr system was comparatively more sensitive to be activated by increased level of LOOH. Promoter analyses revealed that the OhrR was more sensitive to be activated by LOOH than the OxyR. Gel mobility shift assay clearly showed that low concentration of LOOH could release OhrR, a transcription repressor of *ohr*, from P1 promoter and in turn increased expression of *ohr*. Pretreatment *Xp* with sublethal doses of LOOH rendered the bacteria more resistant to a subsequent killing treatment with LOOH. This induced adaptation was existed in the *ahpC* mutant but it was abolished in the *ohr* and the *ohrR* mutants. This study showed that both *ahpC* and *ohrR-ohr* system have the important physiological roles to protect the bacterial from organic hydroperoxide toxicity. However, the *ohrR-ohr* system is more responsive to complex hydroperoxides, CuOOH and LOOH, than the simple one, tBOOH, while *ahpC* system is more sensitive to tBOOH than the complex hydroperoxides.

KEY WORDS: ORGANIC HYDROPEROXIDE / *XANTHOMONAS* / *ohr*/ *ahpC*/ OXIDATIVE STRESS

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การศึกษาหน้าที่ของยีน OHR และ AHPC ในการกำจัดพิษของ ORGANIC HYDROPEROXIDE ใน XANTHOMONAS CAMPESTRIS PV. PHASEOLI (EVALUATION OF THE ROLES OF OHR AND AHPC IN ORGANIC HYDROPEROXIDE DETOXIFICATION IN XANTHOMONAS CAMPESTRIS PV. PHASEOLI)

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บทคัดย่อ

Xanthomonas campestris pv. *phaseoli* (*Xp*) มียีน *ahpC* และ *ohr* ซึ่งทั้งสองยีนนี้ถูกควบคุมด้วยระบบการควบคุมที่ต่างกัน แต่ทั้งสองยีนนี้มีหน้าที่ร่วมกันในการกำจัดพิษของ organic hydroperoxides *Xp* สายพันธุ์ที่ถูกทำลายยีน *ahpC* และ *ohr* ถูกสร้างขึ้นเพื่อศึกษาหน้าที่ของยีนทั้งสอง พบว่าเมื่อยีนทั้งสองถูกทำลาย ทำให้แบคทีเรียมีความไวต่อสาร organic hydroperoxides มากขึ้น และการทดแทนด้วยการแสดงออกของยีน *ahpC* หรือ *ohr* นั้นสามารถทดแทนลักษณะ phenotype ที่ไวต่อสาร organic hydroperoxides ได้ไม่สมบูรณ์ การวิเคราะห์ความสามารถของโปรตีน Ohr และ AhpC ในการทำลายสาร *tert*-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CuOOH) and linoleic acid hydroperoxide (LOOH) พบว่าโปรตีนทั้ง Ohr และ AhpC สามารถทำลาย peroxidies ที่นำมาทดสอบได้ทุกชนิดโดยที่โปรตีน Ohr สามารถทำลาย CuOOH และ LOOH ได้ดี การวิเคราะห์การแสดงออกของยีนทั้งสองใน *Xp* สายพันธุ์ที่ยีน *ahpC* หรือ *ohr* ถูกทำลาย พบว่าไม่มีการแสดงออกที่ทดแทนกันของยีนทั้งสอง อย่างไรก็ตาม CuOOH สามารถกระตุ้นการแสดงออกของยีน *ahpC* ใน *Xp* สายพันธุ์ที่ยีน *ohr* ถูกทำลายได้ดีกว่าสายพันธุ์ปกติ ในทางกลับกันเมื่อยีน *ahpC* ถูกทำลายไปนั้นไม่ส่งผลกะทบต่อการแสดงออกของยีน *ohr*

เมื่อแบคทีเรียถูกกระตุ้นด้วย LOOH ที่ความเข้มข้นต่ำพบว่าสามารถกระตุ้นการแสดงออกของยีนทั้งสองเพิ่มขึ้นหลายเท่า แต่ว่าระบบการผลิตโปรตีน Ohr มีความไวต่อ LOOH มากกว่า จากการวิเคราะห์ promoter พบว่าโปรตีนตัวควบคุม OhrR มีความไวต่อสาร LOOH มากกว่าโปรตีนควบคุม OxyR จาก Gel mobility shift assay พบว่า LOOH ที่ความเข้มข้นต่ำทำให้โปรตีนควบคุม OhrR ซึ่งเป็นตัวยับยั้งการถอดรหัสของยีน *ohr* นั้นไม่สามารถทำหน้าที่ได้และทำให้การถอดรหัสของยีน *ohr* เกิดขึ้นได้ การกระตุ้นแบคทีเรียสายพันธุ์ปกติด้วยสาร LOOH ที่ความเข้มข้นต่ำสามารถเหนี่ยวนำให้แบคทีเรียต้านทานการฆ่าด้วยสาร LOOH ความเข้มข้นสูงๆได้มากขึ้น โดยการตอบสนองเหล่านี้ยังสามารถพบได้ในสายพันธุ์ที่ยีน *ahpC* ถูกทำลายไป แต่ไม่พบในสายพันธุ์ที่ยีน *ohr* และ *ohrR* ถูกทำลาย ความรู้ที่ได้จากงานวิจัยครั้งนี้แสดงให้เห็นว่าระบบของยีน *ahpC* และ *ohrR-ohr* มีหน้าที่สำคัญในการป้องกันแบคทีเรียจากความเป็นพิษของ organic hydroperoxides อย่างไรก็ตามระบบของยีน *ohrR-ohr* มีความไวต่อสาร hydroperoxides ที่มีความซับซ้อนเช่น CuOOH และ LOOH ได้ดีกว่าสาร hydroperoxides ที่ไม่ซับซ้อนเช่น tBOOH ในขณะที่ระบบของยีน *ahpC* มีความไวต่อสาร tBOOH มากกว่าสาร hydroperoxides ที่มีความซับซ้อน

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LIST OF ABBREVIATIONS

Abbreviation	Term
A	Absorbance
AhpR	Alkylhydroperoxide reductase
Ap	Ampicillin
bp	Base pair
^{o}C	Degree Celsius
cfu	Colonies forming unit
cm	Centimetre
DNA	Deoxyribonucleic acid
Dps	Non specific DNA binding protein
E. coli	*Escherichia coli*
et al.	*et alii* (Latin), and other
g	Gram
Gm	Gentamicin
G6PD	Glucose-6-phosphate dehydrogenase
GR	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
h	Hour
HP	hydroperoxidase
HR	hypersensitive response
kat	catalase
kb	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani medium
M	Molar

LIST OF ABBREVIATIONS (cont.)

Abbreviation	Term
m	Milli
µ	Micro
µl	Microlitre
µM	Micromolar
min	Minute
mol	Mole
NEM	N-ethyl maleimide
nm	Nanometre
O_2	oxygen molecule
$O_2^{\bullet-}$	Superoxide anion
O_2^{2-}	peroxide
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
sp.	Species
TAE	Tris-acetate / EDTA electrophoresis buffer
TE	Tris-EDTA buffer
Tet	Tetracycline
tBOOH	*tert*-butylhydroperoxide
U	Unit
UV	ultra violet
volt	Volt
v/v	volume/volume
w/v	weight/volume

CHAPTER I
INTRODUCTION

Xanthomonas campestris pv. *phaseoli* (*Xp*), a gram negative aerobic soil bacterium, and belongs to an important genus of plant pathogenic bacteria causing common blight disease in dry beans (*Phaseolus vulgaris and Phaseolus lunatus*). The bacteria usually affect leaves, causing leaf spots that may coalesce and result in leaf blight. They are also capable of invading the vascular tissue of the plant and infecting stem, pods and seeds. Controlling of this disease is difficult because the pathogen is seed-borne and remains viable in seeds for years (1, 2).

Plant-microbe interactions could be divided into two different interactions defined as incompatible or a resistant plant reaction and compatible or a susceptible plant reaction (3). In incompatible interaction, plants activate defense response both passively and actively to prevent bacterial proliferation and eventually lead to elimination of invading microbes. Critical to development of plant disease during compatible interactions is the ability of the pathogens to multiply and colonize plant tissues. The initial responses to microbial infection, plants increase production and accumulation of reactive oxygen species (ROS) which are an important part of plant defense response (4). These ROS include superoxide anions ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and organic hydroperoxide. Organic peroxides are highly toxic, partly due to the abilities of these compounds can react with metals or with metalloproteins leading to the production of secondary free radicals (5, 6), which may be related to the fact that organic peroxides posses bactericidal activity (7).

One major target of ROS attack on unsaturated lipids, leading to autocatalytic lipid peroxidation. This is a significant source of membrane damage. During lipid peroxidation, reactive lipid and fatty acid hydroperoxide are formed, and these contribute to ongoing autoxidation. Evans *et al*. have been found that LOOH is toxic to wild type *Saccharomyces cerevisiae* at a very low concentration (0.2mM) relative to other peroxides (8). Plants have a defense mechanism that is involved lipoxygenase

pathway, which catalyzes the formation of fatty acid hydroperoxides. The first step in this pathway is the reaction of linoleic acids with molecular oxygen, catalyzed by lipoxygenase. These fatty acid hydroperoxides are the precursors of an array of compounds involved in the regulation of plant development, wound healing, pest resistance, signaling and antimicrobial activity (9). Moreover, in normal aerobic growth, by products of aerobic metabolism are large amounts of ROS. These toxic molecules need to be rapidly removed before their concentrations build up to harmful levels and inhibit cell growth. For surviving and proliferating in plant, bacterial have to detoxify these ROS by both enzymatic and non-enzymatic mechanisms.

Oxidative stress response in *Xp* is complex. Many genes involved in this response have been cloned and characterized. The best characterized bacterial organic peroxide detoxification system involves the alkyl hydroperoxide reductase (AhpCF). The enzyme consists of two subunits, a catalytic AhpC, and a reductase AhpF (10). This enzyme catalyzes the reduction of organic peroxides to their corresponding alcohols (11). *ahpC* is highly conserved and can be found in organisms from bacteria to man (12). This suggests that it serves important physiological roles. Inactivation of *ahpC* resulted in increased sensitivity to organic peroxides and pleiotropic alterations in oxidative stress response (13, 14). The expression of *ahpC* is dependent on OxyR, a peroxide sensor and transcription regulator (15).

A second mechanism for organic hydroperoxide protection is *organic hydroperoxide resistanec* (*ohr*) gene which has been firstly discovered in *Xanthomonas campestris* pv.*phaseoli* (16). It involves in organic peroxide resistance phenotype and inactivation of this gene leads to increase sensitivity only to organic hydroperoxides. *ohr* has unique expression pattern, which is strongly induced by organic peroxide and not by other oxidants (16-18). *ohr* homologues are widely distributes in diverse bacteria (17). The expression of *ohr* is controlled by ohrR encoding a transcription repressor, which senses organic peroxides (19, 20). Ohr possesses thiol-dependent peroxidase activity that catalyses the reduction of an organic hydroperoxide to its corresponding alcohol. The removal of organic hydroperoxides by Ohr is more efficient than the removal of H_2O_2 (21).

This study focuses on characterization of the physiological roles of Ohr and AhpC from *Xanthomonas campestris* pv. *phaseoli* in the detoxification of organic hydroperoxides including tBOOH, CuOOH and LOOH.

The objectives of this thesis were;

(a) To determine the resistance to organic hydroperoxides in *Xp ahpC*::*kan* (*Xp ahpC*), *Xp ohr*::*tet* (*Xp ohr*) and the *ahpC-ohr* double mutant strains.

(b) To analyze the functions of Ohr and AhpC from *Xp* in organic hydroperoxide degradations.

(c) To investigate the expression patterns of *ohr* and *ahpC* from *Xp* in the presence of organic hydroperoxides.

(d) To study the effect of LOOH to *ahpC* and *ohrR-ohr* promoters.

(e) To investigate the induced adaptive responses against LOOH killing in *Xp*, *Xp ahpC* mutant, *Xp ohr* mutant and *Xp ahpC-ohr* mutant strains.

CHAPTER II
LITERATURE REVIEW

1. Background of *Xanthomonas* spp.

Xanthomonas is a typical genus of plant pathogenic bacteria known to cause severe damage to most economical important crops, especially subtropical and tropical areas all over the world. The pathogens cause a variety of diseases including leaf spots, streaks, wilts, cankers, chloroses and soft rots. Naturally, various mixtures of these symptom classes also occur in particular diseases. Disease may occur on foliage (hypocotyls, stems and leaves) and reproductive growth (flowers, fruits and seeds). The host range of *Xanthomonas* includes at least 268 dicotyl and 124 monocotyl plant species (22). A critical point in the life cycles of Xanthomonads, as with other plant pathogens, is transmission to a new host, particularly if a period of survival in the absence of the host is necessary. Such survival may be achieved in many ways, such as with seed, plant residues, perennial hosts, epiphytically, and prophytically in soil. Many Xanthomonads solve this problem by transmission with the seed of their host. The bacteria may be carried in detritus with the seed, on or in the seed coat, or deeper inside the tissues of the seed itself. In agriculture many pathogens are most effectively carried by the activities of man, either in infected planting material or on tools, wheeled vehicles and grazing animals (23).

Xanthomonas cells are obligate aerobic organisms, having a strictly respiratory type of metabolism with oxygen as terminal electron acceptor, non-spore forming gram negative bacilli with a monotrichous flagellum. Their cells are predominantly single short straight rods with round ends, usually within the range 0.4-0.7 wide 0.7-1.8 mm long. The structure of *Xanthomonas* spp. is shown in Fig.1.

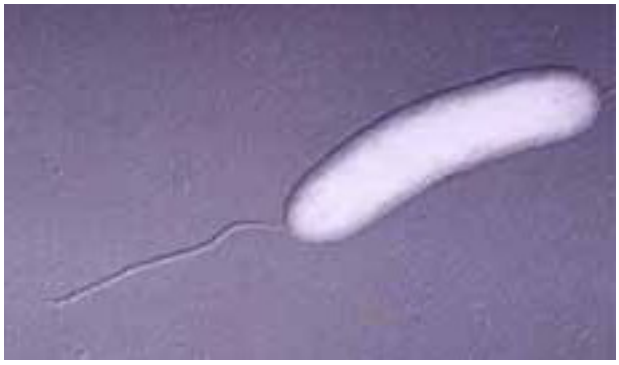


Fig.1 Structure of *Xanthomonas* spp. (24).

One of typical characteristics of *Xanthomonas* genus is the appearance of mucoid colonies when cultured on media supplemented with glucose. The slimy appearance is the result of copious amounts of extracellular polysaccharide (EPS), also known as xanthan gum, produced by the cells. This EPS is thought to play a protective role that helps to maintain the viability of bacterial cells in unfavorable environments. No denitrification or nitrate reduction occurs. Colonies are usually circular, convex, whitish yellow to straw yellow when they become old, smooth surfaced, and opaque against transmitted light. The yellow pigments, insoluble in water, are mono or dibromo arylpolyenes called Xanthomonadins. The oxidase test of *Xanthomonas* is negative or weakly positive but the catalase test is positive. Xanthomonads are chemoorganotrophic and are able to use a variety of carbohydrates and salts of organic acids as carbon sources. The optimal temperature and pH for growth are 25-30^{o}C and 6.0-7.0, respectively. Growth in some strains may be slow. The time taken to produce the colonies about 1 mm in diameter varies from 2 or 3 days to a week or more. Required growth factors usually include methionine, glutamic acid, nicotinic acid or a combination of these. The G+C composition of these bacteria is quite high at 63-71 mol % of the DNA (25). According to Bergey's Manual of Determinative Bacteriology (25), the genus *Xanthomonas* comprises of *Xanthomonas albilineans, X. axonopodis,*

X. campestris, X. citri, X. fragariae, X. graminis, X. oryzae, X. maltophilia, X. phaseoli, and *X. populi.*

The species *X. campestris* includes of numerous pathovars (pv.). These strains are similar with respect to biochemical and bacteriological characteristics but are different with respect to pathogenicity on one or more host plants. For example, *X. campestris* pv. *campestris* is a pathogen affecting plant from the family Cruciferae and it is responsible for one of the most serious diseases called black rot in certain *Brassica* spp. such as cabbage and cauliflower (25).

X. campestris pv. *phaseoli* (*Xp*), the model bacteria in this study, is important in the epidemiology of bacterial common blight in dry beans (*Phaseolus vulgaris* and *Phaseolus lunatus*) worldwide. The bacterium infects many types of beans including snap, field, hyacinth, lima, moth, mung, Scarlet runner and Tepary. *X. campestris* pv. *phaseoli* overwinters primarily in and on infected seed. Bacterial cells in the seed are able to remain viable longer that the seed itself. In temperate climates it may also overwinter on infected plant debris, with the best rate of survival occurring in debris remaining on the soil surface under dry conditions. Bacteria associated with the seed provide a source of primary inoculum for both local and long distant spread of the disease while infected debris (including cull piles) is more important in local spread of the disease. Infection and disease development are favored by warm (28-32^{o}C), wet weather. Seedlings grown from infected seed develop lesions containing bacteria on their stems, cotyledons and first set of primary leaves. The bacteria in these lesions are commonly spread by wind blown rain and infect other bean plants via stomata, hydathodes and wounds. Other means of bacterial dispersal include wind blown dirt particles, contaminated irrigation water, physical contact between plants, humans and animals and insects including white flies and certain other leaf feeding insects. Once the disease is established, numerous lesions are produced on aerial plant parts as serve as an inoculum source for secondary spread of the disease. Seed may become infected in two ways: the bacterium may enter the seed via the vascular system or by external pod infections. The bacterium is also capable of growing epiphytotically on weedy hosts.

Common blight symptoms are initially visible as small water-soaked spots on leaf margins or between the veins. Spots eventually dry and turn brown. They are surrounded by narrow band of lemon yellow tissue. Spots enlarge with age and blight large areas of leaf tissue. Heavily infected leaves may become tattered when wind whipped. In severe cases they die and remain attached to the plant. Pod infections also begin as water-soaked spots. Pod lesions may be reddish-brown at the margins or in entirety. A slimy bacterial exudate may be present in pod lesions. The exudate eventually dries giving the lesion a glazed appearance. Pod lesions ultimately turn brown and vary in size depending on the age of the pod when infection occurs. In severe cases the entire pod shrivels. Seed produced from infected plants may be shriveled, discolored or nonexistent if a severe pod infection occurs. Infected seed produced on white-seeded varieties generally shows some degree of yellow discoloration and appears shiny. Some very distinctive symptoms are associated with this disease, however, it important to note that the bacterium can be present in large numbers on symptomless hosts and can still serve as a source of secondary inoculum from these hosts (26). Fig. 2 (A) and (B) show the common blight symptoms in infected leaves and pods.

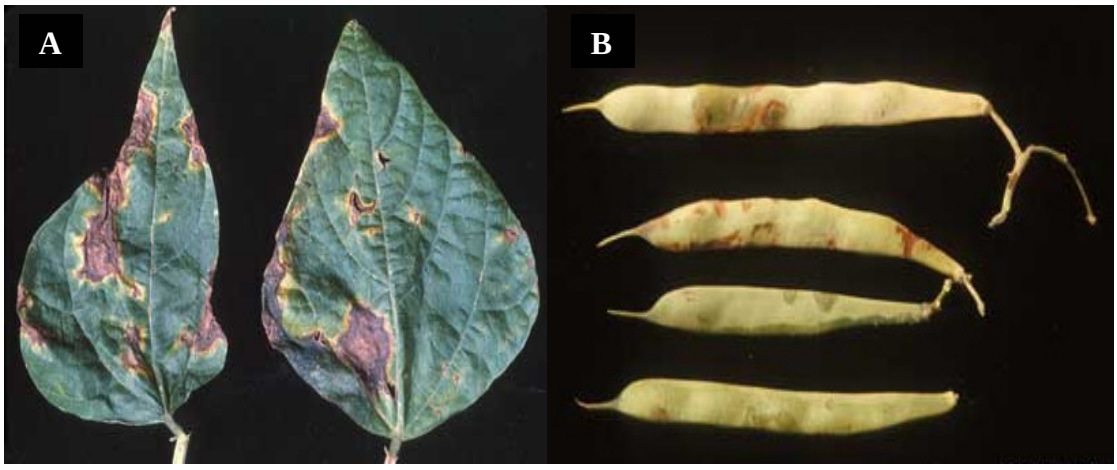


Fig. 2 Common blight symptoms in infected leaves (A) and pods (B) by *Xanthomonas* spp. (26).

2. Plant -pathogen interaction and plant defense responses

Interactions between specific plant cultivars and defined races of potential pathogenic microbes are classified in 2 categories. The first is a compatible reaction (virulent pathogen, susceptible host); the proteins of the pathogenic organism are virulent for the given plant genotype. Pathogen either eludes or suppresses recognition and plant defense responses, which lead to successful colonization and leading to disease. The interactions involve regulation of microbial pathogenically genes by exchange signal molecules that allow for partner recognition (27). Successful infection by microbial pathogens requires surface attachment, degradation of host chemical and physical barriers, production of toxins, and inactivation of plant defenses. The disease symptoms may be considered as the end result of the stages of infection including escape of resistant process, establishment of the nutrition source, colonization on plant tissues and then development of disease symptoms (28). An overall, up to one hundred genes may be needed for bacterial pathogenicity and regulation of these genes involves a complex interplay of signal between host and pathogen (29). The second is incompatible (avirulent pathogen, resistant host). In this reaction, early molecular recognition is followed by rapid expression of defense responses, which are efficiently activated interactions leading to resistance but not are activated or are activated after a significant delay. This mechanism prevents further spread of pathogen to other cells. In many cases, incompatibility is characterized by hypersensitivity response (HR), which defined as a rapid and irreversible increase of membrane permeability lead to loss of structural integrity and confluent tissue necrosis within 24 h (30) With the onset pathogenesis gene transcription, reactive forms of oxygen are formed which cause the death of infected plant cells; the cell wall is reinforced in the region of pathogen penetration by deposition of lignin, lignin-like polymers and structural proteins; the production of proteinase inhibitors and lytic enzymes such as chitinase and glucanase, which can attack microbial cell wall is increased; hydroxyproline-rich glycoprotein (HRGPs) accumulate; and pathogenesis-related (PR) protein accumulates (28, 31 and 32).

The genetics of many plant-pathogen interactions are described by the gene-for-gene hypothesis, such that a dominant plant resistance gene (*R*) will confer resistance to particular race only if the pathogen expresses the corresponding avirulence (*avr*) gene (28, 29). Host resistance, triggered by the interaction of *R* and *avr* products, is often based on an active HR involving the death of challenged host cells and coordinated activation of defense responses in surrounding cells to restrict pathogen spread to healthy tissues (33). Receptors activate signal transduction mechanisms which launch several protective systems. In the case of pathogen penetration, strong oxidants such as H_2O_2, $O_2^{\bullet-}$, $^{\bullet}OH$ are synthesized in an oxidative burst that causes death of infected cells by a mechanism similar to apoptosis in vertebrates (34). A hypersensitive response develops instead of pathogen penetration into the neighboring cells. A rapidly developing local process produces signal molecules spreading along the vascular system of a plant. Independent of local events, a set of signal molecules is synthesized, which turns on a generalized response, resulting in pathogenesis-related genes (PR-genes) being activated, cell walls being strengthened, and the accumulation of protective substances, for use in combating a particular pathogenic form. In plant cells, salicylic acid (SA) is produced in considerable amounts and causes activation of SA-induced genes. These events are referred to collectively as systemic acquired resistance (SAR) (35).

Plants have some mechanisms to defense the microbial infection, which both passively and actively prevent bacterial proliferation and eventually lead to elimination of invading microbes. Plant tissues are known to protect themselves using many means of passive and active defense. Active defense has been characterized as inhibiting the growth of invaders through the expression of inducible genes leading to accumulation of novel compounds such as phytoalexins, lignin, chitinase or glucanase (28, 36 and 37). Passive defenses include fortification against pathogen entry and spread out by using pre-existing passive physical barriers. One active plant defense response to microbial invasion is the increased production of reactive oxygen species (ROS). There is evidence that correlates increased production of ROS with the ability of plants to successfully defend against pathogen attack. Thus, the production of ROS is an important part of the plant defense response. However, microbes must detoxify

the ROS in order to survive and proliferate. Microbial defenses against the damaging effects of oxidative stress involve both enzymatic and nonenzymatic mechanisms to rapidly detoxify ROS and repair ROS induced damage. A summary of oxidative stress response to ROS in *Xanthomonas*, an aerobic soil bacteria and bacterial phytopathogen, is shown in Fig. 3 (38-41).

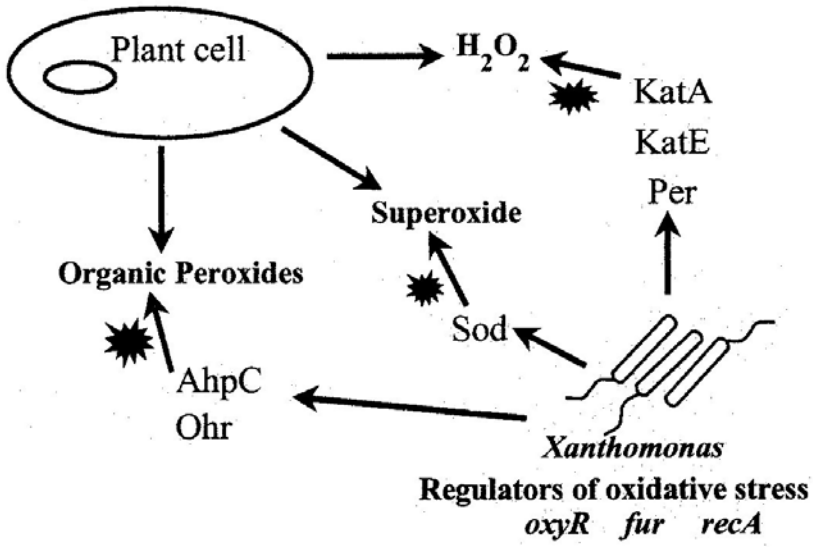


Fig. 3 Summary of the *Xanthomonas* oxidative stress response to encounter plant generated ROS (41).

The level of ROS in infected plants show biphasic increases as shown in Fig. 4 that comprised of an initial oxidative burst (phase I) and a second oxidative burst (phase II). Phase I is non-specific and can be observed in both compatible and incompatible interactions. Phase II is observed only in incompatible interactions (40, 42). These observations indicate that production of ROS is an important part of the plant defense response. Moreover, transgenic plants that have either reduced ability to break down ROS or increased levels of ROS producing enzymes show increased resistance to microbial invasion and altered disease development (43, 44).

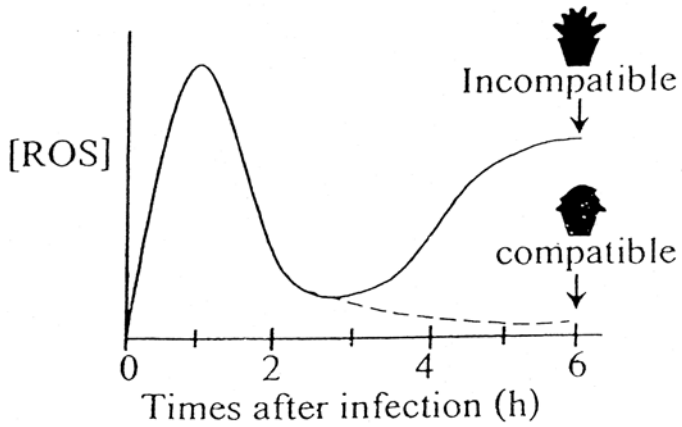


Fig. 4 Plant defense response and ROS concentration during compatible and incompatible between plants and microbes (41)

ROS also play an important role in plant defense system (45).The first reaction during pathogen-induced oxidative burst is believed to be the one-electron reduction of molecular oxygen to form $O_2^{\bullet-}$. $O_2^{\bullet-}$ also plays a part in directly killing of the pathogen. In aqueous solution, $O_2^{\bullet-}$ undergoes spontaneous or superoxide dimutase-catalyzed dismutation to H_2O_2 (46). A rise in ROS level is detected within seconds to minutes following treatment with elicitor or pathogen suggesting that oxidative burst involves the activation of pre-existing oxidase component rather than de novo synthesis of the oxidative burst machinery (33).

In the HR, lipid peroxidation is often a late process occurring at the same time as the appearance of necrosis. Since ROS production preceded lipid peroxidation, it is generally admitted that ROS are implicated in the initiation of membrane damage, and hence hypersensitive cell death. Indeed, inhibition of oxidative burst by exogenous supplied enzymes, scavengers, or inhibitors of ROS generator systems suppresses or delays both lipid peroxidation and hypersensitive cell death. Lipid peroxidation might also be due to lipoxygenases (LOXs, EC.1.13.11.12) (47). Enzymes catalyze the addition of molecular oxygen to polyunsaturated fatty acids to produce an unsaturated

fatty acid hydroperoxide (Fig. 5). In plant, linoleic or linolenic acids are the most common substrates for LOX (48). In the case of linoleic or linolenic acids, this leads to two possible products, the 9- and 13-hydroperoxy fatty acids (48). In tobacco, 9-LOX activity is induced upon infection by *Phytophtora parasitica* var *nicotianae*. Its activity appears earlier in an incompatible interaction than in a compatible one, thus supporting a role for this 9-LOX in plant defense against fungal infection (49) The expression of these enzymes has been shown to be induced in response to microbial invasion and has been linked to the plant microbial defense response. (50).

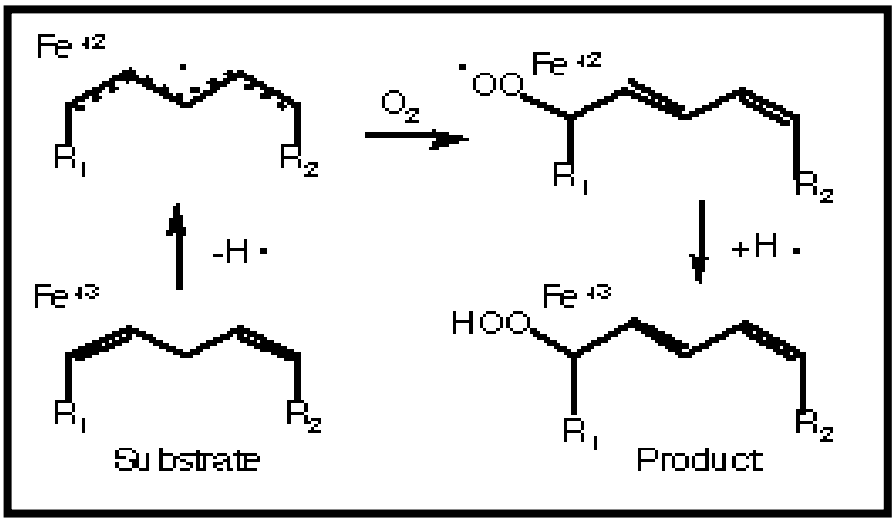


Fig.5 The reaction catalyzed by lipoxygenases to form lipid hydroperoxides (51).

3. Oxidative stress

Oxygen is the primary oxidant in metabolic reactions designed to obtain energy from the oxidation of a variety of organic molecules. Oxidative stress results from the metabolic reactions that use oxygen, and it has been defined as a disturbance in the equilibrium status of pro-oxidant/ anti-oxidant systems in intact cells. This definition of oxidative stress implies that cells have intact pro-oxidant/ anti-oxidant systems that continuously generate and detoxify oxidants during normal aerobic metabolism. When additional oxidative events occur, the pro-oxidant systems outbalance the anti-oxidant, potentially producing oxidative damage to lipids, proteins, carbohydrates and nucleic acid, ultimately leading to cell death in severe oxidative stress. Mild, chronic oxidative stress may alter the anti-oxidant systems be inducing or repressing proteins that participate in these systems.

A disturbance in pro-oxidant/ anti-oxidant systems results from a myriad of different oxidative challenges, including radiation, metabolism of environment pollutants and administered drugs, and immune system response to disease or infection. The immune response is especially interesting since many toxic oxidative materials are generated in order to kill invading organisms. Evidence for the role of a variety of chemicals called radicals in these processes has led to interest in the reactions of partially reduced oxygen products, radical and non-radical species derived from them. A variety of reactive nitrogen species derived from the reactions of nitric oxide play important roles as well. A radical species is specifically understood to be any atom that contains one or more orbital electrons with unpaired spin states. The radical may be a small gas molecule such as oxygen or nitric oxide, or it may be a part of a large biomolecule such as protein, carbohydrate, lipid, or nucleic acid. Some radical species are very reactive with other biomolecules and others like the normal triplet state of molecule oxygen are relatively inert.

Oxidative stress has been implicated in human disease by a growing body of facts. However, cells have multiple protective mechanisms against and succeed in preventing cell damage to the extent that these protective mechanisms are effective. To counter oxidative stress, cells constitutively express enzymes that detoxify reactive oxygen species and repair the damaged cause by them. In addition, bacterial, yeast,

and mammalian cells have adaptive responses to elevate levels of oxidative stress, indicating that these cells sense increased levels of reactive oxygen species and transduce the signal into increased expression of defense activities. Beside, genes encoding antioxidant enzymes can be detected in the sequences of most complete genomes, showing that defenses against oxidative stress are critical to many organisms.

4. Reactive oxygen species (ROS)

One of the paradoxes of life on this planet is that the molecule that sustains aerobic life, oxygen, is not only fundamentally essential for energy metabolism and respiration, but it has been implicated in many diseases and degenerative conditions. All aerobic organisms must cope with ROS such as $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$. ROS or activation of oxygen may occur by two different mechanisms namely, absorption of sufficient energy to reverse the spin on one of the unpaired electrons, or monovalent reduction. The biradical form of oxygen is in a triplet ground state because the electrons have parallel spins. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet state, in which the two electrons have opposite spins (Fig. 6). This activation overcomes the spin restriction and singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two electrons. Since paired electrons are common in organic molecules, singlet oxygen is much more reactive towards organic molecules than its triplet counterpart (52, 53).

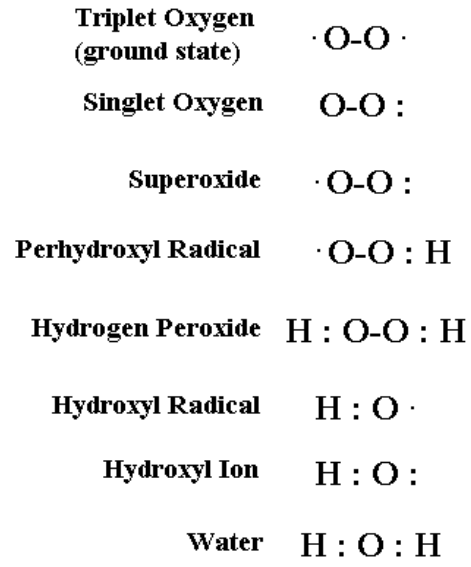


Fig. 6 Nomenclature of the various forms of oxygen (53)

Molecular oxygen is relatively unreactive, yet it is capable of giving rise to lethal reactive excited states as free radicals and derivatives. Utilization of O_2 proceeds most readily via a complete stepwise, four-electron reduction to water during which partially reduced reactive intermediates are generated that showed in the Fig. 7. Some initiators of oxyradicals and the biological dysfunction and cell death showed in Fig. 8. Host generated ROS are thought to function directly in both growth inhibition, killing of pathogen and signal transduction pathways to enhance defense response (40). Most ROS are highly toxic to biological systems and must be removed before reaching harmful levels. In order to survive and proliferate, microbes evolve both enzymatic and non-enzymatic mechanisms to rapidly detoxify ROS and repair ROS induced damage. In many microbes, sub-lethal exposure to a chemical or stress can confer resistance to a lethal exposure to the same agent (an adaptive response) or non-related agents (a cross-protection response) (52, 54). They are essential components of microbes' survival strategies against stresses (55).

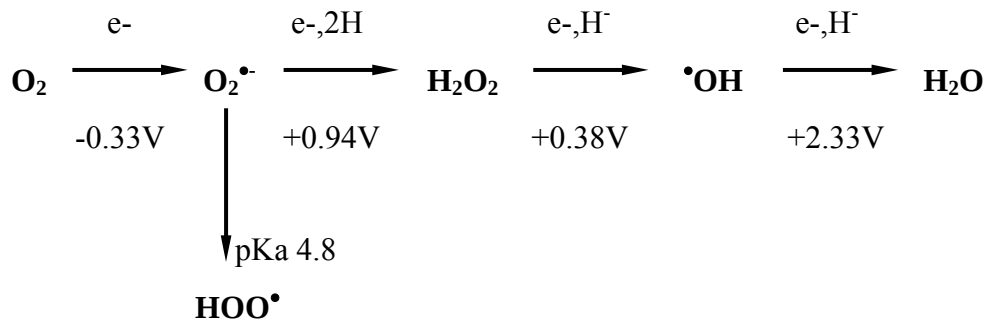


Fig. 7 Pathways in the reduction of O_2 to water leading to the formation of various intermediate ROS (56, 57)

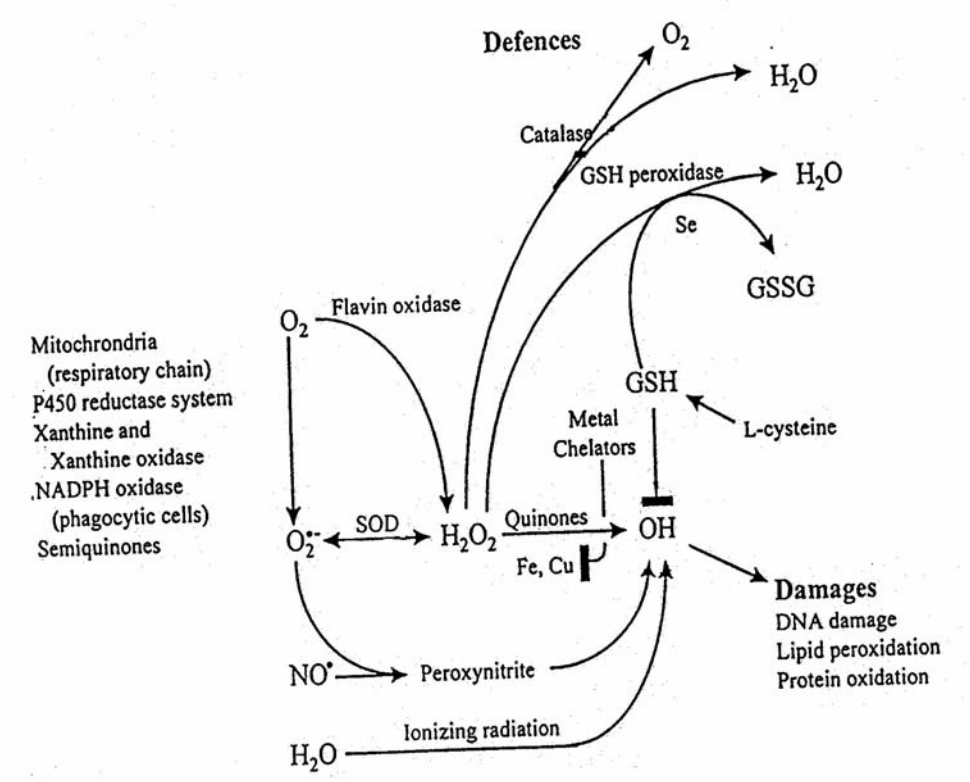


Fig. 8 Some sources of ROS, antioxidant defenses, and biological effects (58).

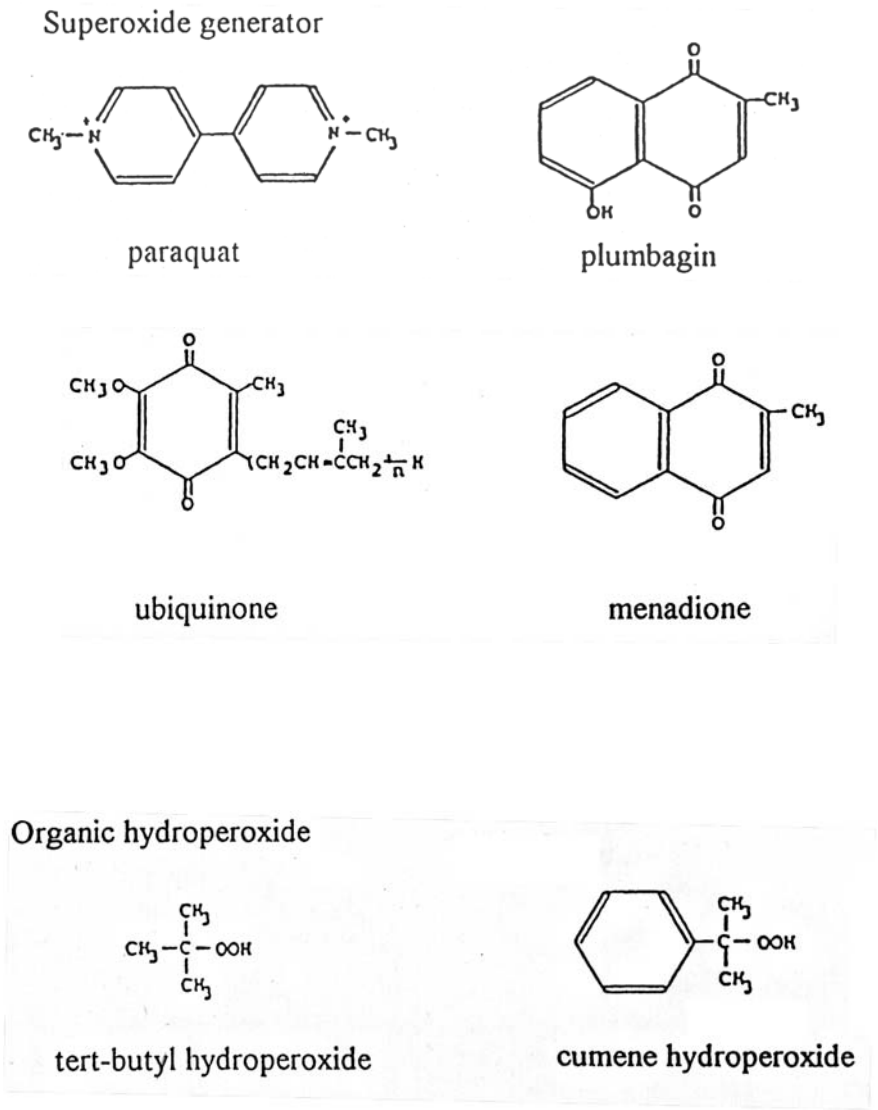


Fig. 9 The chemical structure of commonly used oxidants (7).

4.1 Source of superoxide anion ($O_2^{\bullet-}$)

The first ROS generated during electron transport, mainly produced by NADH dehydrogenase (NDH) is $O_2^{\bullet-}$ (38). Membrane-associated respiratory chain enzymes such as NADH dehydrogenase, succinate dehydrogenase and D-lactate dehydrogenase are major source of $O_2^{\bullet-}$ in *E.coli* (59). Glutathione reductase is cytosolic enzyme thought to be capable of generating significant amounts of $O_2^{\bullet-}$, which uses NADH as an electron source. Enzymes such as xanthine oxidase, aldehyde oxidase and other flavin dehydrogenase are capable of generating superoxide as catalytic by-products (60). Cytochrome P-450s have been shown to be important sources of active oxygen species in mammalian tissues. In certain mammalian tissues, specifically the liver, P-450s can represent up to 4% of total cell protein (61). Autooxidation of several cellular components including ubiquinols, catechols, thiols and flavins have been shown to generate $O_2^{\bullet-}$. In plants, $O_2^{\bullet-}$ are commonly produced in illuminate chloroplasts by the occasional transfer of an electron from an exited chlorophyll molecule or PSI components under conditions of high NADPH/NADP ratio to molecular O_2 (56). Electrophilic quinone compounds both natural cellular constituents (such as ubiquinone) and exogenous sources (such as plumbagin and menadione) are easily reduced to semiquinones, which in turn readily reduced O_2 to $O_2^{\bullet-}$, regenerating the oxidized quinone. The oxidized quinone can undergo this cycle numerous times and is thus referred to as a redox- cycling agent. Paraquat (methyl viologen), plumbagin and menadione are also a very effective redox-cycling agent.

$O_2^{\bullet-}$ will oxidize thiols, ascorbate, tocopherol, catecholamine, and proteins containing an iron-cluster $(Fe\text{-}S)_4$. Most of dehydratases are highly sensitive to attacks by $O_2^{\bullet-}$. Probably the most important reactions of $O_2^{\bullet-}$ are its spontaneous dismutation to H_2O_2 and O_2, and its ability to reduce transition metals (in vivo: Fe^{3+} and Cu^{2+}) even if this metals are in complex form (cytochrome C). The reaction is as followed (60, 62):

$$O_2^{\bullet-} + M^{(n+1)} \rightarrow O_2^{\bullet-} + M^{n+}$$

Further interaction of these reduced transition metals with other ROS will give rise to very highly toxic ROS such as the hydroxyl radical ($OH^{\cdot}$). Spontaneous dismutation of $O_2^{\bullet-}$ in aqueous neutral pH environments produces H_2O_2. As mentioned

above, when $O_2^{\bullet-}$ is protonated, the hydroperoxy radical ($HOO^{\cdot}$) is produced. The $HOO^{\cdot}$ radical is much more reactive than $O_2^{\bullet-}$ because the negative charge has been neutralized.

4.2 Source of hydrogen peroxide (H_2O_2)

The major sources of H_2O_2 come from respiratory chain. The $O_2^{\bullet-}$ is detoxified by superoxide dismutase (SOD) or by two electron reduction of oxygen change to H_2O_2. The reaction of H_2O_2 with organic molecules is still unclear because it reacts very quickly with contaminating metals to form more ROS toxicity. H_2O_2 can act as a weak oxidizing agent and will attack thiol groups of proteins or reduced glutathione (63). Most importantly, H_2O_2 will react with Fe^{2+} or Cu^{+} ions to generate $HO^{\bullet}$ in the Fenton reaction, which take place only in the presence of catalytic amounts of iron salts (39).

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + H_2O + Fe^{3+}$$

The dismutation of $O_2^{\bullet-}$ generates H_2O_2. Then, the simultaneous presence of $O_2^{\bullet-}$ and H_2O_2 can result in the generation of the most potent oxidant $^{\bullet}OH$. Since $O_2^{\bullet-}$ will reduce both Fe^{3+} and Cu^{2+} and since its dismutation produces H_2O_2, it is likely that when the intracellular concentration of $O_2^{\bullet-}$ increases, the concentration of H_2O_2 and $^{\bullet}OH$ will also rise (64). Killing of *E. coli* by exogenous H_2O_2 revealed that *E. coli* was sensitive to both low concentrations of H_2O_2 and high concentrations of H_2O_2 but less sensitive to intermediate concentration (57). In mode-one killing, actively growing cells were sensitive to low dose of H_2O_2, particularly in mutants lacking enzymes required for recombination or base-excision DNA repair pathway. *In vitro* studies of Fenton reaction show that H_2O_2 at concentration causing mode-one killing could generate DNA strand brakes. Therefore, H_2O_2 may constitute a significant stress in vivo by generating DNA damage radicals such as hydroxyl or ferryl radicals ($^{\bullet}OH$ radical formed complex with irons) in the presence of metal ions (65).

4.3 Source of hydroxyl radicals (•OH)

Sources of Hydroxyl radicals ($^{\bullet}OH$) are numerous. $^{\bullet}OH$ are the most toxic ROS that can react with biomolecules such as DNA, RNA at diffusion-limited rates. The significant source of $^{\bullet}OH$ is the reaction of H_2O_2 with reduced metal ions in the Fenton reaction or with $O_2^{\bullet-}$ in the Haber-Weiss reaction. They are extremely reactive and probably react with and damage nearby molecules as soon as the radicals are generated (66). The overall reactions involved the generation of ROS are listed below.

1)	$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$	(Dismutation by SOD)
2)	$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$	
3)	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$	(Fenton reaction)
2) + 3)	$O_2^{\bullet-} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2$	(Haber-Weiss reaction)

$^{\bullet}OH$ is extremely reactive and cytotoxic in all macromolecules, resulted in damage to cellular components, DNA lesions, mutations, and often leading to irreparable metabolic dysfunction and cell death (67, 68). High reactivity of H_2O_2 property, the average diffusion distance of $^{\bullet}OH$ is only a few nanometers and thus its effect on biomolecules will depend on the location of its formation. Within the cell they will react with most biomolecules such as lipids, proteins and nucleic acids at diffusion-limited rates. The reactivity of $^{\bullet}OH$ is due its very high standard electrode potential. It will oxidize almost everything. Because of its reactivity, the average distance of reaction is only a few nanometers, thus its effects on any given biomolecule will depend largely upon the location of its formation. $^{\bullet}OH$ is also produced by ionizing irradiation occurring from the radiolysis of water (39).

4.4 Source of singlet oxygen ($^1O_2$)

The singlet oxygen is formed by oxidation of other partially reduced oxygen species, resulting in oxygen with paired elections in the reactive orbital (69). This chemical form of oxygen is not a true radical but is reported to be an important ROS in reactions related to ultraviolet exposition (UVA, 320-400 nm). Its toxicity is reinforced when appropriate photoexcitable compounds (sensitizers) are present with molecular oxygen. Several natural sensitizers are known to catalyzed oxidative reactions such as tetrapyrroles (bilirubin), flavins, chlorophyll, hemoproteins and reduced pyridine nucleotides (NADH). Some of these sensitizers are also found in foods and cosmetics. Some others are used for therapeutic purposes (anticancer treatments) and are sensitive to visible light. The presence of metals contributes to increase the production of singlet oxygen, as well as anion superoxide, and thus accelerates the oxidation of unsaturated lipids generating hydroperoxides. It has been suggested that singlet O_2 may be formed during the degradation of lipid peroxides and thus may cause the production of other peroxide molecules. This singlet O_2 formation may account for the chemiluminescence observed during lipid peroxidation.

4.5 Source of peroxyl radical

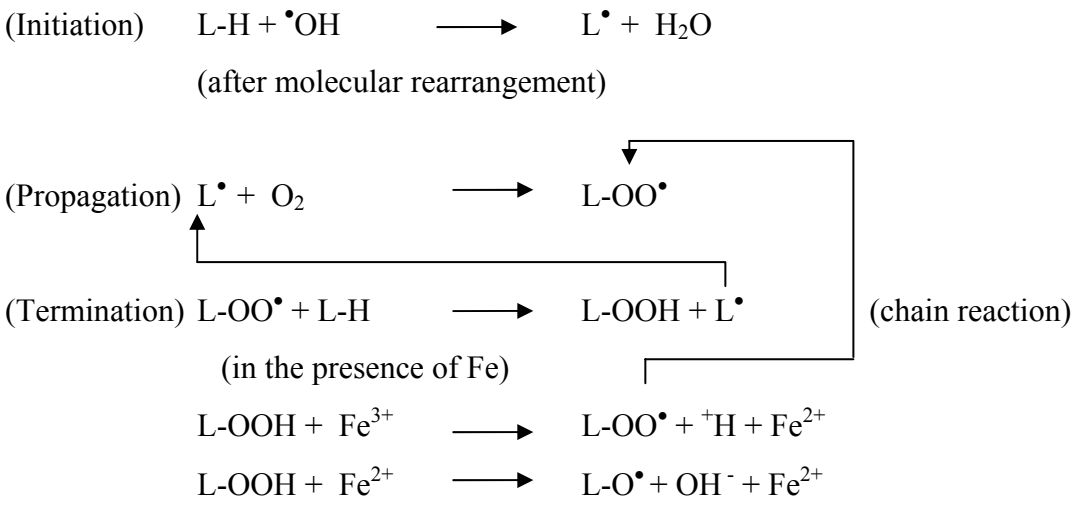
This radical occur during the oxidation of lipids or other organic molecules in oxidative stress. They are formed by addition of oxygen to alkyl radicals (carbon radicals). The peroxyl radical species, which are not very reactive, may diffuse a considerable distance. They have been shown to react with sulfhydryl group (thiols) to generate the thiyl radical (69).

5. Oxidative damages caused by ROS

All of the activated oxygen species are extremely reactive and cytotoxic in all organisms. The mechanisms by which oxygen radicals damage membrane lipids are well accepted, and consequently oxidative damage is often exclusively associated with these peroxidation reactions in membrane lipids. The activated forms of oxygen also degrade proteins and nucleic acids, reactions which can also be very lethal.

5.1 Membrane damage and lipid peroxidation

The reactions of oxygen free radicals with polyunsaturated lipids are the most frequently cited consequence of oxygen radical production in plant cells. Lipid peroxidation entails 3 steps: initiation, propagation and termination. Initiation of lipid peroxidation in a membrane or free fatty acid is due to the attack of any species that have sufficient reactivity to extract a hydrogen atom. A hydrogen atom has only one electron, attacks by $^{\bullet}OH$ will leave behind an unpaired electron on the carbon atom. This carbon radical in a polyunsaturated fatty acid tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which rapidly reacts with oxygen to give a lipid peroxyl radical ($LOO^{\bullet}$). The reaction is perpetuated when the lipid peroxyl radical attacks another unsaturated fatty acid and abstracts a hydrogen atom to form a fatty acid hydroperoxide (ROOH) or lipid peroxide (LOOH) and continue the chain reaction of lipid peroxidation (39). This can be summarized by the following chain reaction:



The hydroperoxides thus produced will break-down thermally or reduced transition metals to form lipid peroxy radicals ($LOO^{\bullet}$) or lipid alkoxy radicals ($LO^{\bullet}$), both of which can initiate new rounds of peroxidation. $LO^{\bullet}$ can undergo cleavage of C-C bonds to form unsaturated fatty acids, aldehyde, and alkyl radicals. When fatty acid chains shorten or gain charges, their ability to rotate within the membrane is altered and membrane becomes more fluid. An increase in membrane fluidity results directly in a loss of structural integrity (39). A second hazard caused by lipid peroxidation is that peroxidation intermediates and end products are mutagenic (70). But aerobic organisms have evolved detoxification mechanism including chemicals such as α-tocopherol and enzymes to deal with these end products.

5.2 DNA damage

The presence of oxidized DNA bases is often used as a marker for ROS-mediated DNA damage. Oxidized DNA bases are considered an important event in chemical carcinogenesis (57). ROS induce numerous lesions in DNA that cause deletions, mutations and other lethal genetic effects. Characterization of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein. Degradation of the base will produce numerous products, including 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened and -saturated products. In general, oxidized DNA exhibits an increased propensity for genetic mutations and alterations in transcription. These include effects on hydrogen bonding, a decreased fidelity of DNA and / or RNA polymerase, and conformational changes in the DNA template. By damaging DNA directly, interfering with DNA repair, affecting cell division or the promotion process or mediating the activation of carcinogen, ROS have the potential to modulate the development of cancer at several levels as well as to disrupt cell functions during non-carcinogenic toxicity events (57). $^{\bullet}OH$ makes a particularly important player in free-radical-mediated toxic processes. It can alter the purine and pyrimidine bases with the guanine base being oxidized by ROS produce to 8-hydroxydeoxyguanosine. Thymine residues in DNA can be hydroxylated to produce 5-hydroxymethyluracil or oxidatively degraded to produce thymine glycol. $^{\bullet}OH$ attacks on the C_4 of deoxyribose lead to

cleavage of the phosphodiester backbone. In addition, the interaction of $^{\bullet}OH$ with C_1 of deoxyribose results in loss of the base (39).

5.3 Protein damage

At the cellular level, when proteins are exposed to ROS, modifications of amino acid side chains occur and, consequently, the protein structure is altered. These modifications lead to functional changes that disturb cellular metabolism (71). ROS can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges. Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives. Reduction of both of these may be accomplished in microbial systems by thioredoxin and thioredoxin reductase (39). The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as Fe. In these cases, the metal binds to a divalent cation binding site on the protein. The metal then reacts with H_2O_2 in a Fenton reaction to form a $^{\bullet}OH$ that rapidly oxidizes an amino acid residue at cation binding site of the protein. It would appear to be generally true that metal-binding sites in proteins are especially sensitive to attack by ROS (66, 39 and 53).

5.4 Mutagenicity

Oxygen radicals cause DNA damage, and DNA repair-deficient mutants are hypersensitive to oxidative stress, it is not surprising that oxidative stress lead to mutagenesis (39). The 5-Hydroxycytosine has already been shown to be mutagenic in the phage system (72). 5-Hydroxyuracil and uracylglycol are both pre-mutagenic lesions, since they would pair with adenine in GC-AT transitions. All of these oxidized derivatives are substrates for *E.coli* endonuclease III.

6. Prevention of oxidative stress damages

6.1 Enzymatic antioxidant

6.1.1 Catalase (Kat)

Catalase (EC 1.11.1.6) or hydroperoxidase is a heme-containing enzyme that catalyses the dismutation of H_2O_2 into water and oxygen (Reaction 1) or oxidizes an intracellular reductant using H_2O_2 (Reaction 2) (73). Elimination of H_2O_2 may also reduce the potential for transition metal-mediated hydroxyl radical formation in the Fenton reaction.

$$2\,H_2O_2 \xrightarrow{\text{catalases}} 2\,H_2O + O_2 \qquad \text{Reaction 1}$$

$$AH_2 + H_2O_2 \xrightarrow{\text{peroxidase}} 2H_2O + A \qquad \text{Reaction 2}$$

Bacterial catalases can be classified into three different types: monofunctional catalases (HPII), bifunctional catalase-peroxidase (HPI) and non-heme catalases. Both catalase and peroxidase used H_2O_2 as a substrate, but catalase uses two electrons transfer in dismutation of H_2O_2 to oxygen and water, whereas peroxidase requires NADH or NADPH as electron source (39). The monofunctional catalase is encoded by *katE*. In *E.coli*, The levels of monofunctional catalases do not change during logarithmic growth but do increase sharply during growth into stationary phase by the effect of various extracellular metabolites produced by the cell during the exponential growth phase and caused by accumulation of weak acids such as acetate, propionate and benzoate. *kat*E is controlled by a stationary phase sigma factor, σ^s, encoded by *rpo*S gene (74). The bifunctional catalases exhibit organic peroxidase activity utilizing *o*-dianizidine as substrate. They have been found in broad range of bacteria including gram-positive (*Bacillus stearothermophilus* PerA), gram-negative (*E.coli* KatG, *Salmonella typhimurium* KatG, *Klebsiella pnemoniae* KatG) and phototropic bacteria; mycobacteria; halophilic archaebacteria and streptomycetes (75). Most commonly, the enzyme is a homotetramer of 80 kD subunits, but variants with both smaller and larger subunits and with homodimeric structures have been

found. The catalytic activity of bifunctional catalases is pH-dependent with a pH optimum at 6.0-6.5. They are more sensitive to pH, temperature, ethanol/chloroform, and H_2O_2 than the monofunctional catalases, but are insensitive to 3-amino-1,2,4-triazole (75). For Non-heme catalases, they originally referred to as pseudo-catalases, since they are insensitive to common catalase inhibitors, azide and cyanide. The common co-factor is Mn ion. This group is now classified as a distinct group of catalases (75).

6.1.2 Superoxide dismutase (SOD)

Superoxide dismutases (SOD, EC 1.15.1.1) are multimeric metalloenzymes that catalyze the dismutation of superoxide radicals to H_2O_2 and O_2. These enzymes are important in the maintenance of cells and DNA integrity during aerobic metabolism by eliminating superoxide radical, a reactive molecule that induces lipid damage and oxidative inactivation of essential enzymes. More importantly, SODs prevent the formation of the highly toxic hydroxyl radical, a mutagenic product of $O_2^{\bullet-}$ reduced iron and H_2O_2 (76). The two classes of SODs differing in metals found at their active sites: manganese or iron and copper/zinc. The best described bacterial SODs are the cytoplasmic MnSOD and FeSOD, encoded by *sod*A and *sod*B respectively. In *E.coli*, the FeSOD is synthesized constitutively and may be more important for protecting against cytoplasmic damage (76) whereas MnSOD synthesis is induced upon exposure to oxidative stress and may be more effective in preventing ROS dependent DNA damage (77). Six global regulators regulate the transcription of MnSOD. Fur (ferric uptake regulation), ArcA (aerobic respiration control), Fnr (Fumarate nitrate reductase), and IHF (integration host factor) (78, 76 and 60) have negative effects on MnSOD expression. Fur couples *sod*A expression to intracellular iron concentration, whereby high intracellular iron causes, Fur + Fe^{2+} to repress a number of genes involving iron uptake as well as *sod*A. ArcA whose effect on *sod*A is enhanced by IHF and Fnr, couples *sod*A to aerobic metabolism (76). The other two regulators, SoxRS, (superoxide response) and SoxQ, which activate MnSOD expression: SoxRS in response to an increase in superoxide radicals and SoxQ in response to unknown stimuli (79). Cu/ZnSOD, encoded by *sodC*, is localized in periplasm and accumulates only in stationary phase in *E.coli* (80, 81). Since $O_2^{\bullet-}$ is non-permeable, the existence of periplasmic Cu/ZnSOD may be

advantage in defending against $O_2^{\bullet-}$ that is generated either extracellularly or within the periplasm (81).

6.1.3 Glutathione reductase (GR)

Glutathione reductase (GR) (EC 1.6.4.2) is a typical member of flavoprotein disulfide oxidoreductase family. GR catalyzes the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by using NADPH as a reducing cofactor. The only exception is the enzyme from *Chromatium vinosum* which uses NADH (82, 83). GR usually exists as homodimer and this dimeric structure is required for catalytic activity. In *E. coli* and *Salmonella typhimurium*, the *gor* (encoded GR) gene is part of the *oxyR* regulon. Thus, its expression is inducible in response to oxidative stress (66). Alternatively, in the cyanobacterium *Nostoc muscorum* the presence of carbamate insecticide significantly stimulates GR activity (84). The explanation offered was that the use of GSH to detoxify the insecticide led to depletion of GSH pool and a consequent rise in H_2O_2 level causing the observed stimulation of GR activity.

6.1.4 Alkyl hydroperoxide reductase (AhpR)

Alkyl hydroperoxide reductase is the major microbial enzyme responsible for metabolizing organic hydroperoxides into corresponding alcohols and water in a pyridine nucleotide-dependent manner (10, 41).

$$NAD(P)H + H^+ + ROOH \longrightarrow NAD(P)^+ + ROH + H_2O$$

The alkyl hydroperoxide reductase system of *Salmonella typhimurium* is composed of two soluble components, the 57 kDa AhpF flavoprotein and the 21 kDa AhpC protein. The 57 kDa AhpF is a thioredoxin reductase-like pyridine nucleotide-dependent protein that contains two redox-active disulfide centers and one FAD per subunit. The DNA sequence of *ahpF* is shown to be highly homologous to *E.coli* thioredoxin and in particular region is similar to the region containing the redox active cysteine, the active site of other reductase (85, 86).

In *Salmonella typhimurium*, AhpF is a flavoprotein with two redox-active disulfide centers per subunit (Cys129 Cys132 and Cys345 Cys348), that are very important for catalysis of disulfide reductase activity. On the basis of working

with flavoprotein systems, electron transfer is expected to occur from NAD(P)H to FAD to one or both redox-active disulfide center(s) of AhpF then electron was transfer from AhpF to AhpC through thiol-disulfide interchange between the two proteins. Reduced AhpC, which contains two thiol groups per subunit, is proposed to catalyze peroxide reduction through a mechanism; a single cysteine thiolate (Cys-S) attacks the -O-O- bond, releasing ROH and forming a cysteine sulfenic acid (Cys-SOH) which is unstable and can react rapidly with the nascent cysteine thiol to generate the disulfide bond and release a molecule of water (87).

In *Salmonella typhimurium* Cys46 and Cys165 play important role in intersubunit disulfide bond in the peroxidatic AhpC protein of alkyl hydroperoxide reductase (AhpR) system. Cys 46 is clearly identify as the peroxidatic center of AhpC and Cys165 as an important residue for preserving the activity of wild-type AhpC by reacting with the nascent sulfenic acid of the oxidized protein (Cys46-SOH) to generate a stable disulfide bond, thus preventing further oxidation of Cys46-SOH by substrate (88). This enzyme will reduce mainly organic hydroperoxide in vitro, including cumene hydroperoxide and tert butyl hydroperoxide (88).

In addition, the other organic hydroperoxide including benzyl hydroperoxide, *p*-methanehydroperoxide (a mixture of secondary monohydroperoxides) and benzoyl hydroperoxide are also substrates. AhpR is known to be positively regulated by *oxyR* locus which has been confirmed by the DNA sequence of the *oxyR* regulated promoter upstream of the *ahpC* gene (89). This promoter regulates the levels of *ahpC* and *ahpF* gene products that comprise the hydroperoxide reductase activity.

In *Xanthomonas*, alkyl hydroperoxide reductase also has two subunits, a catalytic AhpC (a 22 kDa protein) and a reductase AhpF (a 57 kDa protein). AhpC belongs to the highly conserved family of AhpC/TSA proteins involved in reduction of highly toxic organic hydroperoxide to corresponding alcohols (12). AhpF shares homology to other thioredoxin reductase enzymes, and its main function is to regenerate AhpC (86). The regulation of alkyl hydroperoxide reductase in *Xanthomonas* is quite different from other microorganisms. The genes *ahpC, ahpF, oxyR* and *orfX* are arranged in a head to tail fashion. *ahpC* was transcribed as a

monocistronic, whereas *ahpF*, *oxyR* and *orfX* arranged in an operon. In *E.coli* and *Bacillus subtilis*, *ahpC* and *ahpF* are coregulated and arranged in an operon.

6.1.5 Organic hydroperoxide resistance (Ohr)

Organic hydroperoxide resistance, *ohr*, was firstly found in *X. campertris* pv *phaseoli* which showed to be involved in resistance to organic hydroperoxide. Expression of *ohr* was strongly increased when exposed to organic hydroperoxide not other oxidants or stresses. *ohr* is widely distributed among diverse groups of gram-positive and gram-negative bacteria (16). For example, *Bacillus subtilis* contains two linked *ohr* homologues: *ohrA* and *ohrB*. Expression of *ohrB* is controlled by σ^B, the general stress response σ factor. Like other general stress response genes, *ohrB* is induced by heat shock, entry into stationary phase and various oxidants and organic alcohols (90). In contrast, *ohrA* is selectively induced by organic peroxides (19). Regulation of *ohrA* and also ohr in *X. campertris* pv. *phaseoli* are regulated by an organic hydroperoxide resistance regulator, *ohrR*, a novel transcription regulator (91). A crystal structure of Ohr from *Pseudomonas aeruginosa* has been solved. A dimeric Ohr has a peroxidase function which has a novel oval-shaped structure and lacks significant structure similarity to other known peroxidases. Structural study of Ohr also reveals that the preferential metabolism of organic hydroperoxide over inorganic H_2O_2 is probably due to the fact that the entrance to each active site is surrounded mostly by hydrophobic side chains (92). Study in biochemistry of Ohr in *Xylella fastiodiosa* revealed that two conserved Cys play a role in function of this protein. Cys61 is directly involved on peroxide reduction whereas Cys125 is the resolving cysteine. At low concentration of organic hydroperoxide, Cys61 could be oxidized to sulfenic acid, which should be rapidly converted to the intra-molecular disulfide intermediate. Therefore, Ohr is a thiol-dependent that approximately consumed two thiol equivalents per peroxide removed to a less toxic compound, organic alcohol (21), indicating that Ohr catalyzes the following reaction:

$$2RSH + ROOH \longrightarrow RSSH + ROH + H_2O$$

Although, many organisms have both AhpC and Ohr which can used organic hydroperoxide as a substrate. The *ohr* gene product was demonstrated to contribute the decomposition of organic hydroperoxides (18, 93). Analysis of Ohr primary structure shows that it has homology to proteins from both Gram-positive and Gram-negative bacteria, and that it has moderate homology to an osmotically inducible protein (OsmC) from *Escherichia coli*, which has been shown to localize in periplasmid fraction (94).

6.2 Non-enzymatic antioxidants

6.2.1 Glutathione

The tripeptide glutathione (L-δ-glutamyl-L-cysteinyl-glycine; GSH) is the predominant low molecular weight thiol in many organisms and has been proposed to protect biomolecules against free reactive radical species. GSH is synthesized by two ATP-dependent steps catalyzed by g-glutamylcysteine synthethase (*gshA*) and glutathione synthetase (*gshB*). The high steady-state levels of GSH in cells maintain a strong reducing environment. As a radical scavenger, GSH will react with H_2O_2, $O_2^{\bullet-}$, $HOO^{\bullet}$ or $R^{\bullet}$ ($^{\bullet}OH$) and other free radical site in protein and DNA generated by cascade reaction) to form a stable glutathione radical ($GS^{\bullet}$) which can be dimerized to form oxidized glutathione (GSSG). Finally, glutathione reductase enzyme will transfer electrons from NADPH to the GSSG to regenerate the reduced GSH as shown in reactions below (39).

$$GSH + HOO^{\bullet} \longrightarrow GS^{\bullet} + H_2O_2$$

$$GS^{\bullet} + GS^{\bullet} \longrightarrow GSSG$$

$$GSSG + 2NADPH \rightarrow 2GSH + 2NADP^{+}$$

Another role of GSH involves the formation of glutathine-S-conjugate or mixed disulfide. GSH can react with a variety of compounds containing electrophilic centers. The reaction is catalyzed by the glutathione-S-transferase (82). Moreover, the role of GSH on oxidative damage protection in *E. coli* is still controversial since the *gshA* mutant was found to exhibit normal resistance to exogenous oxidant such as H_2O_2, CuOOH. Such mutants only showed high sensitivity

to the electrophile NEM (95). Similar results were reported in a GR-deficient mutant *E.coli* (strain SG5) which shows no increased sensitivity to paraquat. In addition, over expression of GR in *E. coli* did not lead to any increased tolerance to oxidative stress induce by paraquat (96, 97).

6.2.2 Dps (DNA binding protein)

The expression of Dps, a 19 kDa DNA binding protein from starved cells, is critical to the ability of stationary phase *E. coli* cells to survive oxidative stresses (95). *E. coli* Dps protects DNA from cleavage caused by ROS. While 3-day-old cultures of wild type cells are resistant to high dose of H_2O_2 (45 mM), the mutant strains lacking Dps are killed rapidly. Dps forms stable complexes by binding with DNA without apparent sequence specificity. Dps function is to protect DNA against oxidative damage especially H_2O_2. Additionally, Dps was found to aid the survival of both exponential- and stationary phase cells against thiol depleting agent NEM. The expression of *dps* in log phase is controlled by a global peroxide activator OxyR, whereas the induction of *dps* expression at the onset of stationary phase is σ^S-dependent and requires the function of Dps (99, 100). *A. tumefaciens* Dps reduces significantly the production of hydroxyl radicals generated by the Fenton reaction due to its capacity to bind Fe(II). However, the protective effect against oxidative damage does not appear to depend on the formation of a Dps-DNA complex as it is of very similar magnitude in *A. tumefaciens* and *E. coli* Dps, although the former protein does not interact with DNA (101).

7. Transcriptional regulators of the oxidative stress response in prokaryotes

Multiple defenses are known to protect aerobic organisms form toxic reactive oxygen species produced by aerobic metabolisms. Such defenses can be adjusted in response to imbalances between the production and disposal of toxic oxygen radicals. *E. coli* has independent multigene responses to two kinds of oxidative stress: excess H_2O_2 triggers the *oxyR* regulon, and excess $O_2^{\bullet-}$ or nitric oxide ($NO^{\cdot}$) radicals triggers the *soxRS* regulon which coordinates the transcriptional induction of at least 12 promoters (102). In addition to OxyR and SoxRS transcription factor the *rpoS*-encode σ^S subunit of RNA polymerase and several SoxS homologs such as MarA and Rob have also recently been shown to regulate the expression of antioxidant defense genes (103). The important transcription regulators in response to oxidative stress are summarized in the Table 1.

Table 1 Transcriptional regulator of gene involved in the defense against oxidative stress in *E. coli* (103)

Regulator	Inducer*	Redox-center	Regulated defense gene
OxyR	H_2O_2	Cysteine	*katG, ahpCF, dps, gorA*
SoxR	Superoxide	FeS cluster	*soxS*
SoxS	SoxR		*sodA, nfo, zwf, fumC, micF*
RpoS (σ^S)	Stationary phase		*katE, xthA, dps, katG*
ArcA	Anaerobiosis via ArcB		*sodA*
Fnr	Anaerobiosis	Fe	*sodA*
Fur	Iron	Fe	*sodA*

*It is not known whether these inducers directly affect the activity of regulatory proteins.

7.1 The *oxyR* regulon

Many of H_2O_2 inducible gene expression is regulated by the OxyR transcription factor, including hydroperoxidase I (catalase, *katG*) and alkyl hydroperoxide reductase (*ahpCF*) which protect against the toxic effects of peroxide by directly eliminating the oxidant (104), glutathione reductase (*gorA*), glutaredoxin 1 (*grxA*) and thioredoxin 2 (*trxC*) should help maintain the cellular thiol-disulfide balance (104-106). OxyR induction of *fur,* which encodes a global repressor of ferric ion uptake, should prevent damage caused by $HO^{\bullet}$ generated by H_2O_2 reacting with intracellular iron (the Fenton reaction) (107). OxyR also induces the expression of the nonspecific DNA binding protein Dps, which protects against DNA damage and mutation (99, 108). Dps has been proposed to act by sequestering DNA since studies have shown that Dps forms an extensive crystalline lattice in the presence of DNA *in vitro* and *in vivo* (109). Dps may also provide protection by sequestering iron since the crystal structure of Dps revealed that the protein is a ferritin homologue (110). OxyR activation also leads to high levels of a small RNA denoted OxyS (111). This unique RNA protects against mutagenesis, although the mechanism by which OxyS acts as an antimutator is unknown. The OxyS RNA also activates and represses the expression of numerous genes in *E. coli*.

The *oxyR* gene encodes a 34.4-kDa protein that is a member of the LysR family (112, 113). As is the most LysR members, OxyR negatively autoregulates its own expression, both in presence or absence of H_2O_2 (112, 114). Purified OxyR exists in solution as a tetramer (115) and belongs to the class I activators that interact with the carboxyl-terminal region of the α-subunit of RNA polymerase to promote transcription activation. OxyR acts cooperatively to increase binding of σ^{70}-containing RNA polymerase to the *katG* and *oxyS* promoters, presumably through direct contact with the α-subunit (115, 116).

In *E. coli* OxyR synthesis occurs even in the absence of H_2O_2 treatment, and there is no increased synthesis upon addition of H_2O_2 (65). This indicates that H_2O_2 activation of OxyR regulon is result of modification of preexisting OxyR (65). Direct redox sensing by OxyR was demonstrated by in vitro transcription assays, which showed that addition of 100 mM DTT prevented OxyR from activating transcription of the *katG* and *ahpC* genes, whereas removal of DTT restored its activity (104, 65,

117). DNA footprint analyses also indicated that oxidized OxyR binds to *katG*, *ahpC*, *gorA*, and *dps* promoters, but that reduced OxyR does not (118). Interestingly, both oxidized and reduced OxyR can bind to *oxyR-oxyS* promoter region, but with distinct differences in their binding characteristics (118). When binding to target promoters in an oxidized state, OxyR produces a large footprint (>45 nucleotides) that is typical for members of the LysR family. Hydroxyl radical protection experiments have refined the region of DNA-binding and shown that oxidized OxyR recognizes four ATAGnt elements in four adjacent major grooves and binds on one face of the DNA helix. However, when OxyR is reduced, the footprint pattern to the *oxyR-oxyS* promoter is significantly smaller, encompassing only two major grooves that are separated by one helical turn (118). Beside that, the reduced but not oxidized OxyR also promotes a significant bend in the helix axis (118). All of these studies indicate that OxyR must undergo conformational change upon an alteration in its oxidation-reduction state.

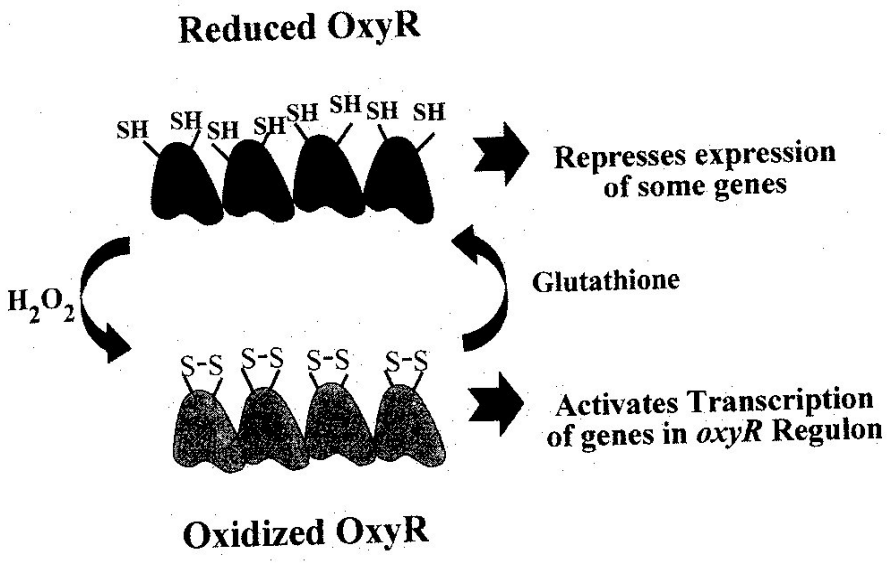


Fig. 10 Oxidation and reduction of OxyR (41).

Fig. 10 showed the mechanism of OxyR activation and its deactivation. The conversion of OxyR from reduced to oxidized form upon the presence of oxidative stress is reversible. Several analyses determined that OxyR did contain neither any metals nor other prosthetic group that could function as a redox active center (106, 65). In vitro analysis demonstrated that mutations in four of the six conserved cysteine residues had no effect on OxyR-dependent oxidative defense (106, 115). However, a

mutation of Cys199 resulted in cells that were as hypersensitive to H_2O_2 killing as were strain-lacking *oxyR*. A mutation in Cys208 was also shown to be partially defective in oxidative resistance (115). This led to a model in which Cys199 is first reacted with H_2O_2 to form a sulfenic acid intermediate (Cys-SOH) that is required for DNA-binding and subsequently reacts with Cys208 to form a stable disulfide bond locking OxyR in an active form (115). More recent MALDI-TOF mass spectrometry analyses of tryptic fragments derived from oxidized OxyR indicated that oxidation of OxyR results in the formation of an intramolecular disulfide bond between Cys199 and Cys208 (106). Presumably, formation of a disulfide bond stabilizes a conformational change that affect DNA-binding. It has also been suggested that the reason why the Cys208 mutation shows little effect on OxyR activity in vivo is that mutation may promote intermolecular disulfide bond formation between two OxyR subunits at Cys199. This could cause a conformational change that partially activates DNA-binding of the Cys208 mutant protein (119).

Zheng et al (106) also used genetic and biochemical analysis to demonstrate that reduction of oxidized OxyR is catalyzed by glutaredoxin1, The redox potential of OxyR was to be –185mV, a value that ensures OxyR is in the thiol form in the reducing environment of the cell (thiol-disulfide redox potential –280mV). Thus direct oxidation of OxyR leading to the disulfide bond formation is the mechanism whereby cells sense H_2O_2 and induce the *oxyR* regulon.

The finding that OxyR was only activated or oxidized for a defined time raised the question as to how the protein was reduced (106). In *E. coli*, the two major disulfide-reduction systems are present in cytosol: which are thioredoxin together with thioredoxin reductase and the tripeptide glutathione (GSH) together with glutathione reductase and the glutaredoxin proteins. Zheng et al (106) also used genetic and biochemical analysis to demonstrate that OxyR regulated responses was found to be prolonged in mutants lacking GSH or glutaredoxin1 but not in mutants lacking thioredoxin or thioredoxin reductase. In vitro transcription assays show that glutaredoxin1 catalyzes the reduction of OxyR by GSH. Using defined concentrations of GSH/GSSG, the redox potential of a reducing the disulfide bond in OxyR has been estimated as –185 mV, which is about 90mV higher the estimated –280 mV potential

of the normal *E. coli* cytosol (106). This provides a basis for explaining why OxyR remains in a reduced state in *E. coli* when it has not been challenged with oxidizing agents. Recently in vivo studies demonstrate that OxyR can be directly activated by H_2O_2 in *E.coli* wild type cells. In the mutants deficient in cellular disulfide-reducing systems, OxyR is oxidized even in the absence of added oxidant because of the change in cellular redox status (120).

These results indicate that reduction of oxidized OxyR is catalyzed by glutaredoxin1in the cell. Since the gene encoding glutaredoxin1 is *grxA*, is itself regulated by OxyR as well as *gorA* that encodes glutathione reductase (65, 105). So oxidized OxyR also activates expression of enzymes required for its in vivo reduction and inactivation, the OxyR response is autoregulated.

Unlike other bacteria *Xanthomonas* OxyR functions as a redox-sensing transcription activator or repressor. It is suggested that *Xanthomonas* OxyR could act as both a peroxide sensor and a transcription activator of its own gene. Exposure to oxidants not only changed the redox state of OxyR, but also increased its cellular concentration (121). Studies on *oxyR* regulated gene, in *Xanthomonas ahpC* has a unique pattern of regulation. Its expression can be increase in response to oxidants in an *oxyR*-dependent fashion (121, 55). Moreover, DNA footprint analyses also indicated that both oxidized and reduced OxyR bind to *ahpC* promoter and affects expression of the gene. The high levels of reduced OxyR lead to repression of *ahpC*, while oxidized OxyR activates expression of *ahpC* (55, 15).

7.2 The *soxRS* regulon

The SoxRS regulon is a key regulator of the adaptive response to superoxide in *E. coli*. When *E. coli* are stressed under elevated levels of the superoxide radical for example, treating cells with superoxide generators such as paraquat or menadione (these agents mediate transfer of electrons from NADH or NADPH to O_2, generating a flux of $O_2^{\bullet-}$ in redox cycling process) result in induction of more than 30 proteins (4, 122 and 123). At least 10 of these $O_2^{\bullet-}$ inducible proteins are encoded by *soxRS*-regulated genes including manganese-containing superoxide dismutase (MnSOD, encoded by *sodA*), the DNA repair enzyme endonuclease IV *(nfo*), glucose-6-phosphate dehydrogenase (*zwf*), fumarase C (*fumC*), NADPH: ferredoxin oxidoreductase (*fpr*), aconitase (*acn*), and a *micF* antisense RNA of *ompF* (outer membrane protein) (122, 123).

The activation of gene expression in this regulon requires two polypeptides of 17 and 13 kDa which were assigned as SoxR and SoxS, respectively. Study in *E.coli*, the *soxRS* locus is organized as two head to head fashion and both genes are transcribed convergently (112, 124). *In vitro* transcription experiments show that transcription of the *soxR* gene initiates in the intergenic region and is itself repressed by SoxR protein. The structure of the overlapping promoters is such that the single SoxR-binding site is located in the -10/-35 spacer of the *soxS* promoter, but just downstream of the -10 element of the *soxR* promoter. Activated and non-activated SoxR bind this site equally well, exerting nearly constant repression of *soxR*; activated SoxR simultaneously stimulates the *soxS* promoter greater than 30-fold. The functional *soxR* promoter depresses *soxS* transcription when SoxR is not activated and enhances *soxS* transcription when SoxR is activated. SoxR is a homodimer of 17 kDa subunits, each of which contains a [2Fe-2S] cluster.. The SoxR FeS centers are not required for DNA binding, but are essential for promoting open-complex formation by RNA polymerase and triggering the expression of *soxS* gene (125-127).

Furthermore, the *soxR* promoter lies within the *soxS* gene. The mechanism by which the two proteins activate transcription of the SoxRS regulon genes is not known. One possibility that the activation involves an increase in the amount of these proteins has been suggested by the observation the *soxS* gene in inducible by paraquat. Since *soxR* is normally expressed at barely detectable levels, the expression of *soxR*

may also be inducible by $O_2^{\bullet-}$ stress. The functional *soxR* promoter depresses *soxS* transcription when SoxR is not activated and enhance *soxS* transcription when SoxR is activated (39, 127).

SoxR is post-translationally activated in response to superoxide or nitric oxide stress. The activation of SoxR triggering by oxidative stress is via the oxidation state of [2Fe-2S] centers (126). In the absence of oxidative stress, the [2Fe-2S] centers are in the reduced form and the protein is inactive, although it still binds the soxS promoter. Agents that generate superoxide in the cell (e.g. paraquat) cause rapid oxidation of the metal centers, which triggers the transcriptional activity of SoxR; removal of the oxidative stress is followed by rapid re-reduction of the [2Fe-2S] centers (128). Thus, the regulation in this regulon is a two-stage control system in which the SoxR iron-sulfur protein senses exposure to superoxide and nitric oxide, and then activates transcription of the *soxS* gene, whose product stimulates the expression of the regulon genes. Interestingly, SoxS was also negatively autoregulated its own expression. SoxS protein activates the promoters of the *soxRS* regulon by a mechanism that involves binding near or at the -35 region. Purified SoxS has been shown to bind to several SoxS-regulated promoters and the consensus sequence proposed for SoxS box is AnnGCAY (128). Recent finding indicates that *soxS* transcription is initiated in a manner dependent on the *rpoS* gene encoding RNA polymerase sigma factor (129).

Detoxification of free radical, especially $O_2^{\bullet-}$, is very important because $O_2^{\bullet-}$ can be converted into much more reactive compounds. This is carried out by SOD, high-capacity enzymes which converted $O_2^{\bullet-}$ to H_2O_2. Subsequently, H_2O_2 is reduced to water by the selenocysteine-containing glutathione peroxidase (GPO) or by catalase (130).

The microbial oxidative stress response is a well-orchestrated set of reactions involving synthesis of many proteins and small molecules. The components of these responses can be broadly divided into three categories namely detoxification of ROS, repair of damaged macromolecules and regulation of the process. Both enzymes and small molecules are involved in detoxification of ROS that are SOD breakdown $O_2^{\bullet-}$ to H_2O_2. H_2O_2 is then broken down by both monofunctional and bifunctional catalases to water (41, 38). Other proteins do not have enzymatic activity

but they can bind to macromolecules and protect them from ROS such as non specific DNA binding protein (131).

7.3 The *rpoS* regulon

Stationary phase cells are constitutively resistant to a variety stress conditions including exposure to high concentrations of H_2O_2, organic hydroperoxide or superoxide (132). In *E. coli*, the expression of genes responsible for this increase resistance is controlled by a stationary phase-specific sigma factor σ^S or σ^{38}. This alternative sigma factor encoded by *rpoS,* formerly named *katF*, controls the expression of a large number of genes involved in cellular responses to a diverse number of stresses, including starvation, osmotic stress, acid shock, cold shock, heat shock, oxidative DNA damage, and transition to stationary phase. A list of over 50 genes whose expression is regulated by σ^S has been compiled. Among these genes are *katE* (HPII monofuctional catalase), *katG* (catalase-peroxidase HPI), *gor* (glutathione reductase), *dps* (DNA binding protein, *rob* (DNA binding protein), *xth* (exonuclease III), all of which are important for resistance to oxidative stresses. Although the transcription factor σ^S acts predominantly as a positive effector, it does have a negative effect on some genes (133). The sequences of various σ^S -dependent promoters have been compared revealing a possible consensus sequence in the -10 region of CTATACT (134). The synthesis and accumulation of σ^S are controlled by mechanisms affecting transcription, translation, proteolysis, and the formation of the holoenzyme complex. The schematic depicting the modulators of σ^S activity is shown in Fig. 11 (133).

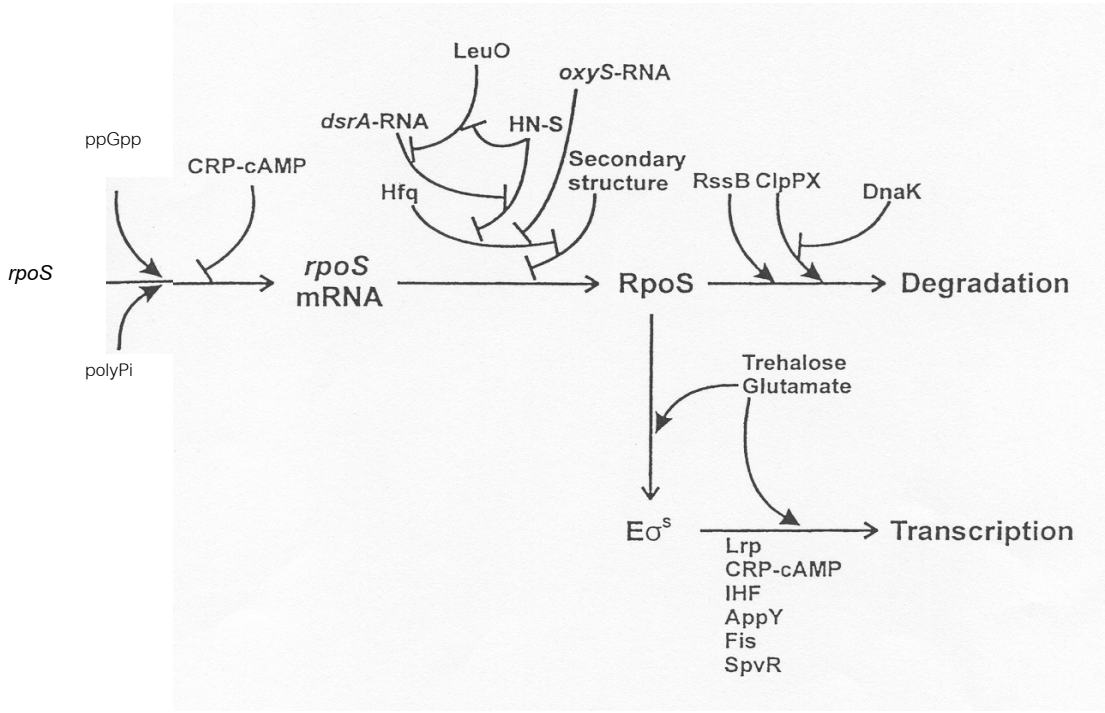


Fig. 11 Schematic depicting the modulation of σ^S activity (133). Lines leading from an effector with arrowheads indicate an activating role or positive effect on the process indicated. Line leading from an effector with a bar at the end indicate inhibiting role or negative effect on the process indicated.

7.4 The *perR* regulon

Bacillus subtilis displays an inducible protective response to challenge with H_2O_2. Treatment of growing cells with sublethal concentrations of H_2O_2 induces the expression of *katA* (vegetative catalase), *ahpCF* (alkyl hydroperoxide reductase), *mrgA* (encodes dps or PexB) and *hemAXCDBL* (heme biosynthesis) (135). The induction of these genes can be repressed by high concentrations of metal ions especially Mn^{2+} and Fe^{2+}. In addition to H_2O_2 induction of exponential phase cells, the expression of this regulon is inducible when cells enter stationary phase. DNA sequence comparisons of the putative promoter regions indicate that the genes of the peroxide regulon contain related operator sites. The *katA* and *mrgA* promoters have very similar Per boxes of 15 bp upstream of their -35 region. The putative consensus sequence of the Per box is CTAt-TTAtAAT-ATTATAAattA. Computer-based DNA sequence analysis identified two similar Per boxes for the heme biosynthesis operon (135) and 3 possible Per boxes at the -10 region of ahpC promoter (13). PerR, the putative peroxide regulon repressor, was postulated to bind these operator Per boxes. Under condition of oxidative stress created either by addition of H_2O_2 to growing cells, or by the elevated activity of electron transport chain accompanying the transition to post-exponential phase. PerR may dissociate from its operator leading to gene induction (13).

The 21-kDa PerR protein has a helix-turn-helix DNA binding motif in its N-terminal region and contains two CXXC motifs in the C-terminal putative metal-binding domain. This regulator also contains two metal binding sites per monomer: one site binds Zn(II) and may a largely structural role, whereas the second site binds a regulatory metal as shown in Fig. 12. The active form of PerR binds to its DNA site containing one Zn and one Fe per monomer. The ability of PerR to sense peroxide stress appears to be affected by the metal cofactor: PerR-Fe dissociates more readily from target operators than does PerR-Mn following exposure to H_2O_2 (20).

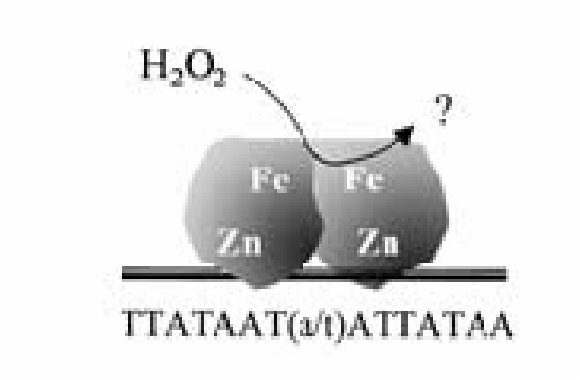


Fig. 12 PerR binding site (20)

Mutant strains de-repressing transcription of *mrgA* in the pesence of high Mn^{2+} ions were selected. The mutants that possess high catalase, AhpCF, and Dps levels were postulated to be defective in PerR repressor. These mutants have recently been analyzed and found that all mutants contain nucleotide changes in *ygaG* gene (136). Since *perR* phenotype of these mutants can be complemented by the *ygaG* gene, Bsat et al. conclude that *ygaG* encoded PerR. The putative amino acid sequence of *ygaG* gene product is homologous to *E.coli* Fur (Ferric uptake repressor) the protein that requires Fe as co-repressor, therefore, PerR is a Fur-homolog. Like other Fur proteins, PerR requires divalent metal ions such as Mn^{2+} or Fe^{2+} to activate DNA binding. The bound metal ions may play a key role in peroxide sensing, either by metal-catalyzed oxidation reaction that could damage PerR or by changing in the oxidation state of the bound metal ions (136).

7.5 The *ohrR* regulon

A novel transcription regulator that regulates the expression of the organic hydroperoxide resistance gene, *ohr*, was identified in *X. campestris* and called *ohrR*. *ohrR* is located upstream of and formed an operon with *ohr*. The high level expression of *ohrR* resulted in the repression of *ohr* expression, therefore, *ohrR* act as a negative transcription regulator. *ohrR* mRNA was found as a bicistronic message with *ohr*, while *ohr* mRNA was found in both bicistronic and monocistronic. *ohrR* is widely distributed in both gram positive and gram negative bacteria. Exposure to organic hydroperoxides both tBOOH and CuOOH can cause a derepression of OhrR resulting in the expression of Ohr (16). The highly conserved cysteine C22 of OhrR is required for organic peroxide inducible gene expression (148).

B. subtilis contains two linked *ohr* homologues: *ohrA* and *ohrB*. Regulation of *ohrA* is mediated by OhrR, encoded by a convergent gene, while ohrB is regulated by σ factor. OhrR binds to a pair of inverted repeat sequences overlapping the *ohrA* promoter site and thereby blocks transcription initiation (19). Insights into the mechanism of peroxide sensing by OhrR have emerged from recent genetic and biochemical studies. OhrR and related proteins in other bacteria have a single, conserved cysteine residue. In *B. subtilis*, mutant strains expressing OhrR proteins with either a Cys15Ser or a Cys15Ala substitution are unable to induce *ohrA* expression in response to organic peroxides. *In vitro*, purified OhrR protein binds tightly to its cognate operator site, and binding is abolished by treatment with peroxides but can be restored by the thiol reductant, dithiothreitol. These results suggest that peroxide sensing requires oxidation of Cys15. Unlike OxyR or SoxR, *in vitro* oxidation of OhrR does not lead to disulfide bond formation and, instead, leads to the formation of a Cys-SOH as shown in Fig. 13.

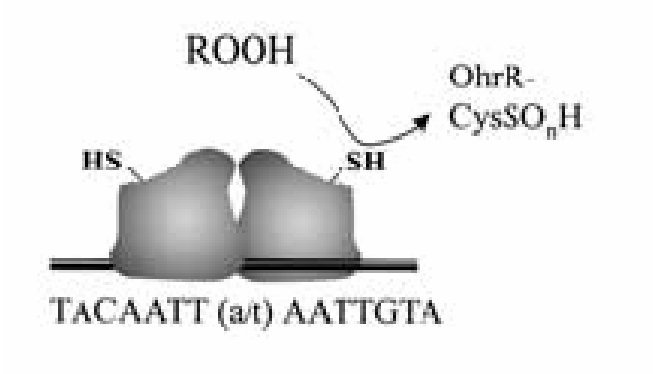


Fig. 13 The responding to organic hydroperoxide of OhrR (20)

The OhrR protein is shown bound on its DNA targets as defined for *B. subtillis*. Oxidation of OhrR upon exposure to organic peroxides leads to oxidation of the single cysteine residue (Cys15), leading to the formation of a Cys–SOH (and Cys–SO2H) derivative and a concomitant loss of DNA-binding activity. Biochemical experiments using both chemical modification and mass spectrometry have demonstrated that oxidized OhrR contains a Cys-SOH (and further oxidized) residue *in vitro*, and the formation of these derivatives is correlated with loss of DNA-binding activity (137). The *ohr* system is conserved in a variety of Gram-negative and some Gram-positive genomes. In addition to *X. campestris* and *B. subtilis*, *ohr* homologues been identified in *Pseudomonas aeruginosa* (18) and *Enterococcus faecalis* (138).

CHAPTER III
MATERIALS AND METHODS

MATERIALS

1. Bacterial Strains and Plasmids

All bacterial strains and their genotypes are listed in Table 2. Plasmid vectors and recombinant plasmids used and constructed in this study are summarized in Table 3.

2. Chemicals and Media

All chemicals used in this study were molecular or analytical grade and purchased from Sigma (USA), Fluka (Switzerland), Merck (Germany), or BDH (UK). All restriction endonuclease and DNA-modifying enzymes were purchased from Promega (USA), BRL (USA), or New England Biolabs (USA). Antibiotics were purchased from Sigma (USA).

All culture media were purchased from Difco (USA), GibcoBRL (UK), or Oxiods (UK). The preparation was performed according to manufacturer' recommendations.

Table 2. List of bacterial strains.

Bacterial strain	Genotype and Phenotype	Source
X. campestris pv. *phaseoli* (*Xp*)	Wild-type, Rif^R, Km^S, Gm^S, Tet^S	Laboratory stock
Xp ohr	*ohr* mutated strain, Tet^R	(16)
Xp ahpC	*ahpC* mutated strain, Km^R	(146)
Xp ahpC-ohr	*ahpC* and *ohr* mutated strain, Km^R, Tet^R	This study
Xp ahpC-ohr/ pOhr	*ahpC* and *ohr* mutated strain, Km^R, Tet^R harbouring *ohr* in pUFR047	This study
Xp ahpC-ohr/ pAhpCF	*ahpC* and *ohr* mutated strain, Km^R, Tet^R harbouring *ahpC* and *ahpF* in pUFR047	This study
Xp TnCP1	Wild-type harbouring *ahpC* promoter integrated into chromosome, Km^R	(15)
Xp oxyR TnCP1	*oxyR* mutated strain harbouring *ahpC* promoter integrated into chromosome, Km^R, Gm^R	(15) (148)
Xp ohrR P1lacZ	*ohrR* mutated strain harbouring *ohrR* promoter integrated into chromosome, Tet^R, Km^R	(148)
Xp ohrR P1lacZ/ pOhrR	*ohrR* mutated strain harbouring *ohrR* promoter integrated into chromosome, Tet^R, Km^R and *ohrR* in pBBR1MCS-5	(148)

Table 3. List of plasmids

Plasmids	**Genotypes or characteristics**	**Source**
pUFR047	Ap^R, Gm^R	(150)
pAhpCF	*ahpC* in pUFR047, Ap^R, Gm^R,	Laboratory stock
pOhr	*ohr* in pUFR047, Ap^R, Gm^R,	Laboratory stock
pOPRX-1	*ohr* in pKS, Ap^R,	(151)
pOhrR	*ohrR* in pBBR1MCS-5, Gm^R	Laboratory stock
pOhrRC22S	*ohrR* C22S in pBBR1MCS-5, Gm^R	Laboratory stock

Table 4. List of primers and nucleotide sequences

Primers	Gene	Sequences
#133	*ahpC* (150 bp)	5’GGATCCATGTCTCTTAT 3’
CS2		5’GGTCGGGCTGTTGAAGG 3’
M13R	*ohrR* (170 bp)	5’GAAACAGCTATGACCATG 3’
BT18		5’ACAACGCAAAGGACAGCTG 3’

METHODS

1. Bacterial growth and maintenance

All *Xanthomonas* strains were cultured aerobically in modified Silva-Buddenhagen (SB) medium (0.5% yeast extract, 0.5% peptone, 0.5% sucrose and 0.1% glutamic acid pH 7.0). The cultures were incubated at 28°C with continuous shaking at 200 rpm. For long term storage of bacterial strains, the log-phase bacterial cultures in an appropriate medium were mixed with 15 % final concentration of sterile glycerol and the aliquots were stored at -70°C. For routine cultivation of bacteria, freshly streaked cells were used as a starting inoculum for *Xanthomonas spp.* broth cultures. This ensured rapid and more synchronous growth.

2. DNA extraction

2.1 Plasmid extraction

Small scale isolation of plasmid DNA use QIAprep® Minipreps (Qiagen)

Isolation of plasmid DNA was performed by the QIAprep® Minipreps (Qiagen) which used to purify a low copy of plasmid. This preparation method was modified from the alkaline lysis method of Birnboim and Doly (139) in that plasmid DNA was prepared from small amounts of many different cultures of plasmid containing bacteria. Bacteria were lyzed by treatment with a solution containing sodium dodecyl sulfate (SDS) and NaOH. SDS denatured bacterial proteins and NaOH denatured chromosomal DNA and plasmid. The mixture was neutralized with potassium acetate, causing the plasmid DNA to reanneal rapidly. The reannealed plasmid DNA was then separated from other residues by use of the specific column to bind plasmid DNA and eluted from the column using buffer or steriled distill water pH 7.0-8.5.

A single bacterial colony was inoculated in 5 ml broth containing 50 μg/ml of amplicilin or other appropriate antibiotic and grown to saturation overnight. The 1.5 ml bacterial cells were spun at 10,000 rpm for 1 min in a microcentrifuge tube at room temperature. The harvested bacterial cells were resuspended in 250 μl of P1 buffer, a resuspend buffer which has 100 μg/ml RNase A. The resuspension was added with 250 μl of lysis buffer (P2) mixed by gently invert tube. The clear-viscous mixture was

neutralized with 350 µl of neutralization Buffer (N3). Cell debris and chromosomal DNA were pelleted by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a miniprep column. The process was carried out according to the instruction provided by the manufacturer. Finally, the plasmid DNA was eluted with buffer EB (10 mM Tris Cl, pH8.5) and was stored in the microcentrifuge tube at 4 °C or –20 °C.

2.2 Bacterial genomic DNA extraction

Genomic DNA was prepared by the method described by Chen and Kuo (140). Bacterial cells (20 ml) were harvested by centrifugation at 7,000 rpm for 5 min. The pellet was resuspended in a small amount of TE buffer. The cell suspension was lysed in 5.0 ml lysis buffer (40 mM Tris-acetate, pH 7.8, 20 mM sodium acetate, 1 mM EDTA and 1% SDS). After complete lysis, 1.7 ml of 5.0 M NaCl solution was added and mixed well. Most protein and cell debris were removed from the mixture by centrifugation at 12,000 rpm for 15 min at 4°C. Clear supernatant was transferred and extracted once with chloroform. Genomic DNA was precipitated by adding an equal volume of isopropanol, incubation at -20°C for 30 min, and centrifugation at 10,000 rpm for 10 min. The DNA pellet was washed once with 70% ethanol, dried and resuspended in TE buffer.

3. DNA Analysis

3.1 Restriction endonuclease digestion

Plasmid and chromosomal DNA were digested with restriction endonuclease enzymes under optimal conditions recommended by manufacturers. The reaction was usually stopped either by adding EDTA and heating at 65°C for 15 min or extraction with phenol-chloroform depending on the nature of each restriction enzyme. When two digesions of DNA with 2 enzymes were required, in the case where buffer conditions were compatible, both enzymes were added simultaneously. However, in the case of the enzymes condition was incompatible; an ethanol precipitation was performed after first enzyme digestion. Alternative way, DNA was firstly digested with a low salt concentration buffer following with a high salt concentration. After restriction

endonucleas digestion, DNA was analyzed by agarose gel electrophoresis as described in method 3.3.

3.2 DNA cloning by polymerase chain reaction

All primers used are concluded in Table 4. The PCR reaction was prepared in 0.6-ml PCR tube by mixing 1μg of DNA template, 0.5 μM of each primer, 5 μl of 10X buffer (20 mM Tris-HCl pH8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween20, 100 μg/ml of autoclaved gelatin or nuclease- free bovine serum albumin), 25 mM of each dNTP and 5 units of *pfu* DNA polymerase. The reaction volume was adjusted to 50 μl with sterile deionized water and overlaid with 20 μl of sterile mineral oil. The tube was placed in a thermal cycle preheated at 94 °C, setting of the thermal cycling condition were set as follows:

Denaturation: 94°C for 30 sec.

Primer annealing: 55°C for 30 sec.

Primer extension: 72°C for 30 sec.

The amplification reaction required 30 cycles to complete. The PCR products were analysed by agarose gel electrophoresis. The amplified DNA fragments were excised from agarose gel.

3.3 Agarose gel eletrophoresis

DNA fragments were analyzed using sub marine gel electrophoresis (141). DNA samples were mixed with 1/6 volume of loading dye (50% sucrose, 50 mM EDTA; pH 8.0, 0.05% bromophenol blue) and loaded into an agarose gel containing 0.1 μg/ml ethydium bromide. The % agarose gel was varied from 0.8% to 2% depending upon the specific size of DNA fragments interested. The electrophoresis was carried out at a constant 100 volts at room temperature in TAE buffer (0.04 M Tris acetate and 2 mM EDTA, pH 8.0). The *Eco*RI and *Hind*III digested Lambda DNA or pGem DNA marker (Promega, USA) were used as molecular weight markers. DNA fragments were visualized under a long wave length UV transluminator (Vilber Lourmat, France) and photographed using a Polaroid camera with Polaroid 667 film or Gel Doc 1000 (BIO-RAD, USA).

3.4 Purification of specific DNA fragment from agarose gel

Excised DNA fragments were purified from agarose gel by using a "QIAquick gel extraction kit" (QIAGEN, Germany) with the protocol recommended by the manufacturer. Purification of DNA fragment with this kit was based on the fact that in the presence of chaotropic salt, DNA bound selectively to silica-membrane in a special centrifuge tube. The DNA remains bound while a series of rapid "wash-and-spin" steps in the presence of high salt to remove other residues. Finally, low salt elution removes DNA from silica-membrane. The first step of DNA purification from agarose was cutting out of the interested band from the gel with a sharp scalpel by cutting of the smallest possible gel slice. The agarose gel slice was placed into a sterile 1.5 ml microcentrifuge tube. Sliced gel was dissolved by addition of buffer QG and incubated at 50 °C for 10 min. There after, DNA was bound selectively to silica-membrane in a special centrifuge tube. Bound DNA was washed once with buffer QG and once time with washing buffer, supplied in the kit. Elution step was performed with low salt elution buffer; buffer EB (Tris HCl pH8.0) to remove DNA from silica-membrane.

3.5 Southern blot analysis (141)

After a gel electrophoresis, the agarose gel containing DNA fragments was photographed under UV light with a ruler as a scale. The DNA fragments were denatured by soaking the gel in a denaturing solution (0.5 M NaOH and 1.5 M NaCl) for 30 min with continuous shaking. The denatured gel was neutralized in a neutralizing solution (0.5 M Tris; pH 7.4 and 1.5 M NaCl) for 30 min with shaking. The DNA was transferred to a nylon membrane by vacuum blotting (Hoeffer, USA). The gel was placed on the top of the nylon membrane and all air bubbles trapped between the gel and the membrane were removed. The transfer was done by applying 100 mm Hg vacuum for 2 h. A 6X SSC (3 M NaCl, 0.3 M sodium citrate; pH 7.0) was used as the blotting buffer. After blotting, the membrane was rinsed briefly with 2X SSC, and the transferred DNA was fixed by UV crosslinking (GS-Gene linker, Bio-Rad, USA).

4. RNA analysis (142)

4.1 Bacterial RNA extraction

All *Xp* strains were grown aerobically on a shaker in SB medium at 28 °C. The experiment were performed by sub-culturing of a 15-hr *Xp* culture into fresh SB medium followed by incubation for 4 hr before cells were harvested by centrifugation at 7,000 rpm for 5 min at 4 °C. The cell pellet was resuspended in 300 µl ice-cold 0.3 M sucrose/ 0.01 M sodium acetate (pH 4.5) and transferred to a new 1.5-ml microcentrifuge tube. Then 300 µl of 0.01 M sodium acetate, pH 4.5/ 2% SDS was added and the bacterial suspension was heated and thoroughly mixed at 65 °C for 5 min or until the solution become clear before addition of 300 µl of hot phenol, pH 4.5 equilibrated with DEPC treated water (distilled water was treated with 0.1% of diethylpyrocarbonate overnight and subsequently autoclaved). The acid phenol extraction facilitated removal of chromosomal DNA. The sample was then briefly vortexed and heated at 65 °C for 3 min before being centrifuged at 10,000 rpm for 5 min at room temperature. The aqueous layer was transferred to a new tube and re-extract twice with hot acid phenol and twice with chloroform. Then, 1/10 volume of 3M sodium acetate solution, pH 4.5 and 2 volume of absolute ethanol was added to aqueous phase to precipitate RNA. The tube was left at -20 °C for 2 hr, then centrifuged at 10,000 rpm for 10 min at 4 °C and the pellet was washed with 70% ethanol. Then RNA pellet was air dried and dissolved in 50 µl DEPC treated water. RNA samples were stored at -70 °C.

4.2 Formaldehyde agarose gel electrophoresis

Formaldehyde agarose gel is a simple denaturing electrophoresis system that allows good size separation and resolution of single-stranded RNA. Formaldehyde was used as denaturant in the gel. Electrophoresis was carried out in a 1.2 % formaldehyde agarose gel prepared by mixing melted gel in DEPC treated water which was cool down to 60 °C before adding 10X MOPS (0.2 M 4-morpholine propanesulfonic acid, 0.05 M sodium acetate, 0.01 M EDTA pH 7.0) and 37% formaldehyde, to give final concentration of 1X and 1.1%, respectively. Total RNA of 10 to 15 µg was mixed with 10 µl of premix buffer (1X MOPS, 6.54 % formaldehyde,

50% formamide and 0.5 mg/ml of ethidium bromide). The mixture was heated at 55°C for 10 min and then 3 μl of loading buffer (1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol) was added and loaded onto a submarine gel covered with 1X MOPS as a running buffer. RNA samples were separated electrophoretically at 4 to 6 v/cm for 2 to 3 hours or until bromophenol blue migrated about three fourths of the gel length. After that, photograph of the gel with ruler on UV transilluminator for recording marker positions was taken.

4.3 Northern blot analysis (141)

After gel electrophoresis was completed. The RNA was transferred by capillary blotting procedure to a nylon membrane by placing the gel on the support (wrap a piece of whatman 3 mm paper around a piece of plexiglass or a stack of glass plate) and place the wrapped support inside a large baking dish and then filled the dish with 6X SSC which was used as the blotting buffer. The gel was inverted and placed on the bridge 3 mm paper without air bubbles between the 3 mm paper and the gel. Nitrocellulose membrane was placed on the top of the gel without air bubbles. After a stack of paper towels (5-8 cm. height) was placed on the 2-3 pieces of 3 mm paper upper nitrocellulose membrane, glass plate was put on the top of the stack and was weighed down with a 500-g weight. The goal is to set up a flow of liquid from the reservoir through the gel and the nylon membrane, so RNA was transferred from the gel deposited on to the nylon membrane by capillary force within 12-24 hrs. After blotting, membrane was rinsed briefly with 2X SSC and fixed by placing under UV light for 1 min.

5. Southern and Northern hybridization (141)

5.1 Labeling DNA fragments by random priming

The Random primed DNA labeling method was performed according to the manufacturer's instruction manual (Pharmacia Biotech). The DNA was heat denatured by boiling for 5 min then transferred to a tube containing room-temperature-stable bead containing buffer, dATP, dGTP, dTTP, FPLC pure Klenow Fragment and random nanomers. The α-^{32}P dCTP (3000 Ci/mmol) 5 µl and distilled water were added to total volume of 50 µl .The mixture was mixed by pipetting up and down and subsequently incubated at 37 °C for 30 min. After that 50 µl of sterile distilled water were added to the reaction tube. The labeled DNA was separated from non-incorporated radioactive nucleotides by spin-column chromatography through a sephadex G-50 (equilibrated in TE buffer or sterile water). The column was centrifuged at 3,400 rpm for 1 min at room temperature. Labeled DNA was used for the following hybridization steps.

5.2 Hybridization procedure (141)

Pre-hybridization step

Membranes carrying DNA or RNA from Southern or Northern blotting were prehybridized in hybridization tube containing 10-15 ml of the hybridization buffer (0.5 M phosphate buffer pH 7.2, 1% BSA, 1 mM EDTA, 7% SDS) and incubated at 60 °C for at least 1 hr.

Hybridization step

Pre-hybridization buffer was removed and replaced with hybridization buffer. Then the radioactive labeled probe was denatured by boiling for 10 min. After that the tube was immediately cooled down on ice, the probe was added to the hybridization tube without air bubbles and hybridized membrane at 55 °C for 8 to 16 hr.

Washing steps

After hybridization, the filter was washed twice with an appropriate volume of 2X SSC + 0.1% SDS at room temperature for 10 min, followed by two more times for 15 min at 65°C in 0.1X SSC + 0.1% SDS. Checked background with

Geiger counter, if the background still high the filters were washed again at a higher temperature, dried on a filter paper to be slightly damp and finally placed in plastic bags and sealed.

Autoradiography

The filters from the washing step were exposed to a X-ray film (Amersham) with an intensifying screen at -70 °C. The film was developed in a developer and a fixer solutions (Kodak) according to manufacturer's recommendations.

Rehybridization of southern blot

For rehybridization of Southern blot, the nylon membrane, must not be dried completely after hybridizing with the first probe. The membrane was boiled in 0.1% SDS for 15 min. Then, the membrane was ready for prehybridization and hybridization with the target probe.

6. Bacterial transformation

***Xanthomonas* transformation by electroporation**

Electroporation was performed as described in the instruction manual of Cell-Porator (BRL) with some modifications (143). *Xanthomonas* from a fresh plate culture was inoculated into 20 ml of nutrient broth and incubated overnight at 28°C with continuous shaking. Cells were harvested by centrifugation at 7,000 rpm for 5 min at 4^{o}C. The cell pellet was washed twice with an equal volume of ice-cold sterile 10% glycerol solution. After washing, cell pellet was resuspended in 0.1ml of ice-cold sterile 10% glycerol solution to give a cell concentration about 10^{10} cells/ml. Plasmid DNA (100 ng) was added to the competent cells. The mixture was left on ice for 30 min, and then 20 l of cell suspension was transferred to the electroporation chamber. The gene Pulser apparatus was adjusted to give final voltage around 8-10 $kVcm^{-1}$ and pulsed for 8 sec. Immediately after the pulse had been applied, the cell suspension was placed in a tube containing 1.0 ml of SB broth for expression at 28°C for 3 h. To increase number of transformants, the suspension was centrifuged at 5,000 rpm for 5 min. The cell pellet was resuspended in 100 l of SB broth and spread onto SB agar plates containing appropriate antibiotics. After 3 days of incubation at 28°C, the transformants were purified for further characterization.

7. Construction of a double mutant of *ohr* and *ahpC*

Xp ahpC-ohr was constructed by electroporation of the chromosome from the single mutants of *Xp ohr* into the *Xp ahpC* cell in order to inactivate the second gene by integration into the chromosome of *Xp ahpC*. This was accomplished via double recombination which occured between the other mutant chromosome and *Xp ahpC* chromosome. The double mutant could be selected by antibiotic resistance phenotype and southern blot hybridization.

8. Effects of oxidant on killing of *Xanthomonas*

8.1 Qualitative analysis by inhibition zone determination.

Qualitative analysis of the killing effects of various reagents on *Xanthomonas* was done using the inhibition zone assay as described by Mongkolsuk et al. (16). Essentially, overnight culture cells were subcultured as 5% inoculum into fresh SB broth. A 500-μl aliquot of log phase cells (4 h, OD_{600} ~ 0.5 culture) or 200 μ l aliquot of stationary phase cells (30 h, OD_{600} ~ 3) were mixed with 3.0 ml top agar (SB containing 0.7% agar) prewarmed at 50^{o}C and overlaid onto SB plates (9-cm-diameter petri dishes and poured with 20 ml of SB agar). The plates were left at room temperature for 15 min to let the top agar solidify. Sterile 6-mm-diameter discs (prepared from Whatman filter paper no.3) soaked with 5 μl of the appropriate concentrations of oxidants were placed on the cell lawn and zones of growth inhibition were measured after 24 h of incubation at 28^{o}C.

8.2 Quantitative analysis by survival curve determination

Killing experiments in *Xanthomonas* were performed according to the method described by Vattanaviboon et al. (132). *Xanthomonas* strains were cultured overnight in SB medium at 28°C with shaking. The overnight culture was subcultured into 20 ml of SB broth to make a 5% inoculum and incubated further at 28°C with shaking for 4 h. For adaptive or cross-protective experiments, the cultures were grown at 28°C with shaking for 3 hr. and subsequently induced with sublethal concentrations of various substances, and growth was allowed to continue for an hour at 28°C with shaking. A 1-ml aliquot of cell culture was treated with killing concentration of tested

substances. Samples (100 µl) were placed in tubes containing 900 µl of SB broth at 10 min interval for 30 min. Treated cells were collected by centrifugation at 7,000 rpm for 2 min, resuspended in 100 µl of SB broth, and immediately diluted a 10 fold in SB broth. The appropriate dilutions were spread onto SB agar plates to determine the viable cells after 3 days incubation at 28°C. The surviving fraction is defined as the number of viable cells after treatment divided by the number of viable cells prior to treatment. The survival curves were determined by plotting the surviving fraction versus times.

9. DNA mobility shift assay

The DNA-binding assay using mobility shift PAGE is based on the difference of the migration between unbound DNA fragment that move faster than DNA-protein complex. This technique is simple, rapid and sensitive for the detection of DNA-protein complex.

9.1 DNA probe preparation

A 170 bp of fragment containing an *ohrR* promoter, P1 from plasmid pBBR*ohrR* was used a template, M13R and BT18 as an upstream and downstream primer, respectively to amplify with *Pfu* DNA polymerase by PCR. The using condition was 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. Then, 280 bp PCR product was digested with *Eco*RI resulting in a digested 170 bp fragment (147). Eluted fragment was end labeled with [α-^{32}P] dCTP using Klenow DNA polymerase (Promega). The labeled DNA fragment was purified through the chromatography of Sephadex G-50.

9.2 Protein-DNA binding reaction

Purified OhrR and OhrRC22S proteins (147) were bound with DNA probe in the mixture of the reaction containing: a DNA probe, protein, poly(dI-dC), a carrier DNA, 0.5X binding buffer (40 mM HEPES pH 7.6, 2 mM EDTA, 20 mM $(NH_4)_2SO_4$, 10 mM DTT, 0.4 % Tween20, 60 mM KCl) and incubated at 30 °C for 30 min. Finally, the protein-DNA complex was separated from unbound DNA probe on 5% native polyacrylamind gels in TAE buffer by electrophoresed at 100 V for 90 min. The gel was then autoradiographed and quatitated by densitometry.

10. Assay of enzyme activity

10.1 Sample preparation

X. campestris pv. *phaseoli* cultures were grown in 5 mL of SB broth supplement with appropriated antibiotic for 16-18 hrs at 28 °C with shaking. There after the overnight culture was inoculated into fresh 20 mL of SB broth in a final concentration of 1% v/v and was moderately shaked at 28°C to obtain an OD_{600} measurement of the cells of 0.3-0.4. The culture was induced with 100 and 200 µM of *t*BOOH (*tert*-butyl hydroperoxide), an organic peroxide oxidant, for 30 min. Then, centrifuge at 8,000 rpm for 5 min. The pelleted cell was kept at -70 until assay. Pellted cell was resuspended in a 100 µl of phosphate buffer pH 7 and 1 mM phenylmethylsulfonyl fluoride (PMSF) and cell was breaked by sonication on ice. After centrifugation at 12,000 rpm for 10 min, the suspension were collected and used to assay an enzyme activity.

10.2 Total protein assay

The total protein content in the lysate was measured by dye-binding method described by Bradford (144) with some modifications (Bio-Rad, USA). The procedure was performed according to the manufacturer's recommendations. A working solution for protein assay was prepared by mixing 1 volume of stock solution to 4 volume of distilled water. To assay the protein concentration, 10 µl of sample (or standard protein) was added to 1.0 ml working solution in a 1-ml disposable cuvette. The reaction was mixed by inversion and left at room temperature for 5 min. After incubation, the absorbance at 595 nm (A_{595}) was determined against reagent blank. The protein concentration in the sample was calculated by the following equation:

$$\text{Protein concentration (mg/ml)} = \frac{A_{sample} \times \text{standard concentration (mg/ml)}}{A_{standard}}$$

10.3 Assay of enzyme activity

10.3.1 Measuring of Cholramphenicol acetyltransferase (CAT) activity

The reacton buffer consist of 100 mM Tris pH 7.8, 0.1 mM Acetyl-coenzyme A, 0.1 mM Cholramphenicol and 10 μM 5,5'-dithio-bis (2-nitrobenzoic acid) or DTNB. Then, 10 μl of sample was added into 1 mL of reacton buffer and an absorbance changes in the reaction was recorded at OD_{412}. The procedure was performed according to the manufacturer's recommendation. The unit of enzyme was defined as μmol of chloramphenicol acetylted per min and the specific activity is expressrd as the number of unit enzyme activity per mg protein.

10.3.2 Measuring of β-galactosidase activity

The reaction buffer consist of 50 mM phosphate buffer, 0.05 M β -mercaptoethanol, 1mM $MgCl_2$ and 1 mg/ML *o*-nitrophenyl-β-D-galactoside (ONPG). Sample was added into 1 mL of reaction buffer and detected a β-galactosidase enzyme to hydrolize the colorless substrate ONPG. The releasing of an *o*-nitrophenol (ONP) which can detect at OD 420 was a product from reaction. An enzyme activity was recorded as ΔA_{420} and the specific activity is expressed as ΔA_{420} per mg protein.

11. Preparation of linoleic acid hydroperoxide (LOOH)

LOOH was prepared by using enzymatic reaction of soybean lipoxygenase and linoleic acid decribed by Evans et al (145). Essentially, 25 ml of 0.5 mM linoleic acid in 0.1 M *tetra*-sodium borate buffer (pH 9.5) in a 250 ml conical flask was mixed with 4,000U soybean lipoxygenase and vigorously stirred at room temperature for 30 min. LOOH was purified by passing through a Sepack C18 cartridge. The coulumn was activated with 5 ml of methanol and 10 ml of water. After passing he sample, column was washed with 20 ml of water and then LOOH was eluted with 1.5 ml methanol. The concentration of LOOH was determined by measuring A_{234} with ε_{234} = 25,000 $M^{-1}cm^{-1}$.

12. Organic hydroperoxide degradation assay

The degradation of organic hydroperoxide was measured as described by Ochsner et al. (18) with some modification. Overnight cultures of various *Xanthomonas* strains were incoculated into 20 ml SB medium to give a final OD_{600} of 0.1. The log phase cultures (after 4 h growth) were adjusted to OD_{600} of 0.5 with fresh medium prior to an addition of organic hydroperoxide to a concentration of 200 µM. Residual organic hydroperoxide concentrations were determined at 5-min intervals by a xylenol orange-iron reaction. At indicated time, 1 ml of the culture was removed and cells were pelleted. Then, 100 µl of the clear supernatant was added to 400 µl of 25 mM sulfuric acid in a 1 ml cuvette. Subsequently, 500 µl of freshly prepared reaction buffer (200 mM ammonium ferrous sulphate, 200 µM xylenol orange in 25 mM sulfuric acid) was added to the mixture. After 10-15 min incubation at room temperature, the absorbance at 540 nm was monitored. The concentration of residual organic hydroperoxide in the culture was calculated using standard organic hydroperoxide in SB medium.

CHAPTER IV
RESULTS

Part I: Evaluations of the roles of *ohr* and *ahpC* in synthetic organic hydroperoxide detoxification

1. Construction of *Xp ahpC-ohr* double mutant

ahpC and *ohr* are recognized as the important genes responsible for degradation of organic hydroperoxides in *X. campestris* pv. *phaseoli*. The first step in an attempt to elucidate the significant of both genes toward organic hydroperoxide protection is the construction of *ahpC-ohr* double mutant.

1.1 Electroporation of *Xp ohr* genomic DNA into *Xp ahpC*

The *ahpC-ohr* double mutant was constructed by transferring *X. campestris* pv. *phaseoli ohr*::*tet* (*Xp ohr*) (16) genomic DNA into *X. campestris* pv. *phaseoli ahpC*::*kan* (*Xp ahpC*) (143) using electroporation method as described in the Material and Methods. The homologous recombination between *Xp ohr* (*ohr::tet*) genomic DNA and functional copy of the *ohr* gene in the *Xp ahpC* mutant will give rise to the *ahpC-ohr* double mutant. The putative double mutant was selected for its ability to grow on medium containing kanamycin (30 μg/ml) and tetracycline (30 μg/ml). Colony that possesses Kan^r and Tet^r phenotypes were rechecked its genotypes in term *ohr::tet* and *ahpC::kan* by Southern blot analysis.

1.2 Southern blot analysis of *Xp ahpC-ohr* mutant

To assure the disruption of both *ahpC* and *ohr* genes in *Xp ahpC-ohr*, Southern blot analyses for both genes were performed.

1.2.1 Confirmation of *ahpC* disruption

The schematic diagram summarized the construction and the physical map of the *Xp ahpC* mutant (143) was illustrated in Fig. 14. For Southern blot analysis, *X. campestris* pv. *phaseoli*, *Xp ahpC*, *Xp ohr* and *Xp ahpC-ohr* were grown in SB and the genomic DNA were extracted as described in the Material and Methods. 10 µg of DNA from each samples were completely digested with *Sac*II restriction endonuclease under condition recommended by manufacturer. The digested genomic DNA samples were separated on 0.8% agarose gel, alkaline denatured, neutralized and subsequently blotted onto a nylon membrane (Fig. 15 A). DNA hybridization was performed using radioactively labeled (^{32}P-dCTP) 150-bp DNA fragment of *ahpC* coding region amplified with #133 and CS2 primers as a forward and reverse primers, respectively and *Xp* genomic DNA as templates. After hybridization, the non-specific binding probe was washed out from the membrane under high stringency condition (0.5X SSC, 0.1 % SDS at 65°C for 15 min). The autoradiogram in Fig. 15 B showed positively hybridized bands in all strains. Only one band of 1.1 kb was detected in *Xp* wild-type and the *ohr* mutant but two hybridized signals of 464 bp and 5.08 kb were detected in the *Xp ahpC* as well as in the *ahpC-ohr* double mutants. The appearance and the size of two positively hybridized bands was in good agreement with the genetic map of *ahpC* mutant and indicated that *ahpC* on the putative *ahpC-ohr* mutant chromosome was inactivated by *kan*.

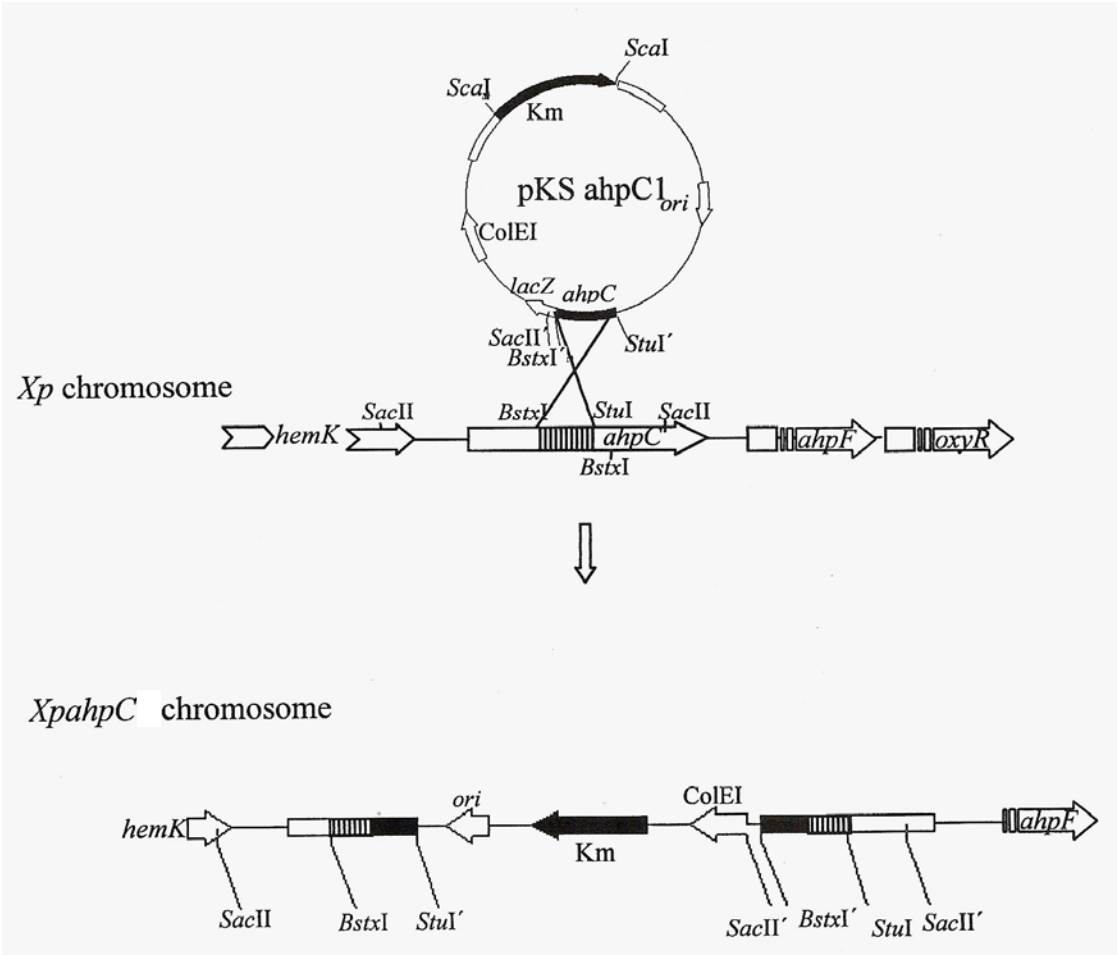


Fig. 14. Schematic diagram summarized the construction and genetic map of *X. campestris* pv. *phaseoli ahpC* mutant (143).

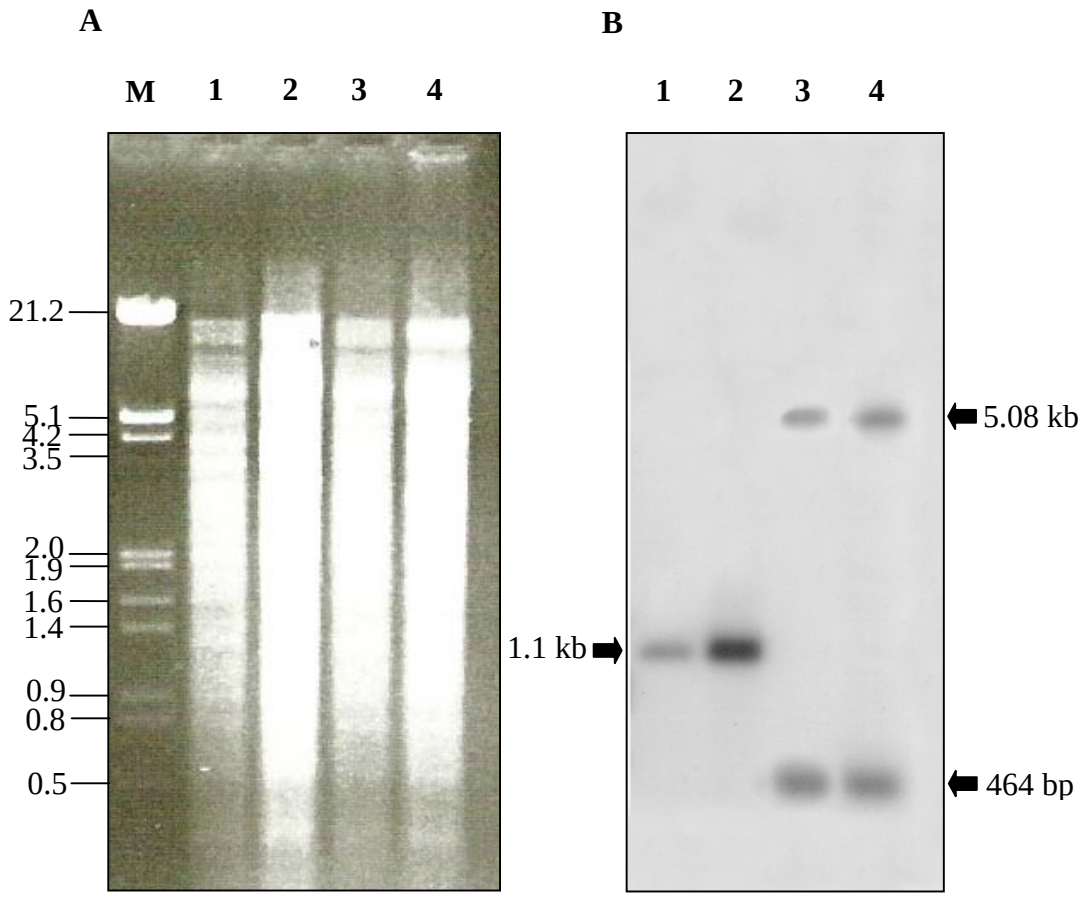


Fig. 15. Southern blot analysis of the *ahpC* mutant.

(A) Agarose electrophoresis gel from *X. campestris* pv. *phaseoli*, *Xp ahpC*, *Xp ohr* and *Xp ahpC-ohr* genomic DNAs digested with *Sac*II.

(B) Autoradiogram of the blotted DNA and hybridized with ^{32}P-labeled 150 bp fragments of *ahpC* coding region.

LaneM Molecular weight marker

Lane1 *Xp* wild-type

Lane2 *Xp ohr*

Lane3 *Xp ahpC*

Lane4 *Xp ahpC-ohr*

1.2.2 Confirmation of *ohr* disruption

The construction of the *ohr* mutant (16) and its physical map of *ohr* locus are shown in Fig. 16. To perform Southern analysis for *ohr* inactivation, 10 µg of genomic DNA from the samples were digested with *Eco*RI restriction enzyme. The digested DNA were separated on 0.8% agarose gel electrophoresis, denatured, neutralized and finally blotted onto a nylon membrane (Fig. 17 A). The membrane was subsequently probed with ^{32}P-labeled 211-bp *Sac*I fragments of pOPRX-1. Non-specific hybridization was removed by washing under a stringency condition (0.5X SSC, 0.1 % SDS at 65°C for 15 min). The autoradiogram was shown in Fig. 17 B. *Xp* wild-type and the *Xp ahpC* mutant showed only one hybridized band of 10.5 kb while *Xp ohr* and *Xp ahpC-ohr* double mutants possessed two bands of 9.3 and 4.35 kb. The size of two positively hybridized bands corresponded to the expected fragments shown in the physical map of *ohr::tet* mutant. The results affirm that *ohr* was disrupted by *tet* in the double mutant.

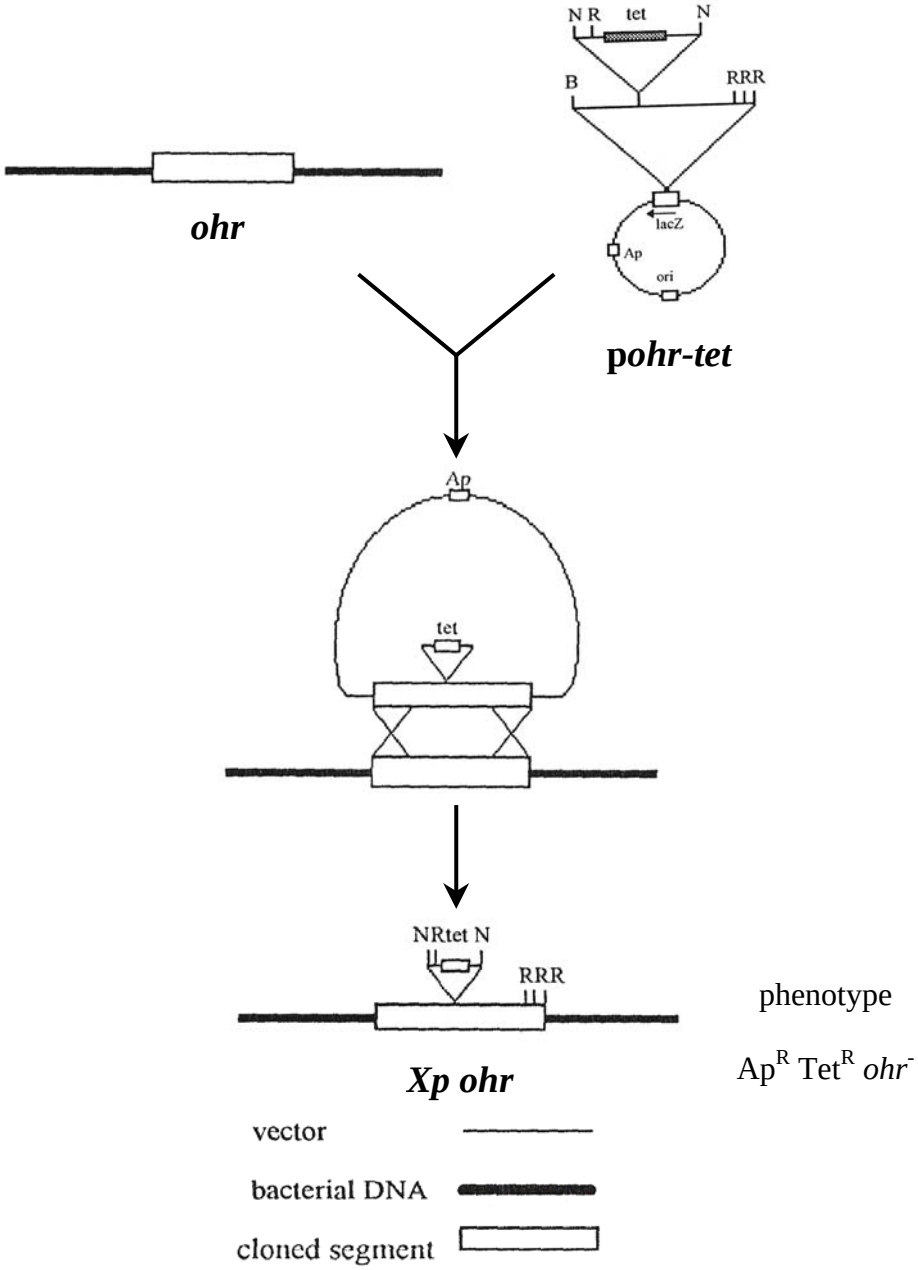


Fig. 16. Schematic diagram summarized the construction and genetic map of *X. campestris* pv. *phaseoli ohr* mutant (16). p*ohr-tet* has been constructed by insertion of *tet* to the *Not*I site of *ohr* coding region in p*ohr*11 (16). B, *BamH*I ; N, *Not*I ; R, *Eco*RI.

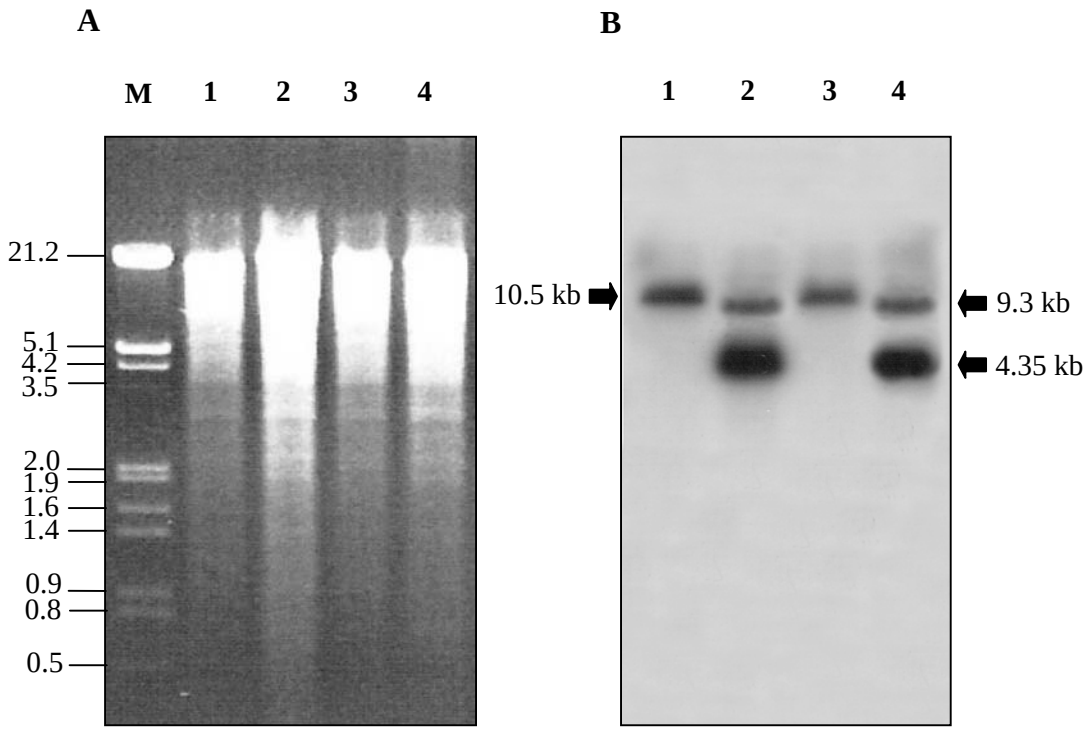


Fig. 17. Southern blot analysis of the *ohr* mutant.

(A) Agarose electrophoresis gel of *X. campestris* pv.*phaseoli*, *Xp ahpC*, *Xp ohr* and *Xp ahpC-ohr* genomic DNAs were digested with *Eco*RI.

(B) Autoradiogram of the blotted DNA and hybridized with 211 bp *Sac*I fragments of *ohr* coding region labeled with α^{32}-P.

LaneM Molecular weight marker

Lane1 *Xp* wild-type

Lane2 *Xp ohr*

Lane3 *Xp ahpC*

Lane4 *Xp ahpC-ohr*

2. Determination of the resistance levels toward synthetic organic hydroperoxides in various *Xanthomonas* strains

Inactivation of genes involving in protection against oxidative stress sometimes causes serious effects to aerobic growth of bacteria by lowering their growth rates. To evaluate the effect of *ahpC* and/or *ohr* mutations on the growth of *X. campestris* pv. phaseoli, overnight cultures of *Xp, Xp ahpC, Xp ohr* and *Xp ahpC-ohr* were inoculated into fresh SB medium to give final OD_{600nm} of 0.1. The cultures were cultivated at $28^{o}C$ with continuous shaking at 150 rpm. Bacterial growth was monitored by measuring OD_{600nm} at 2 hr interval for 10 hr. The aerobic growth rate in SB medium of *Xp ahpC, Xp ohr* and *Xp ahpC-ohr* were identical to the parental strain *Xp* (Fig. 18). These data indicated that lack of functional *ahpC* and *ohr* produced no detectable effect on bacterial growth in rich medium under aerobic conditions. Experiments were then extended to test the effects of low concentration of oxidants on growth of *ahpC-ohr* mutant strains. The growth of the double mutant was severely retarded in the presence of 100µM tBOOH or 100µM CuOOH. The doubling time for the single *ohr* or *ahpC* mutant and the parental *Xp* strain in SB medium containing tBOOH (Fig. 19) and CuOOH (Fig. 20) were identical at 2 and 2 h, respectively while those of the *ahpC-ohr* double mutant were 6.0 and 4.2 h when tBOOH and CuOOH was added to the medium, respectively. These data suggested lacking of both *ahpC* and *ohr* rendered *X. campestris* pv. *phaseoli* highly sensitive to organic hydroperoxides.

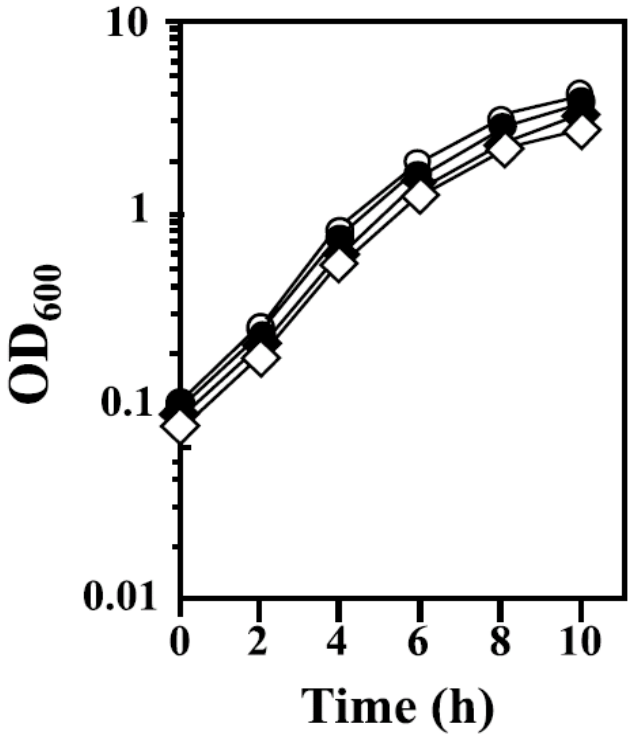


Fig. 18 Growth of *X. campestris* pv. *phaseoli* (o), *ahpC* mutant (■), *ohr* mutant (•), and *ahpC–ohr* double mutant (□) was monitored spectrophotometrically in the absence of organic peroxide. The experiments were repeated three times and typical results are shown.

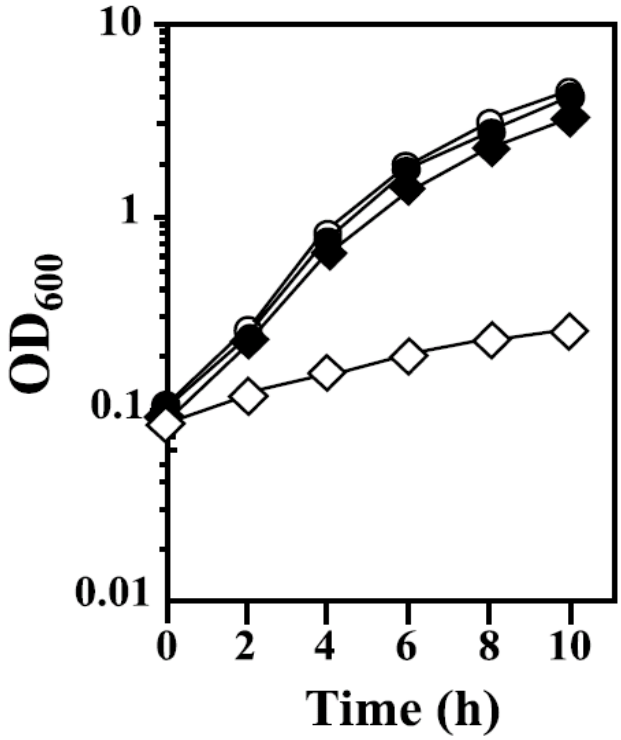


Fig. 19 The effects of low concentrations of tBOOH on growth of *ahpC*, *ohr*, and *ahpC–ohr* double mutant. Growth of *X. campestris* pv. *phaseoli* (o), *ahpC* mutant (■), *ohr* mutant (•), and *ahpC–ohr* double mutant (□) was monitored spectrophotometrically in the presence of 100 µM tBOOH. The experiments were repeated three times and typical results are shown.

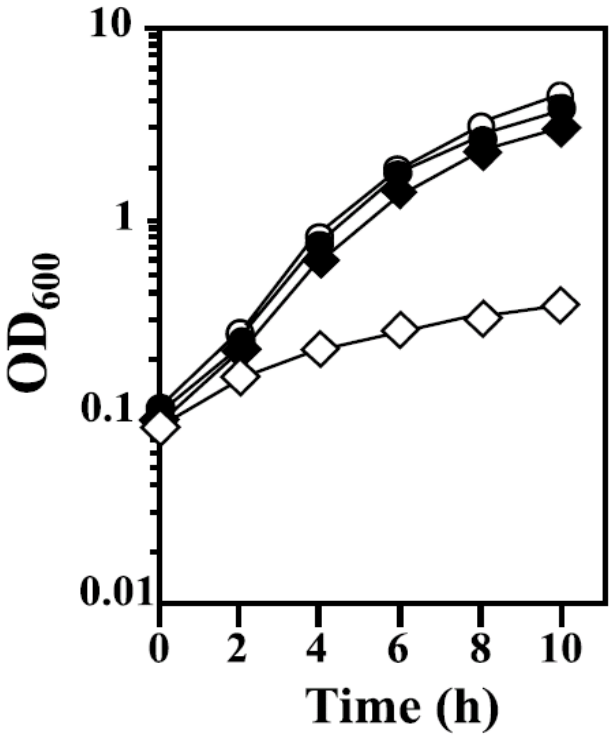


Fig. 20 The effects of low concentrations of CuOOH on growth of *ahpC*, *ohr*, and *ahpC–ohr* double mutant. Growth of *X. campestris* pv. *phaseoli* (o), *ahpC* mutant (■), *ohr* mutant (•), and *ahpC–ohr* double mutant (□) was monitored spectrophotometrically in the presence of 100 µM CuOOH. The experiments were repeated three times and typical results are shown.

The resistance levels against killing concentrations of tBOOH and CuOOH in the *ahpC-ohr* mutants were determined by using the growth inhibition zone assay. Fig. 21 and 22 show that *ahpC-ohr* double mutant was drastically more sensitive to killing treatments with both tBOOH and CuOOH than the mutant lacking *ahpC* or *ohr* alone.

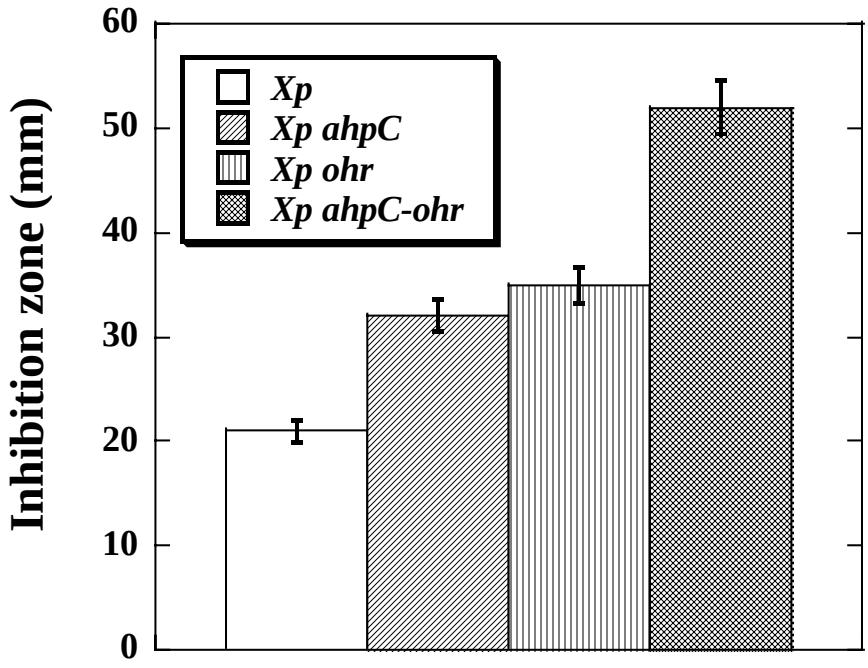


Fig. 21 Determination of resistant levels to 0.5 M tBOOH in the *Xp* parental strain, the *ohr* mutant (*Xp ohr*), *ahpC* mutant (*Xp ahpC*), and *ahpC–ohr* double mutant (*Xp ahpC-ohr*) using the growth inhibition zone method. Values shown are means of four replicates and error bars indicate SD.

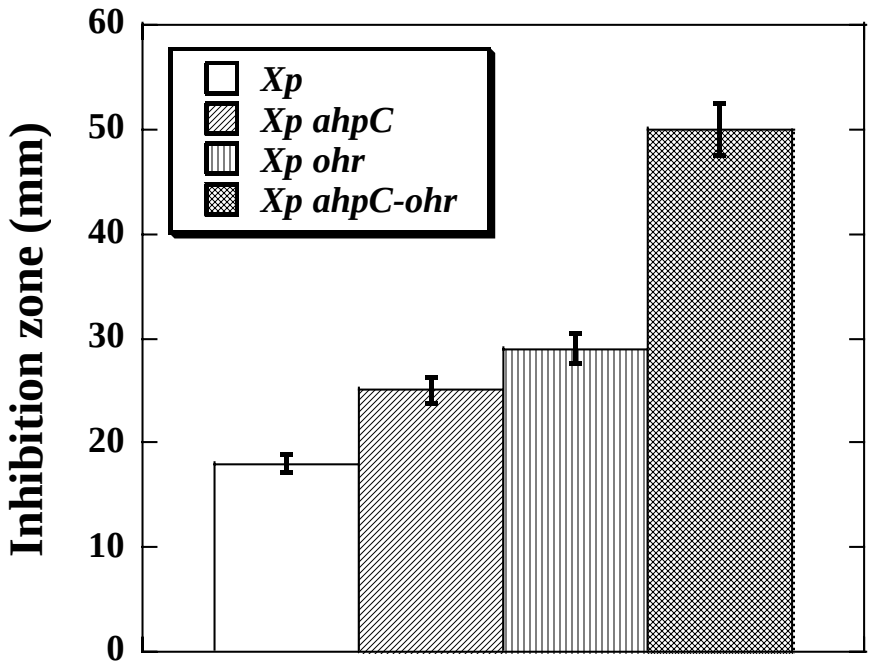


Fig. 22 Determination of resistant levels to 0.5 M CuOOH in the *Xp* parental strain, the *ohr* mutant (*Xp ohr*), *ahpC* mutant (*Xp ahpC*), and *ahpC–ohr* double mutant (*Xp ahpC-ohr*) using the growth inhibition zone method. Values shown are means of four replicates and error bars indicate SD.

3. Complementation of *ahpC* and *ohr* in *Xp ahpC-ohr* double mutant

The abilities of a plasmid-borne expression of *ahpC* and *ohr* to restore organic hydroperoxide hypersensitive phenotypes of the *ahpC-ohr* mutant were tested. Recombinant plasmids, pAhpC (a broad-host-range plasmid vector pUFR047 containing full-length *ahpC*) and pOhr (pUFR047 containing full-length *ohr*) were transformed into the *ahpC-ohr* double mutant. The resistance level toward killing concentration of tBOOH and CuOOH in the *ahpC-ohr* mutant harboring pUFR047 vector control, pAhpC, pOhr and the *Xp* parental strain harboring pUFR047 were determined using inhibition zone assay. The results were summarized in Table. 5. The *ahpC-ohr* double mutant with pOhr (*Xp ahpC-ohr*/ pOhr) was more resistant to tBOOH and CuOOH than the mutant with pAhpC (*Xp ahpC-ohr*/ pAhpC) as judged by the zone of growth inhibition of 25.0 and 29.5 mm for tBOOH, and 23.0 and 30.0 mm for CuOOH, respectively. This suggests that high expression of *ohr* from the plasmid was better than high level expression of *ahpC* at protecting the *ahpC-ohr* mutant from both tBOOH and CuOOH toxicities, although the level of resistance conferred by each of these genes in double mutant was still less than that of the parental strain. Thus, high level of either *ohr* or *ahpC* could not fully complement the organic hydroperoxide hypersensitive phenotype of the double mutant. The results also implied that *Xanthomonas* required both *ahpC* and *ohr* to be fully protected from organic hydroperoxide.

Table. 5. Determination of levels of resistance to killing treatment with organic peroxides in various *X. campestris* pv. *phaseoli* (*Xp*) strains.

X. campestris pv. *phaseoli* strains/ plasmid	Zone of growth inhibition (mm)*	
	0.5 M tBOOH	0.5 M CuOOH
Xp/ pUFR047	21.0 ± 2.5	18.0 ± 1.5
Xp ahpC-ohr/ pUFR047	52.0 ± 5.0	50.0 ± 4.5
Xp ahpC-ohr/ pOhr	25.0 ± 3.0	23.0 ± 2.5
Xp ahpC-ohr/ pAhpC	29.5 ± 3.0	30.0 ± 3.0

* Values are means ± SD of four replicates.

4. Functional analyses of Ohr and AhpC in synthetic organic hydroperoxide degradation

The ability of the various *X. campestris* pv. phaseoli mutant strain to degrade organic hydroperoxides *in vivo* was determined in order to evaluate the physiological role of *ahpC* and *ohr* in term of organic peroxide detoxification. Exponential phase cultures (OD_{600} of 0.5) of *Xp ahpC*, *Xp ohr* and *Xp ahpC-ohr* and the parental wild type *Xp* in SB medium were challenged with 200 µM tBOOH or CuOOH. The treated cultures were incubated with continuously shaking. At 5 min interval, the residual tBOOH or CuOOH in the cultures was monitored using xylenol orange colorimetric method (FOX assay) as described in Materials and Methods. Percentage residue of tBOOH or CuOOH for each time point was calculated by comparing with the started concentration of organic hydroperoxide and plotted against incubation time. As shown in Fig. 23 and 24, *Xp* wild type strain rapidly degraded both tBOOH and CuOOH to non-detectable level within 15 min after addition of organic hydroperoxides. The ability of tBOOH and CuOOH degradation in the *ahpC-ohr* double mutant was drastically decreased as may be seen that only 20% of tBOOH and 15% of CuOOH were degraded after 20 min incubation. The roles of each system in the degradation of tBOOH and CuOOH were evaluated using single *ohr* or *ahpC* mutants. The results in Fig. 23 illustrated that the rate of tBOOH degradation was higher in the *ahpC* mutant than the *ohr* mutant but these rates were significantly above the rate of tBOOH degradation in the double mutant. For CuOOH degradation, the results in Fig. 24 showed that the rate in *ohr* mutant was comparable to the basal rate in the *ahpC-ohr* mutant. In contrast, the rate of degradation in the *ahpC* mutant was approaching the parental strain level. Taken together, the results indicated that *ohr* accounts for majority of organic hydroperoxides either tBOOH or more hydrophobic CuOOH.

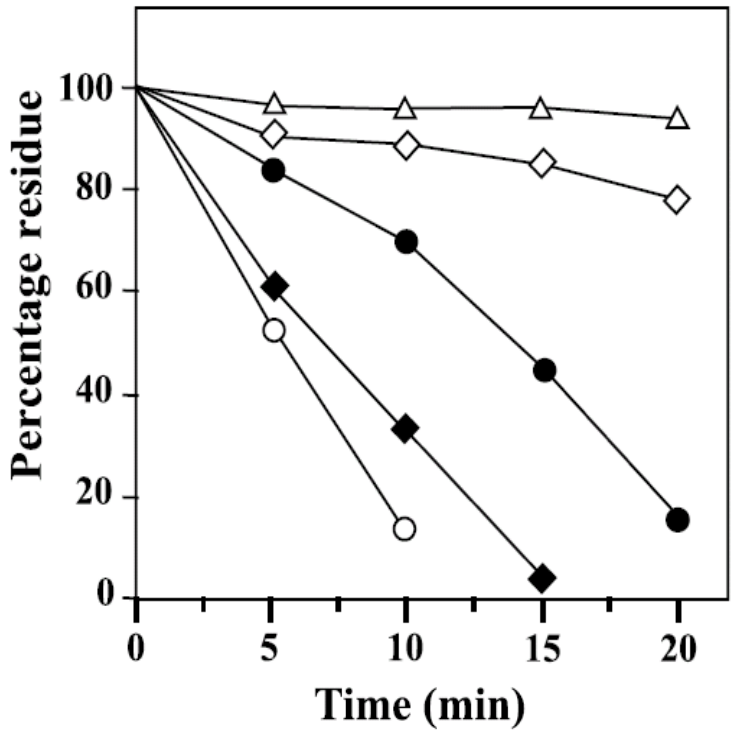


Fig. 23. Functional analyses of organic hydroperoxide degradation of AhpC and Ohr. The rates of tBOOH degradations in *X. campestris* pv. *phaseoli* (o), *ahpC* mutant (■), *ohr* mutant (•), and *ahpC–ohr* double mutant (□) were measured and compare to the control without bacterial culture (Δ). The experiments were independently repeated three times and typical results are shown.

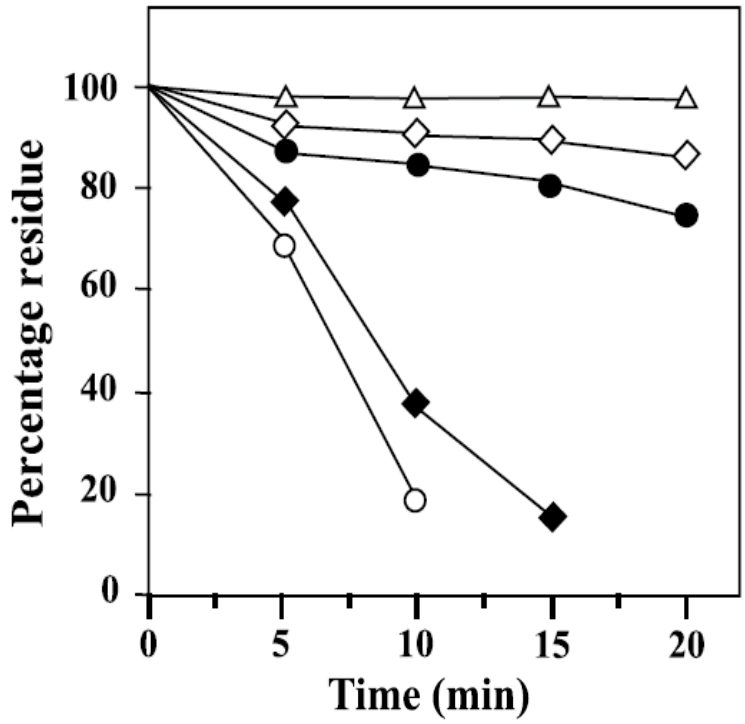


Fig. 24. Functional analyses of organic hydroperoxide degradation of AhpC and Ohr. The rates of CuOOH degradations in *X. campestris* pv. *phaseoli* (o), *ahpC* mutant (■), *ohr* mutant (•), and *ahpC–ohr* double mutant (□) were measured and compare to the control without bacterial culture (Δ). The experiments were independently repeated three times and typical results are shown.

5. CuOOH induced expression of *ahpC* and *ohr* in the mutants

ahpC and *ohr* were regulated by different transcriptional regulator. The former is a member of OxyR regulon while the latter is controlled by OhrR, a transcriptional repressor. However, *ahpC* and *ohr* expressions are highly induced by challenging the *Xanthomonas* cultures with sublethal concentrations of tBOOH and CuOOH (15, 16). Increased expression of these genes is essential to protect cells from organic peroxide toxicity as show by analysis of an *oxyR* mutant that is unable to up regulate *ahpC* and that, as a consequence is sensitive to organic peroxide killing. Inactivation of either gene could affect organic peroxide induced gene expression of another. Northern blot analysis was undertaken to investigate the inducibilities of *ahpC* and *ohr* expressions by the presence of various concentrations of CuOOH in different mutants and the parental strain. Total RNA were prepared form exponential cultures of *ahpC*, *ohr*, *ahpC-ohr* mutants and the parental *Xp* induced with indicated concentrations of CuOOH. The RNA samples were separated using a denaturing formaldehyde gel electrophoresis and subsequently transferred to nylon membrane (Fig. 25 A, 26 A). Northern hybridization were performed with 211 bp *Sac*I fragments of pOPRX-1 as a probe for *ohr* and 150-bp PCR fragment of *ahpC* coding region by using #133 primer as forward and CS2 primer as reverse primers for *ahpC*. The results are shown in Fig. 25 B and 26 B, The magnitudes of *ahpC* and *ohr* induction showed a CuOOH dose-dependent response. At 10 µM, significant induction of both genes was detected, while full induction required at least 100 µM CuOOH. Interestingly, determination of the levels of *ahpC* mRNA in *Xp* and the *ohr* mutant treated with 10, 50 and 100 µM CuOOH showed that *ahpC* expression in the *ohr* mutant was higher at all CuOOH concentrations tested (Fig. 25 B), while *ohr* mRNA levels were similar in both the parental and the *ahpC* mutant strains at all CuOOH concentrations tested (Fig. 26 B)

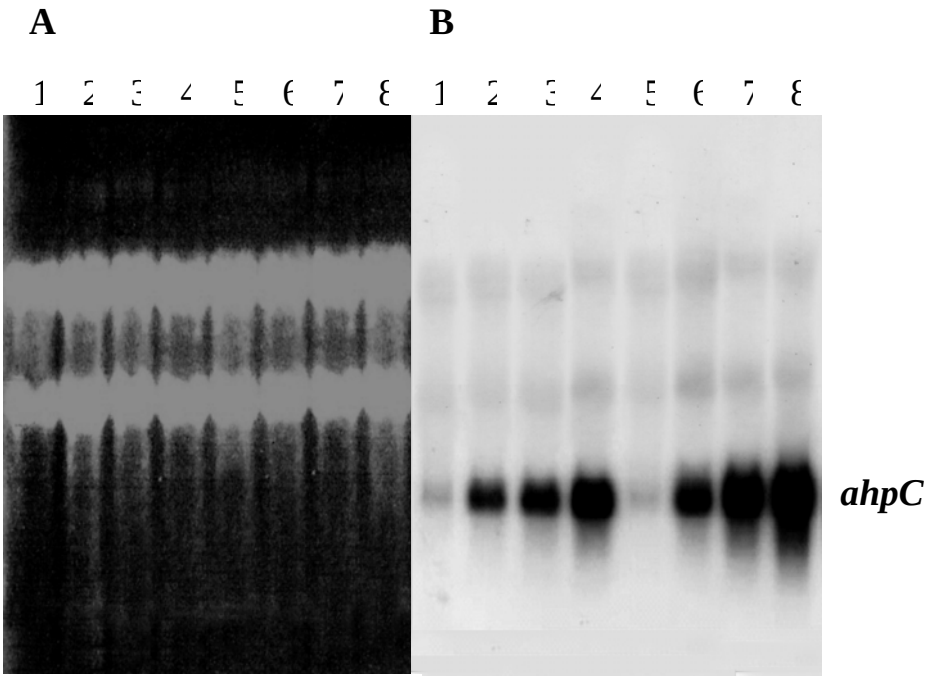


Fig. 25. Induction of *ahpC* expression in *Xp* parental and the *ohr* mutant strains by various concentration of CuOOH. The membrane was hybridized with radioactively labeled *ahpC* probe.

Lane1 *Xp* uninduced;

Lane2 *Xp* induced with 10 µM of CuOOH

Lane3 *Xp* induced with 50 µM of CuOOH

Lane4 *Xp* induced with 100 µM of CuOOH

Lane5 *Xp ohr* uninduced

Lane6 *Xp ohr* induced with 10 µM of CuOOH;

Lane7 *Xp ohr* induced with 50 µM of CuOOH.

Lane8 *Xp ohr* induced with 100 µM of CuOOH

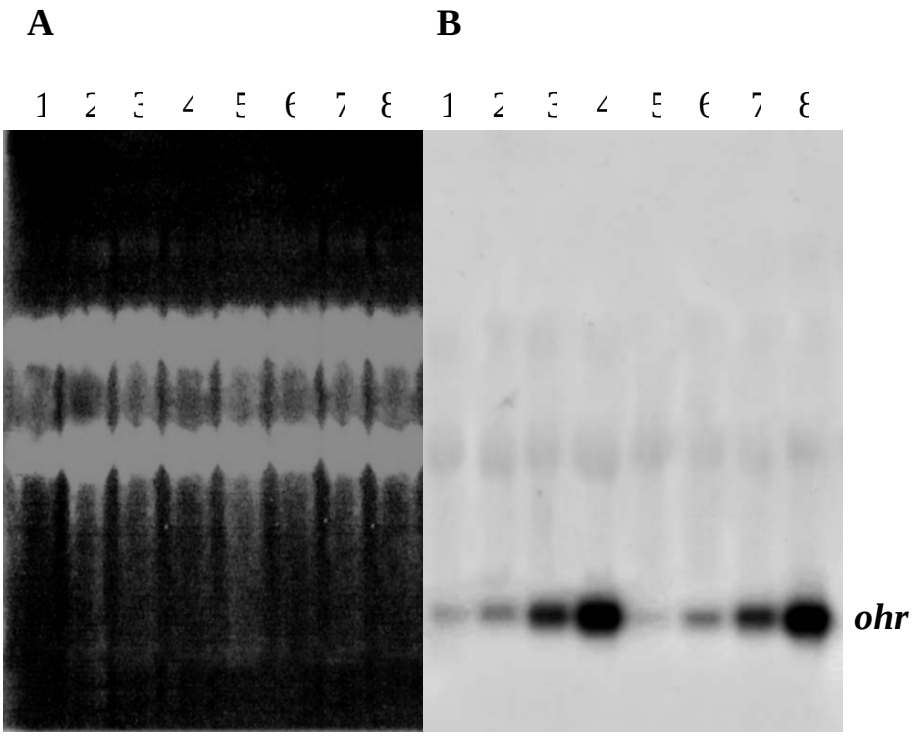


Fig. 26. Induction of *ohr* expression in *Xp* parental and the *ahpC* mutant strains by various concentration of CuOOH. The membrane was hybridized with radioactively labeled *ohr* probe.

Lane1 *Xp* uninduced;

Lane2 *Xp* induced with 10 µM of CuOOH

Lane3 *Xp* induced with 50 µM of CuOOH

Lane4 *Xp* induced with 100 µM of CuOOH

Lane5 *Xp ahpC* uninduced

Lane6 *Xp ahpC* induced with 10 µM of CuOOH;

Lane7 *Xp ahpC* induced with 50 µM of CuOOH.

Lane8 *Xp ahpC* induced with 100 µM of CuOOH

Part II: Functional analysis the role of Ohr and AhpC in linoleic acid hydroperoxide protection

1. *ahpC* and *ohr* had differential protective roles against LOOH toxicity

X. campestris pv. *phaseoli*'s ability to metabolize lipid hydroperoxide is important for its survival, especially during plant infection. Many organic hydroperoxide metabolizing systems have been studied in bacteria, however, none of these studies addressed the efficiency of different systems in metabolizing lipid hydroperoxide or the integrated roles gene regulation, enzymology and bacterial physiology play in lipid hydroperoxide defense. To elucidate lipid hydroperoxide defense in *X. campestris* pv. *phaseoli*, the resistance levels of *Xp ohr*, *Xp ahpC*, *Xp ahpC-ohr* and the parental strain (*Xp*) against linoleic hydroperoxide (LOOH) were investigated by an inhibition zone assay using 6 mm paper disks soaked with 50mM LOOH. *Xp ohr* was much more sensitive to LOOH than *Xp* as shown by the inhibition zone of the former was 12 mm while the latter was non-detectable (Fig. 27). *Xp ahpC* gave a small inhibition zone size of 6.5 mm which is a little bit more than that of *Xp* (no zone). The hyper-sensitive phenotype to LOOH in the *ahpC-ohr* mutant could be complemented by plasmid-borne expressions of *ahpC* and *ohr* genes from pAhpC and pOhr, respectively. However, it could not be concluded that the level of resistance to LOOH in the double mutant complemented with pAhpC and pOhr was equal to the wild type level since no inhibition zone was observed in all strains. An attempt to increased concentrations of LOOH could not be accomplished due to a limitation of the LOOH preparation.

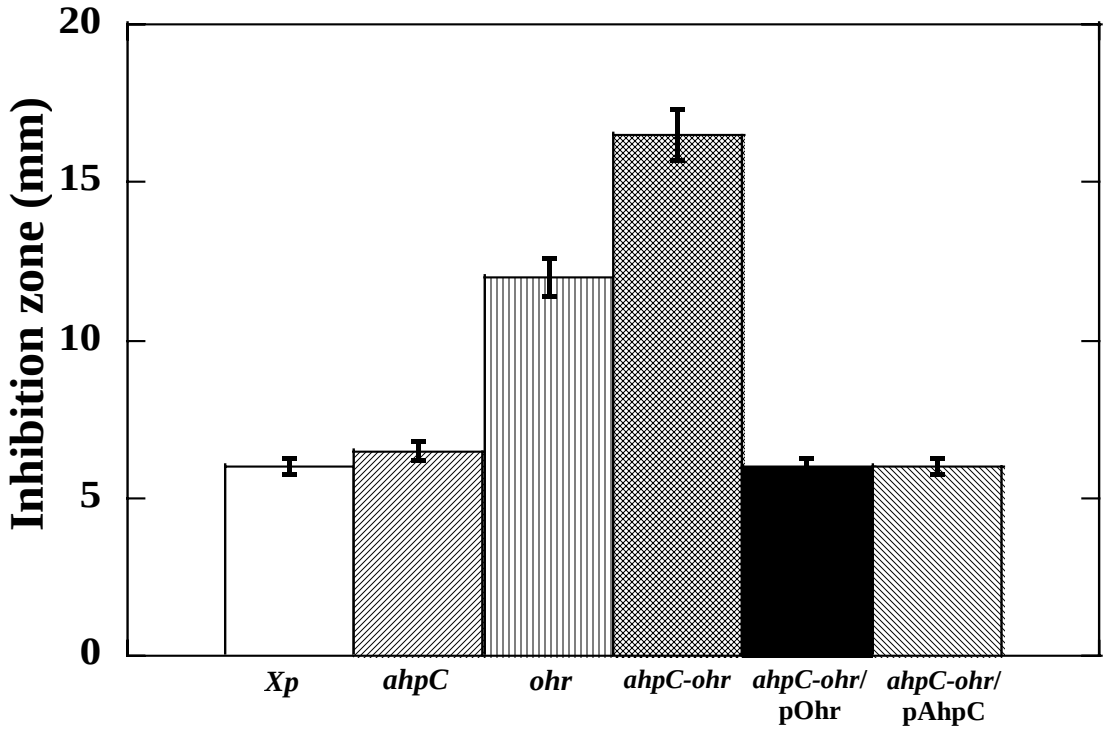


Fig. 27. Inhibition zone assays of *Xp ahpC Xp oh r*, *Xp ahpC-ohr*, *Xp ahpC-ohr/* pOhr, *Xp ahpC-ohr/* pAhpC and the parental strain (*Xp*) against LOOH.

2. Linoleic acid hydroperoxide degradation assay in various *Xanthomonas* strains

The abilities of Ohr and AhpC to degrade LOOH in *ahpC-ohr* mutants were investigated to evaluate the role of both enzymes on in vivo reduction of LOOH toxicity. The exponential phase cultures (OD_{600} of 0.5) of *Xp ahpC*, *Xp ohr*, *Xp ahpC-ohr* and the parental *Xp* strains in SB medium were subjected to 200 µM LOOH. The amounts of LOOH in the medium were measured at indicated time interval using the xylenol orange colorimetric method. The rate of LOOH degradations were determined by plotting the percentage LOOH residue against time. The results in Fig. 28 illustrated that the wild-type rapidly reduced LOOH level to about 20% residue at 30 min after addition of the peroxide. The rate of LOOH reduction in the *Xp ahpC-ohr* double mutant was significantly decreased. In this double mutant, only 25% of LOOH was degraded after 30 min incubation. When *Xp ohr* and *Xp ahpC* were tested instead, as expected, *Xp ohr* could degrade LOOH to the level of 60% residue at 30 min or only 15% below the double mutant level. While the rate of reduction in *Xp ahpC* mutant was only about 10% lower than the *Xp* parental strain. Next, the ability to degrade LOOH by cells expressing high levels of *ahpC* or *ohr* was investigated. The double mutant harboring either pAhpC or pOhr was highly efficient at degrading LOOH (Fig. 29) However, in cells harboring pOhr, 75% of LOOH was degraded compared with 55 % after 20 minutes in cells harboring pAhpC (Fig. 29) After 30 min incubation, the mutant harboring pOhr completely degraded LOOH while the mutant containing pAhpC, 20% LOOH remained (Fig. 29). Data support an idea that *ohr* plays a major role in degradation of LOOH in *X. campestris* pv. *phaseoli*.

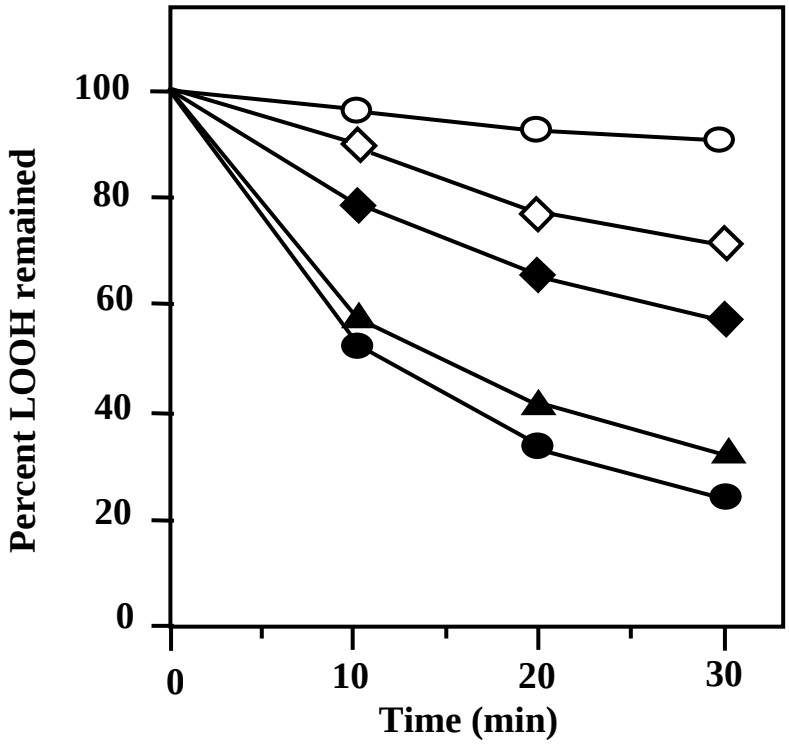


Fig. 28. The rates of LOOH degradations in *X. campestris* pv. *phaseoli* (•), *ahpC* mutant (▲) *ohr* mutant (■), and *ahpC–ohr* double mutant (□) were measured and compare to the control without bacterial culture (o). The experiments were independently repeated three times and typical results are shown.

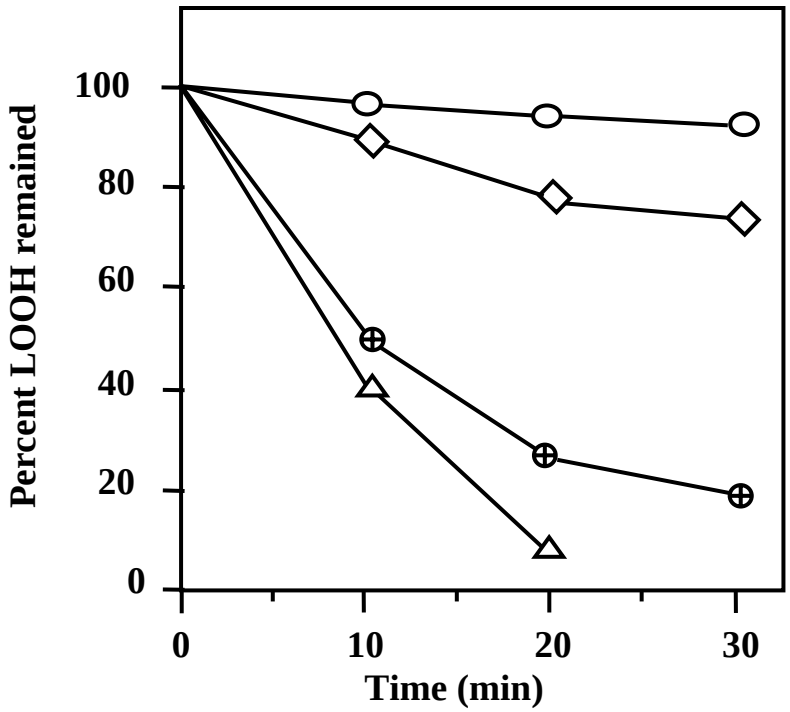


Fig. 29. The effect of high expressions of *ahpC* and *ohr* genes on LOOH degradations. The rate of reduction in *Xp ahpC-ohr* harbouring pOhr (Δ) and pAhpC (⊕) were measured and compared to *Xp ahpC-ohr* double mutant (□) and the control without bacterial culture (o).

3. The effect of LOOH on induction of *ohr* and *ahpC* expression

The expressions of *ahpC* and *ohr* are regulated by different transcription regulators namely OxyR and OhrR respectively. OxyR could be oxidized to an active form of transcription activator by exposure to peroxides including H_2O_2, tBOOH and CuOOH. In contrast to OxyR, OhrR could be oxidized and deactivated its function as transcriptional repressor only by treating the bacterial cultures with organic hydroperoxides, tBOOH and CuOOH. To determine whether pretreatment of bacterial cultures with sublethal concentration of LOOH, a hydrophobic fatty acid hydroperoxide, could alter level of *ahpC* and *ohr* expressions in *X. campestris* pv. *phaseoli*, Northern blot analyses were performed. Total RNA prepared from the exponential *Xp* wild-type cultures exposed to 10, 50 and 100 µM of LOOH and tBOOH (as control) for 15 min were separated on formaldehyde agarose gel electrophoresis, transferred to nylon membranes (Fig. 30 A and 31 A) and hybridized with ^{32}P-labeled *ohr* and *ahpC* DNA probes prepared as previous described. The results in Fig. 30 B showed that the level of *ohr* expression in LOOH-induced cultures were markedly increased even expose to 10 µM LOOH whereas a similar treatment with 10 µM tBOOH did not induce expression of the gene. As inducing concentrations of LOOH increased, there was a parallel increase in the magnitude of induction of *ohr* expression that reached a maximum level about 80-fold as judged from densitometric measurements, relative to the level in uninduced cells, following treatment with 100 µ M LOOH. *ohr* expression was also induced by tBOOH, but to a lesser degree. Treatment with 100 µM tBOOH induced *ohr* expression by less than 10-fold. The results in Fig. 31 B showed that the situation of observed *ahpC* expression was reversed. As was the case of *ohr*, both peroxides were able to induce *ahpC* expression. However, tBOOH was more effective than LOOH. Treatment with 100 µM tBOOH produced an 80-fold induction in *ahpC* expression levels compared to a 30-fold increase in the *ahpC* levels following treatment with 100 µM LOOH.

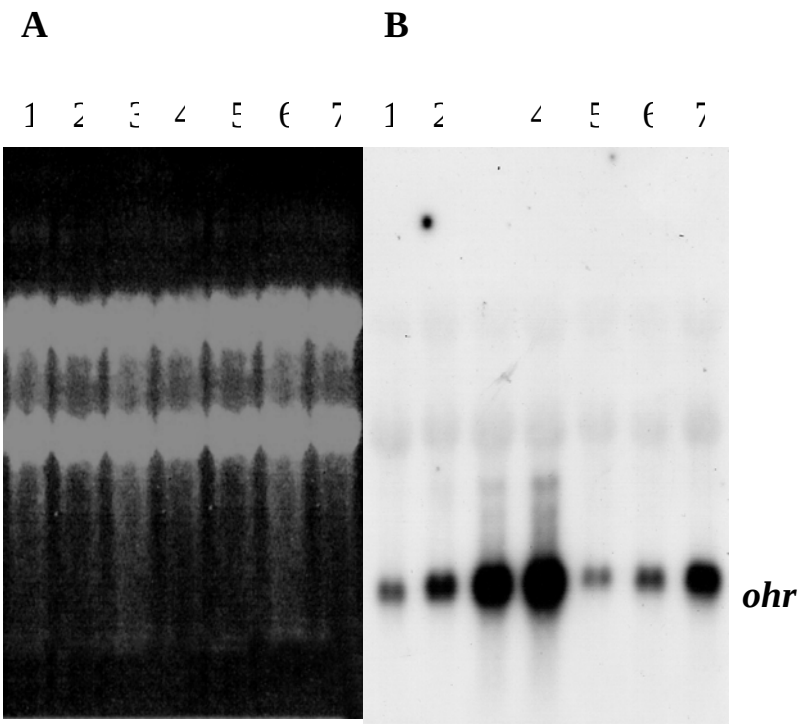


Fig. 30. Induction of *ohr* expression in *X. campestris* pv. *phaseoli* by LOOH and tBOOH. In Fig. A the membrane was hybridized with radioactively labeled *ohr* probe.

Lane1 uninduced;

Lane2 induced with 10 µM of LOOH

Lane3 induced with 50 µM of LOOH

Lane4 induced with 100 µM of LOOH

Lane5 induced with 10 µM of tBOOH;

Lane6 induced with 50 µM of tBOOH.

Lane7 induced with 100 µM of tBOOH

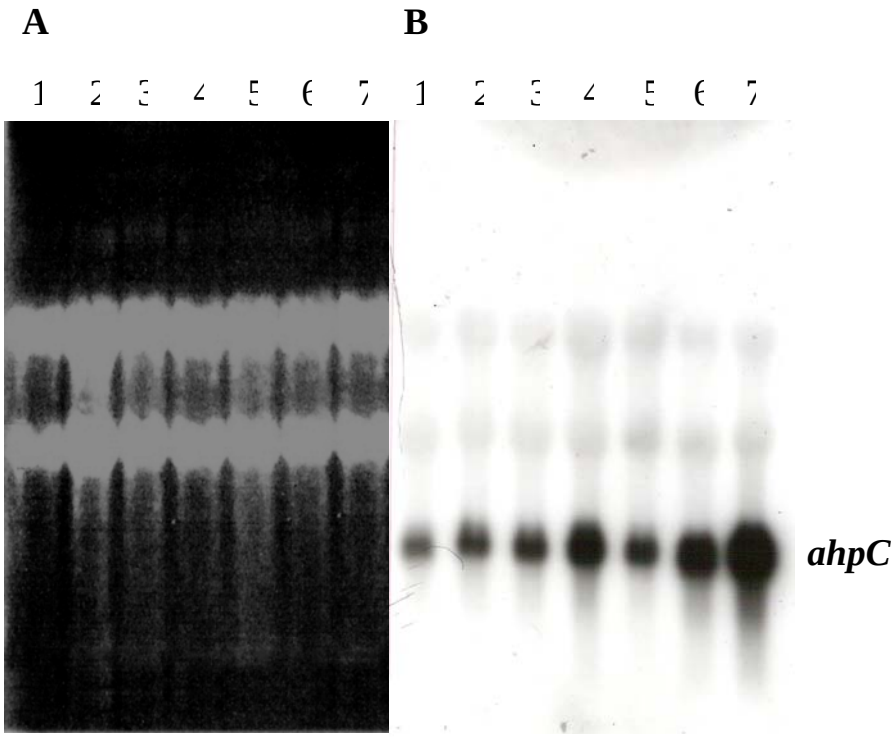


Fig. 31. Induction of *ahpC* expression in *X. campestris* pv. *phaseoli* by LOOH and tBOOH. In Fig. A the membrane was hybridized with radioactively labeled *ahpC* probe.

Lane1 uninduced;

Lane2 induced with 10 µM of LOOH

Lane3 induced with 50 µM of LOOH

Lane4 induced with 100 µM of LOOH

Lane5 induced with 10 µM of tBOOH;

Lane6 induced with 50 µM of tBOOH.

Lane7 induced with 100 µM of tBOOH

4. The effect of LOOH to the *ahpC* and *ohrR-ohr* promoters

The effects of pretreatment bacterial cultures with LOOH and tBOOH on the transcriptions of *ahpC* and *ohr* genes were determined by monitoring the promoter activities of these genes in *X. campestris* pv. *phaseoli* strains containing transcriptional fusions of the *ahpC* promoter with chloramphenical acetyltransferase (*cat*) (*Xp* TnCP1) (15), and the *ohrR* P1 promoter with β-galactosidase (*Xp ohrR* P1lacZ) (148) that were constructed by insertion of the reporter gene cassette within the chromosomal copy of *ahpC* or *ohrR*. *Xp* TnCP1 and *Xp ohrR* P1lacZ were grown to exponential phase in SB medium before the cultures were subjected to treatment with inducing concentrations of tBOOH and LOOH at indicated. Cells were harvested for preparation of crude extracts. *ahpC* and *ohr* promoter activities were monitored by determining CAT and β-galactosidase, respectively. The results in Fig. 32 showed that the *ahpC* promoter was more efficiently induced by tBOOH. Treatment of *Xp* TnCP1 with 200 µM tBOOH resulted in a 4.5-fold increase in *ahpC* promoter activity, relative to an uninduced culture, compared with only a 2.2-fold increased in the presence of 200 µM LOOH. Furthermore, induction of the *ahpC* promoter by either organic hydroperoxide depended on the presence of functional OxyR since no induction of the *ahpC* promoter was observed in an *oxyR* mutant background (*Xp oxyR* TnCP1). Analysis of the hydroperoxide dependent induction of *ohrR* P1 promoter activity was complicated by the fact that the *lacZ* reporter gene insertion in this strain inactivates *ohrR*, encoding the *ohr* repressor (148). Thus, it was necessary to first complement this strain with a plasmid-borne copy of *ohrR* (pOhrR) (147). The results were shown in Fig. 33 LOOH was more efficient at inducing P1 promoter activity than tBOOH. Treatment of the strain containing the pOhrR (*Xp ohrR* P1lacZ/ pOhrR) with 100 and 200 µM LOOH induced P1 promoter activity by 6.8- and 9.7-fold, respectively, while treatment with the same concentration of tBOOH resulted in respective increase in P1 promoter activity of 4.5- and 6.4-fold. The induction of the P1 promoter was found to be dependent on the presence of functional OhrR since the uncomplemented *ohrR* mutant strain (pBBR) did not show hydroperoxide-specific induction of the P1 promoter.

In *Xanthomonas* the mechanism of organic hydroperoxide-dependent derepression of *ohr* transcription is thought to proceed via the oxidation of the highly

conserved peroxide sensing cysteine residue, Cys-22, of OhrR (148). In order to test whether Cys-22 is required for LOOH inactivation of OhrR, a plasmid carrying a copy of the mutant *ohrR* (pOhrRC22S), in which Cys-22 has been changed to serine (C22S), was transformed into *Xp ohrR* P1lacZ and the ability of LOOH to induce the P1 promoter in this strain was evaluated. The results in Fig. 36 show that LOOH-dependent induction of the P1 promoter was abolished in *Xp ohrR* P1lacZ harboring pOhrRC22S.

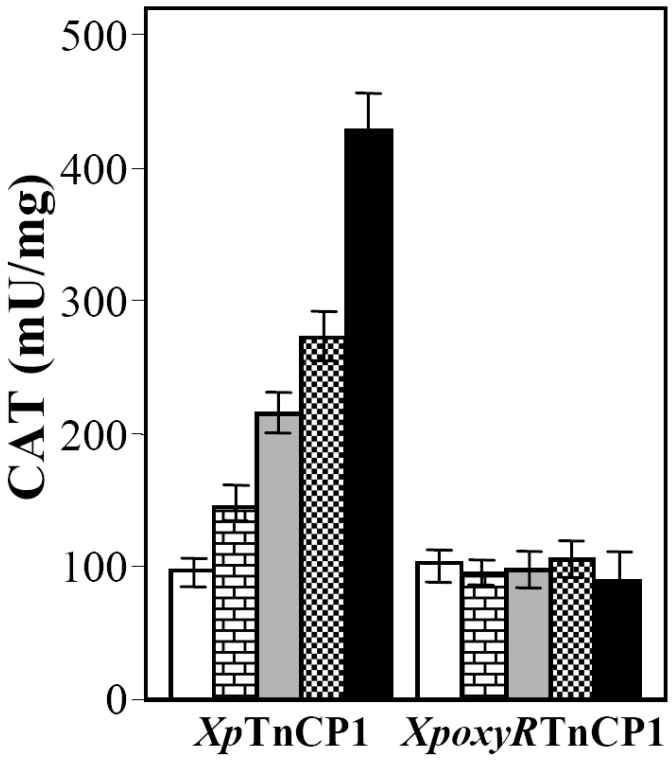


Fig. 32. Induction of the *ahpC* promoter fused to *cat* was monitored by determination of CAT activity in *X. campestris* pv. *phaseoli* TnCP1 (*Xp* TnCP1) and an *oxyR* mutant containing TnCP1 (*Xp oxyR* TnCP1). Exponential-phase cultures were untreated (open bars) or treated with LOOH (100 µM; brick bars, and 200 µM; gray bars), or tBOOH (100 µM; checkered bars and 200 µM; black bars) for 30 min. The CAT specific activities from induced and uninduced cultures are shown.

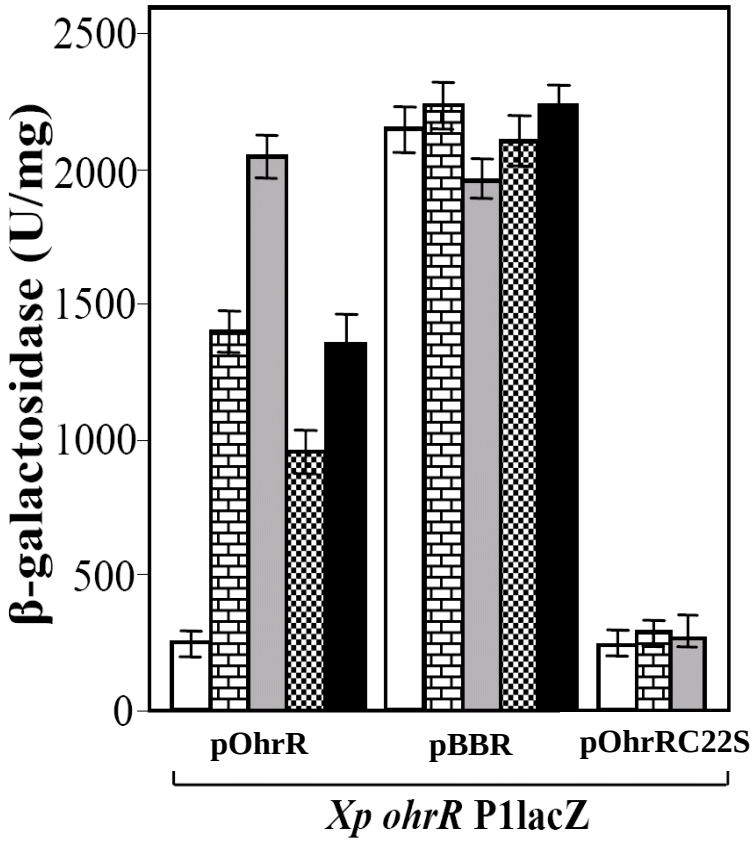


Fig. 33. Induction of the *ohrR* P1 promoter fused to *lacZ* was monitored by determination of β-galactosidase activity in an *ohrR* mutant containing a P1 *lacZ* fusion (*Xp ohrR* P1lacZ) harbouring pOhrR, pBBR1MCS-5 (pBBR) (146) and pOhrRC22S. Exponential-phase cultures were untreated (open bars) or treated with LOOH (100 µM; brick bars, and 200 µM; gray bars), or tBOOH (100 µM; checkered bars and 200 µM; black bars) for 30 min. The CAT specific activities from induced and uninduced cultures are shown.

5. LOOH activated *ohr* by directly release OhrR from the promoter

Although OhrR regulates the expression of *ohr*, the two genes are transcribed in the same operon (Fig. 34). Under normal condition, OhrR regulates the expression of *ohr* by binding to the P1 promoter and thereby represses transcription of *ohrR-ohr*. As exposure to organic hydroperoxides such as tBOOH and CuOOH, the conserved cysteine residue (Cys22) of OhrR is oxidized leading to a release of the repressor from the cognate operator region (P1) and increase in transcription (Fig. 34). Elevated expression of *ohr* stimulated by challenging *X. campestris* pv. *phaseoli* with LOOH could occur directly as a result of oxidation of Cys22 of OhrR. To clarify this possibility, the DNA mobility shift assay was performed to test the *in vitro* effect of LOOH on the ability of purified OhrR and OhrRC22S to bind to P1 promoter. Purified proteins (30-60 ng) were incubated with the radioactively labeled 170-bp P1 promoter fragment (from -95 to +50) in the presence and absence of LOOH. The results in Fig. 35 showed that the absence of LOOH, OhrR strongly bound to the P1 promoter fragment as shown by the slower-migrating P1 promoter fragment OhrR complex. Addition of 3 µM LOOH to the binding reaction completely negated OhrR binding to the P1 promoter fragment. The concentration of LOOH required to completely inhibit the binding of OhrR to the P1 promoter was 100-fold lower than that previously determined for tBOOH. The inhibitory effect of LOOH on the binding of OhrR to the P1 promoter was reversed by treatment with reducing agent 10 mM of a reducing agent (dithiothreitol: DTT). As shown in Fig. 36 the mutant OhrRC22S had no binding defect as shown by its ability to efficiently bind to the promoter fragment as wild-type OhrR. However, treatment of OhrRC22S with increasing concentrations of LOOH had no effect on the binding ability of the mutant protein to the P1 promoter.

Since LOOH is highly hydrophobic molecule, the question was raised whether LOOH could enter the bacterial cells. To clarify this, a strategy previously used to test the role of phytanic acid to release Bm3R1 repressor (159) was applied. Essentially, crude lysates were prepared from uninduced and LOOH induced cultures. The wild-type strain was grown to exponential phase in SB medium and the cultures were treated with inducing concentrations of LOOH (200 µM). Cells were harvested for preparation of crude lysates. Purified OhrR about 30-60 ng was incubated with the radioactively labeled 170-bp P1 promoter fragment in 1, 5 and 10 µg of crude lysates

prepared from either uninduced or induced cultures. The results in Fig. 37 show that addition of a lysate prepared from LOOH induced cells lead to release of OhrR from the P1 promoter fragment while a control lysate prepared from an uninduced culture had no effect. This indicates that LOOH or it derivatives do find the way to get into the cells to oxidized OhrR.

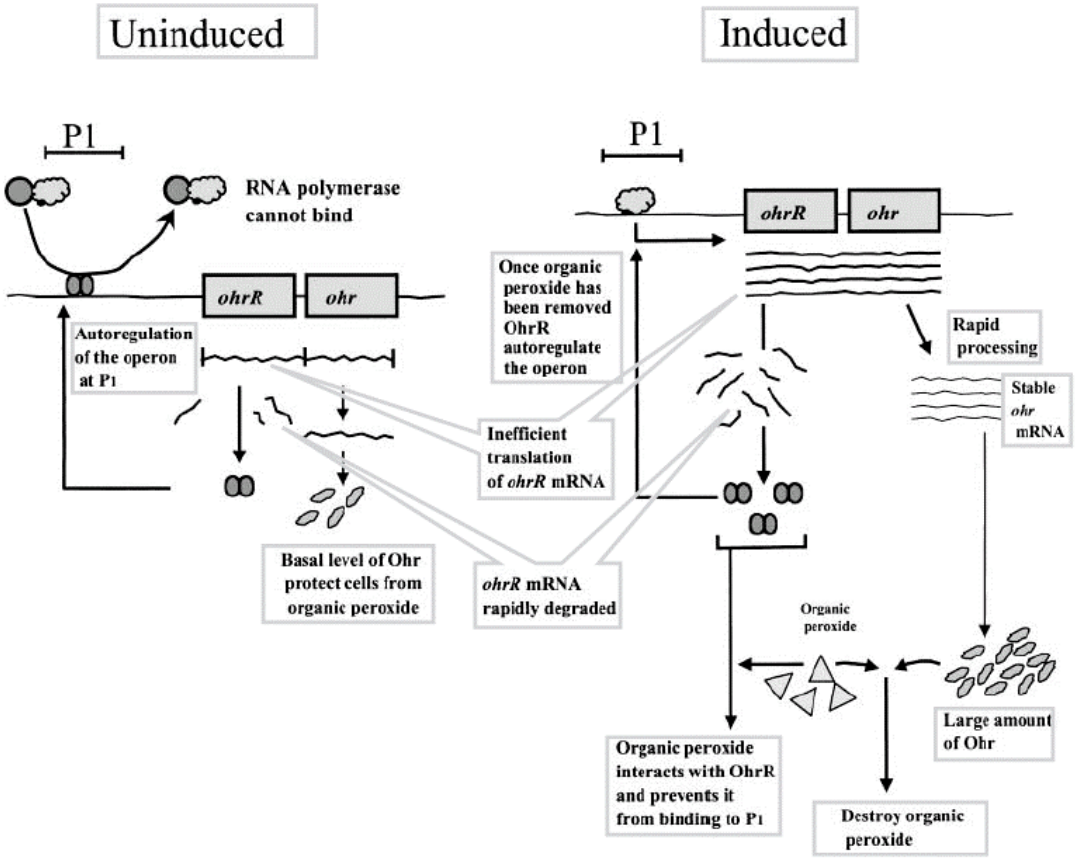


Fig. 34. A model for *ohrR* regulation at transcriptional and post-transcriptional levels.

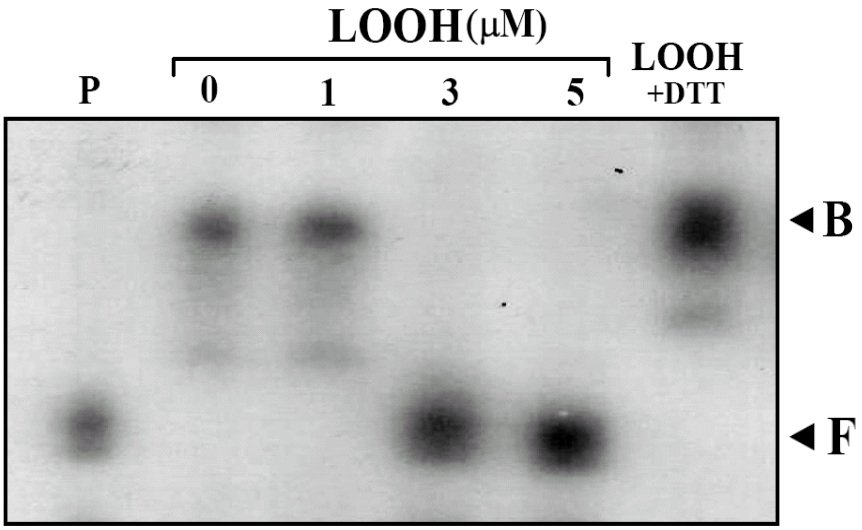


Fig. 35. The effects of LOOH and DTT on OhrR binding to the *ohrR-ohr* promoter. The binding reactions containing the P1 promoter fragment and 3 nmol OhrR were treated with either various concentrations of LOOH or 5 μM LOOH followed by 10 mM DTT treatment (LOOH+DTT). The numbers on the top of each lane indicated the concentrations of LOOH added. P indicates free probe. The positions of bound (B) and free (F) probes are indicated.

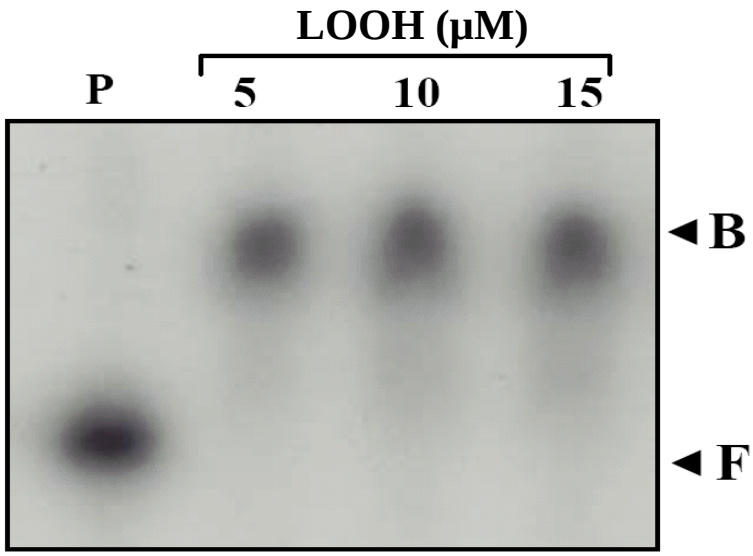


Fig. 36. The binding of P1 promoter to 3 nmol of purified OhrRC22S before 5, 10 and 15 μM LOOH were added to the binding reactions. The numbers above each lane indicated the concentrations of LOOH added. P indicates free probe. The positions of bound (B) and free (F) probes are indicated.

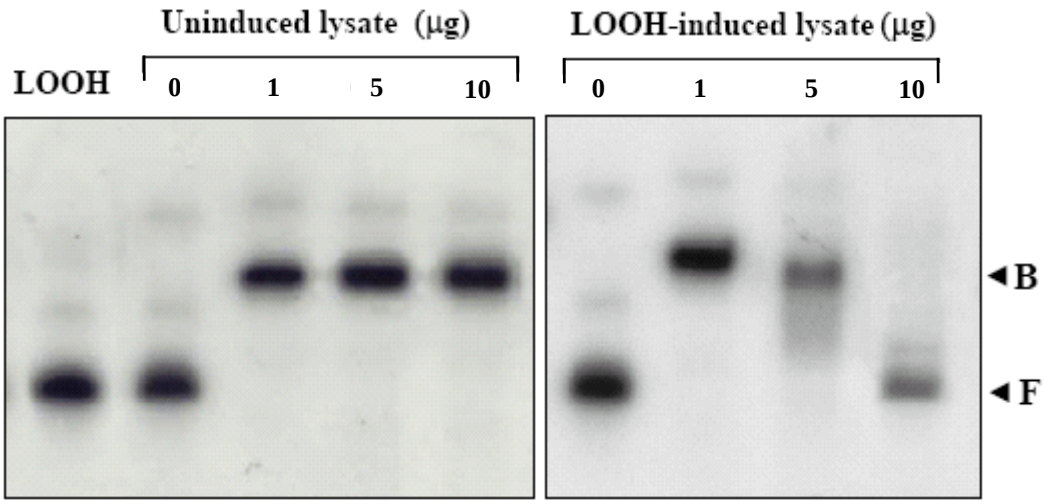


Fig. 37. The binding reactions containing the P1 promoter fragment and 3 nmol OhrR were treated with crude lysates prepared from either uninduced or LOOH induced cell cultures. The numbers on the top of each lane indicated the concentrations of crude lysates added. LOOH indicates the binding reaction was treated with 5 µM of LOOH. The positions of bound (B) and free (F) probes are indicated.

6. Adaptive protection against LOOH required functional *ohr*

Induced adaptation is an important strategy for bacteria to survive stressful environment. Experiments were done to test if *Xanthomonas* have he capacity to mount an adaptive response to LOOH and whether *ahpC* and *ohr* were involved in the process. The *Xp* wild-type was cultivated to mid-exponential phase and pretreated with 50 µM LOOH for 30 min. The induced cultures were then exposed to lethal concentrations (1, 2 and 3 mM) of LOOH for 30 min. The bacteria survived the treatment were counted by plating on SB agar plate. Colonies were scored after 48 h incubation at 28^{o}C and the fractions of surviving cells were determined by the number of cells after treatment divided by the number of cells before the treatment. The results in Fig. 38 show that LOOH induced cells were 50-fold more than resistant to LOOH killing than uninduced cells. Experiments were then performed using *ohr* and *ahpC* mutants in order to determine the roles of *ohr* and *ahpC* in the LOOH adaptive response. Pretreatment of an *ahpC* mutant with LOOH could induce high-level resistance to subsequent LOOH killing (Fig. 39). By contrast, a similar preexposure of the *ohr* mutant to LOOH failed to induce increased protection, relative to uninduced cells, against subsequent LOOH killing treatment (Fig. 40). Clearly, *ohr*, but not *ahpC*, is required for the LOOH adaptive response. The investigation by determining whether proper regulation of *ohr* or simply the presence of functional *ohr* was required for LOOH adaptive response was performed. The LOOH adaptive response experiment was repeated using the *ohrR* mutant. The results are in Fig. 41. Pretreatment of the *ohrR* mutant with LOOH did not induce adaptive protection against subsequent LOOH killing indicating that proper regulation of the operon is required for the LOOH-induced adaptive response.

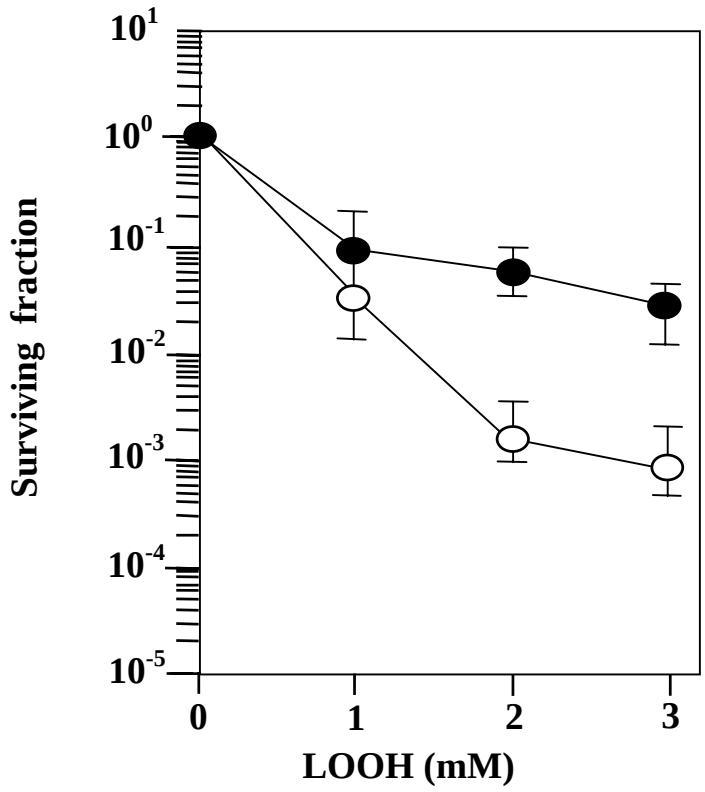


Fig. 38. Induced adaptive protection response to LOOH in *X. campestris* pv. *phaseoli*. Plots of surviving fraction of the cells in cultures that did (•) and did not (o) receive a pretreatment are shown.

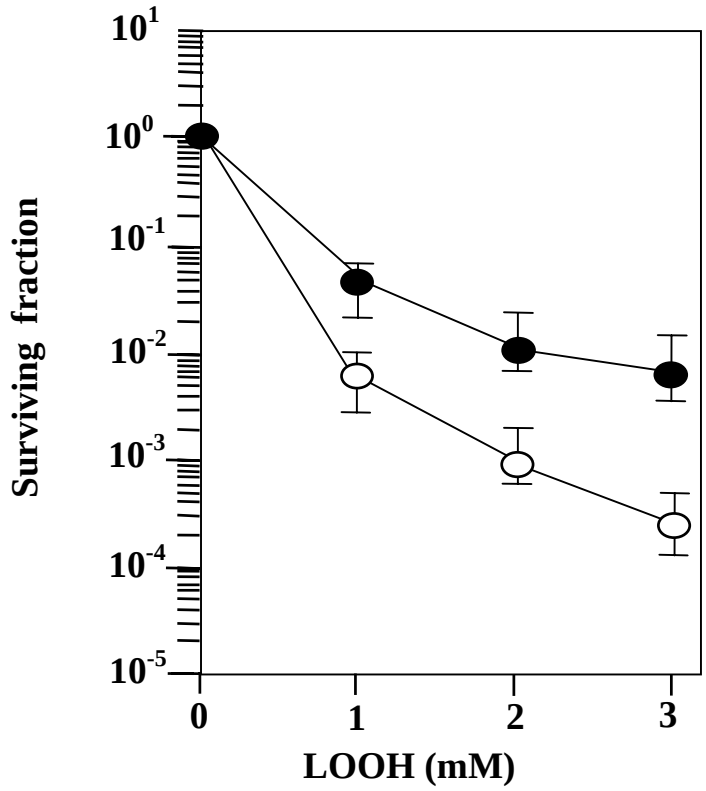


Fig. 39. Induced adaptive protection response to LOOH in *ahpC* mutant. Plots of surviving fraction of the cells in cultures that did (•) and did not (o) receive a pretreatment are shown.

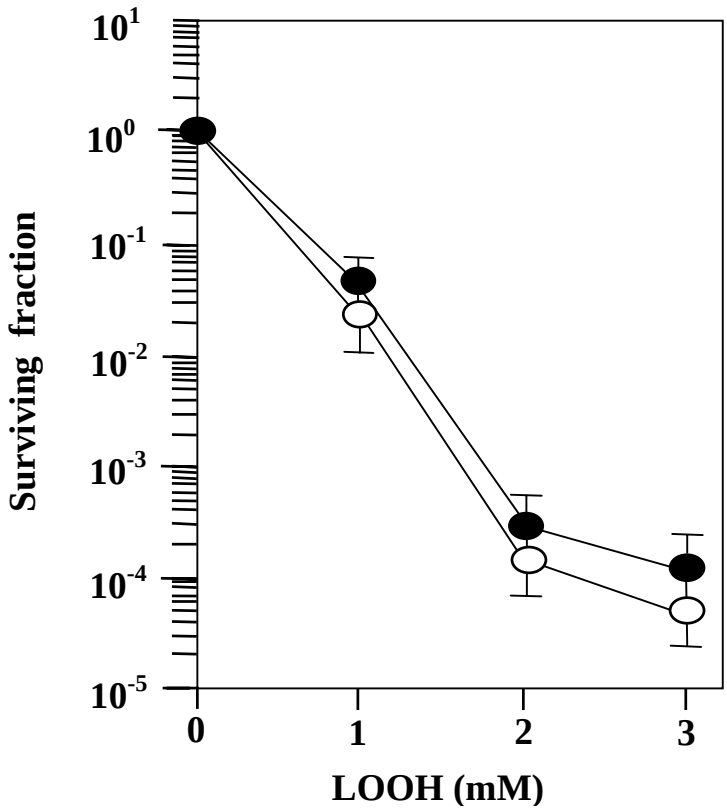


Fig. 40. Induced adaptive protection response to LOOH in *ohr* mutant. Plots of surviving fraction of the cells in cultures that did (•) and did not (o) receive a pretreatment are shown.

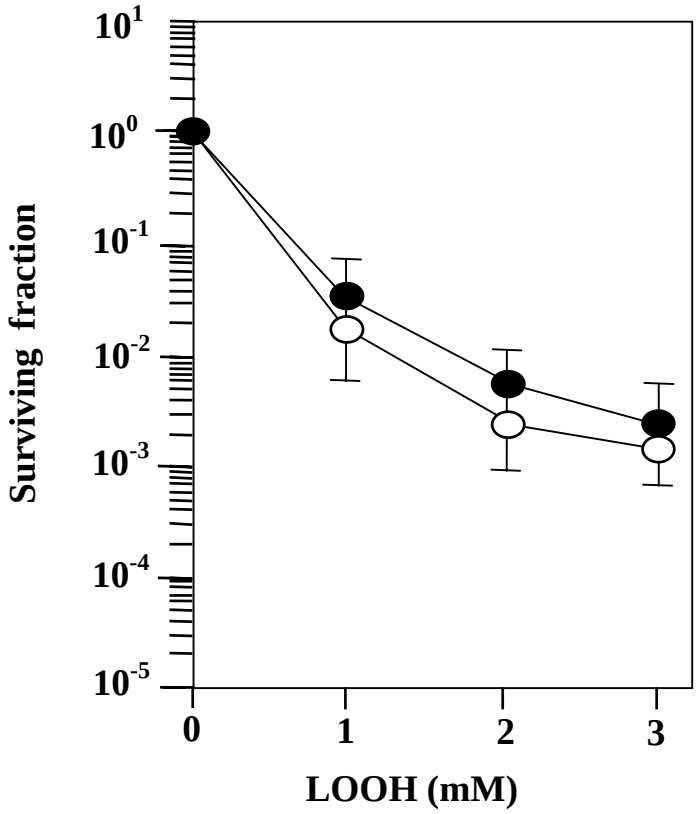


Fig. 41. Induced adaptive protection response to LOOH in *ohrR* mutant. Plots of surviving fraction of the cells in cultures that did (•) and did not (o) receive a pretreatment are shown.

CHAPTER V
DISCUSSION

X. campestris pv. *phaseoli* (*Xp*) is important in the epidemiology of bacterial common blight in dry beans (*Phaseolus vulgaris* and *Phaseolus lunatus*) and can be present as an epiphyte on bean seeds, leaves and pods. This pathogen has to encounter reactive oxygen species (ROS) including H_2O_2, $O_2^{\bullet-}$ and organic hydroperoxides from its aerobic metabolism and host initial defense systems during plant-microbe interactions. Organic hydroperoxides are highly toxic to biological macromolecules including proteins, membranes, and nucleic acids. They can participate in free radical reactions by reacting with lipid molecules to generate more toxic organic radicals. Lipid hydroperoxides are also produced during plant defense response by lipoxygenase enzymes. These enzymes catalyze the formation of fatty acid hydroperoxides through the reaction of fatty acid precursors such as linoleic and linolenic acids, which are the common fatty acids in plant membranes, with molecular oxygen. Many enzymes have been reported to contribute to the protection of bacteria from organic hydroperoxides such as glutathione peroxidase-like (Gpx-like), bactoferritin comigratory protein (BCP), and thiol peroxidases (9, 156). The best-characterized bacterial system for the detoxification of organic hydroperoxides is the alkyl hydroperoxide reductase (AhpR), which consists of two subunits, a catalytic AhpC and a reductase AhpF (10). Homologues of *ahpC* are widely distributed in diverse organisms (12, 154). It has been shown to be involved in converting organic hydroperoxide into the corresponding alcohols in NADH dependent manner (11). In many bacteria, inactivation of *ahpC* results in increased sensitivity to organic hydroperoxides and pleiotropic alterations in the oxidative stress response (13, 14, 146, 152, 153). A second system for organic hydroperoxide protection, designated *ohr*, has been discovered in *Xanthomonas* (16). *ohr* homologues are wildly distributed in both gram-negative and gram-positive bacteria (17, 19, 92, 93, 138). The structure and mechanism of Ohr have been elucidated (92, 155). Ohr is a dithiol-dependent

peroxidase that catalyzes the reduction of an organic hydroperoxide to its corresponding alcohol (21). Thus, AhpC and Ohr appear to have similar biochemical properties and possibly overlapping physiological functions. Unlike *E. coli* whose possesses only AhpC, *X. campestris* pv. *phaseoli* produces both AhpC and Ohr. Although the expression of *ahpC* and *ohr* could be induced in response to the presence of sublethal concentrations of organic hydroperoxides, the genes are independent regulated. *ahpC* is a member of *oxyR* regulon regulated by a peroxide sensor and transcription regulator, OxyR (15, 153), whereas *ohr* is controlled by the OhrR, a transcription repressor in the MarR family (20). These make the expression pattern of both genes differ upon exposure to different stimuli. The expression of *ahpC* is induced by various oxidants including H_2O_2, superoxide generating agents and organic hydroperoxides but *ohr* is strongly induced only by organic hydroperoxide (15, 16). Additionally, H_2O_2 induces increased expression of *ohr* has been reported in *Pseudomonas aeruginosa* using DNA microarray technique. This induction has never been observed in *X. campestris* with Northern hybridization.

In this study, the physiological and biochemical roles of AhpC and Ohr in the protection against organic hydroperoxides including synthetic and lipid hydroperoxides were evaluated. In order to elucidate the significance of *ahpC* and *ohr* in protection of organic hydroperoxides, the *Xp ahpC-ohr* double mutant was firstly constructed. Inactivation of genes involved in oxidative stress protection, in many cases, leading to a reduction of bacterial ability to cope with stresses thereby affecting their growth rates under aerobic condition. The results (Fig. 18) clearly illustrated that functional loss of a single *ahpC* or *ohr* and double *ahpC-ohr* in *X. campestris* pv. *phaseoli* caused non-detectable defect on aerobic growth rate in rich medium (SB). This implied that under culturing condition, the concentration of intracellular organic hydroperoxides generated from aerobic metabolism is below harmful level that could be capable of retarding bacterial growth. However, addition of sub-inhibitory concentration of tBOOH and CuOOH (100 µM) to the cultures severely inhibited the growth rate of *Xp ahpC-ohr* double mutant. This concentration of organic hydroperoxide did not cause any effect on the growth of the single *Xp ahpC* or *Xp ohr* mutant and the parental wild type. These data indicated that the *Xp ahpC-ohr* mutant

was extremely susceptible to organic hydroperoxides (Fig. 19, 20). The phenotype was good agreement with observations from the inhibition zone assays (Fig. 21 and 22) that the *Xp ahpC-ohr* mutant highly enhanced sensitivity to killing concentrations of synthetic organic hydroperoxides, tBOOH and CuOOH (500 µM) than the single *Xp ahpC* or *Xp ohr* mutant. When the zone sizes which inversely correlated with the resistance level of the bacteria are taken into consideration, the data (Fig. 21 and 22) revealed that the *ohr* mutant was slightly more sensitive to both tBOOH and CuOOH suggesting that the Ohr system play at least equal or a more important role in protection of exogenous organic hydroperoxide as compare to the AhpC. The results were consistent with data derived from complementation experiments where high expression of *ohr* from pOhr plasmid in the *ahpC-ohr* double mutant was capable of protecting the cells from tBOOH and CuOOH better than that of *ahpC* (from pAhpC). Interestingly, the full protection required both gene products since the level or resistance conferred by each of these genes in double mutant was still less than that of the parental strain (Table. 5). When experiments were performed with long chain fatty acid hydroperoxide, LOOH, *Xp ohr* was much more sensitive to LOOH than the parental wild type *Xp*, while the zone from *Xp ahpC* was just slightly but non-significant wider than its parental strain. Similar to observation in synthetic organic peroxide, *ohr* seems to be more important than *ahpC* in term of protection against hydrophobic organic hydroperoxide. Moreover, *ahpC* also appears to have a physiological role on protection against LOOH since the *Xp ahpC-ohr* double mutant was comparatively more sensitive than *Xp ohr* (Fig. 27). These data emphasized that the *Xp ahpC ohr* mutant highly increased in sensitivity to both synthetic organic hydroperoxides and lipid hydroperoxides. High expression of either *ahpC* or *ohr* failed to fully restore the synthetic organic hydroperoxide resistance level of the *Xp ahpC-ohr* back to the wild type *Xp* level suggest that these genes would function synergistically but in separate pathway to protect this pathogen from organic hydroperoxide toxicity. In addition to *Xanthomonas*, many Gram negative and Gram positive bacteria such as *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Streptomyces coelicolor*, have at least one homologous gene from each of the *ahpC* and *ohr* families (17). However, some bacteria including *Agrobacterium tumefaciens* contains only an

ohr homologue. The ubiquitous distribution of *ohr* and together with the observation in *X. campestris* pv. *phaseoli* lead to a belief that Ohr has primary role in organic hydroperoxide detoxification in bacteria (12, 146). More recent report in *X. campestris* pv. *phaseoli* revealed that AhpC play a crucial function in protecting bacterial cells from exogenous H_2O_2 (164).

The *in vivo* ability of *ahpC* and *ohr* in organic hydroperoxide degradation was evaluated in the *Xp ahpC* and *Xp ohr* mutants. The rates of CuOOH and LOOH degradations in the *ohr* mutant were comparable to the basal rates in the *Xp ahpC-ohr* double mutant, while the rates of the peroxides reductions in *Xp ahpC* mutant were only slightly lower than the parental strain levels (Fig. 24, 28). The tBOOH degradation rate of the *ahpC* mutant was higher than that of the *ohr* mutant (Fig. 23). The data suggest that AhpC and Ohr systems are capable of degrading both synthetic organic (tBOOH and CuOOH) and lipid hydroperoxides (LOOH), but through different pathways. However, these could be difference in the efficiency in which each system handled various substrates. Ohr is more efficient at degrading complex organic peroxides such as CuOOH and LOOH than AhpC, while both proteins efficiently removed simple organic peroxide (tBOOH). The Ohr is also slightly more efficient at tBOOH degradation. A possible explanation for this observation could be due to a difference in the cellular location of the two enzymes. Ohr is structurally related to OsmC, a putative thiol peroxidase that is localized in the periplasmic space (92, 157). By contrast, AhpC is likely to be a cytoplasmic protein (10). Thus, periplasmic Ohr could efficiently detoxify CuOOH and especially polyunsaturated fatty acid hydroperoxide, LOOH, before they entered the cytoplasm, thereby limiting damage to intracellular macromolecules. Additionally, *Xp ahpC-ohr* double mutant could still reduce peroxides in small amounts at 30 min comparing with the control experiment without bacterial cells. These data implied the presence of other mechanisms in this pathogen, which are able to degrade organic hydroperoxides (Fig. 23, 24, 28, 29). Glutathione peroxidase (Gpx) has been shown to display the ability to reduce fatty acid hydroperoxides in the eukaryote systems (158). Recently, *in vitro* functional analysis of the Gpx-like protein in cyanobacteria, *Synechocystis* sp. revealed that this enzyme is capable of reducing several unsaturated fatty acid hydroperoxides including

LOOH (9). The genes encode proteins homologue to Gpx-like are also existed in the *X. campestris* genome (XCC1413, XCC4213 and XCC1501). Thus, Gpx-like could be one of the possible mechanisms accounted for reduction of peroxides in *X. campestris* pv. *phaseoli*. This hypothesis needs intensive investigation.

ahpC and *ohr* expressions are highly induced by tBOOH and CuOOH (15, 16). The expressions of *ahpC* and *ohr* genes are regulated by different transcriptional regulators namely OxyR and OhrR as described earlier. The sensitivity of each system to different organic hydroperoxides was evaluated using Northern blot analyses probed with radioactively labeled *ohr* or *ahpC*. The expression levels of *ahpC* gene in the *ohr* mutant at all CuOOH concentrations (10, 50 and 100 μM) were higher than those obtained from the parental strain, while *ohr* expressions did not show the different degree of inductions in both the parental strain and the *ahpC* mutant (Fig. 25 and 26). These findings are in contrast to those made with *Bacillus subtilis* and *P. aeruginosa*, where inactivation of *ahpC* led to higher induction of *ohr* at lower concentrations of organic peroxide (18, 19). The data imply that *ohr* prevents intracellular accumulation of CuOOH by limiting its entry into the cells and/or facilitating removal of the peroxide, for the reason that inactivation of *ohr* leads to a higher intracellular concentration of CuOOH that either directly or indirectly converts OxyR from a reduced to an oxidized form and, consequently, activates the *ahpC* promoter (15). This idea is further supported by findings that the *ahpC* promoter is differently regulated by OxyR and the oxidized OxyR activates transcription from the promoter (15). In contrast, inactivation of *ahpC* did not alter the level of CuOOH induced *ohr* expression (Fig. 26). This suggests that the level of Ohr in the *Xp ahpC* mutant is sufficient to prevent an excessive intracellular accumulation of CuOOH that would be expected to inactivate OhrR, a negative regulator of *ohr*, and enhance expression of *ohr*.

Northern experiments were extended to assess the ability of fatty acid hydroperoxide LOOH to induce expression of *ahpC* and *ohr* and compared with the less hydrophobic organic hydroperoxide tBOOH. The magnitudes of inductions of *ohr* expressions are dose dependants by LOOH and tBOOH. However, LOOH is a stronger inducer than tBOOH (Fig. 30). The situation is reversed in *ahpC* expression

(Fig. 31). In the similar manner for *ohr*, both organic hydroperoxides could induce *ahpC* expression but tBOOH is more effective than LOOH (Fig. 31). The data clearly show that the regulation of *ohr* is more sensitive to the complex and hydrophobic organic hydroperoxide, LOOH, than to the simple organic hydroperoxide, tBOOH. In contrast, the *ahpC* regulation is more responsive to tBOOH than to LOOH. These observations were extended by determining the effect of LOOH and tBOOH on the regulation of *ahpC* and *ohrR-ohr* by monitoring the promoter activities of these genes using strains containing transcriptional fusions of the *ahpC* promoter with chloramphenical acetyltransferase (*cat*) (*Xp* TnCP1) (15) and the *ohrR* P1 promoter with β-galactosidase (*Xp ohrR* P1lacZ) (148). The result supports the Northern blot results. tBOOH could induce the *ahpC* promoter better than LOOH (Fig. 32) but the *ohrR* P1 promoter is more efficiently induced by LOOH (Fig. 33). These data confirmed that the observed increase in the levels of *ahpC* and *ohr* mRNA, in response to organic hydroperoxide treatments, are due to increased rates of *ahpC* and *ohr* transcription. The data also show that in the presence of peroxides, the *ahpC* and *ohr* promoters are induced by separate peroxide sensing regulatory systems. It appears that both OxyR and OhrR can sense lipid hydroperoxide levels with the latter being more sensitive to the presence of the more complex and hydrophobic hydroperoxide, LOOH, while OxyR is more sensitive to the simple organic hydroperoxide molecule, tBOOH. This may be due to the difference in the biochemical property and three dimensional structures of both transcriptional regulators. The crystal structure of *E. coli* OxyR has been dissolved. The sensing redox-active Cys-199 resides in the narrow hydrophobic pocket and its side chain directs toward the interior (161). This makes the protein more susceptible to be oxidized by small molecular weight peroxide than the complex hydroperoxides. Unfortunately, the structure of OhrR has not been characterized yet and the mechanism of which the active cysteine senses hydroperoxides is still unclear. Studies in *X. campestris* pv. *phaseoli* OhrR reveals that the redox active Cys-22 is required for deactivation of OhrR induced upon exposure to tBOOH and CuOOH (148). Experiment has been carried out to find whether Cys-22 is essential for LOOH induction. The results illustrated that the P1 promoter (P1::*lacZ*) loses the LOOH-dependent induction when Cys-22 of the OhrR

has been mutated to serine (OhrRC22S) (Fig. 33). This indicates that a derepression of OhrR on the P1 promoter induced by LOOH requires Cys-22 as in cases of tBOOH and CuOOH. Cys-22 locates in the highly conserved region and the mechanism of organic hydroperoxide dependent derepression of *ohr* transcription is thought to proceed via the oxidation of this cysteine residue. The question could be raised whether OhrR is directly oxidized by LOOH. In the absence of LOOH, OhrR strongly binds to the P1 promoter fragment as shown by the slower migrating P1 promoter fragment OhrR complex (Fig. 35). Addition of comparatively low concentration of LOOH (3 μM) to the binding reaction completely negates OhrR binding to the P1 promoter fragment. The concentrations of LOOH require to completely inhibit the binding of OhrR to the P1 promoter are 100-fold lower than that previously determined for tBOOH (148). Next, to test whether the inactivation of OhrR by LOOH is due to direct oxidation of the protein by assessing whether the process could be reversed by treatment with a reducing agent (DTT) and determined the effect of LOOH on a non-sensing mutant protein OhrRC22S. The results show that 10 mM DTT reversed the inhibitory effects of LOOH on the binding of OhrR to the P1 promoter (Fig. 35). In addition, the mutant OhrRC22S has no binding defects as shown by its ability to efficiently bind to the promoter fragment at a similar concentration as wild type OhrR (Fig. 36). However, treatment of OhrRC22S with increasing concentrations of LOOH has no effect on the mutant protein's ability to bind to the P1 promoter (Fig. 36). The results support the idea that LOOH inactivated OhrR through the direct oxidation of the sensing Cys-22 residue. The available *in vitro* DNA binding data from this and previous studies (148) support the *in vivo* promoter analyses in showing that OhrR is 80-fold more responsive to the complex hydroperoxide, LOOH, than to the simple hydroperoxide, tBOOH. This favors the idea that OhrR may have evolved to preferentially sense complex organic hydroperoxides such as lipid hydroperoxides via oxidation of the highly conserved peroxide sensing residue Cys-22. The *in vivo* and *in vitro* regulatory characteristics of the *ohrR-ohr* operon support its role as the major system for the sensing of and protection from lipid hydroperoxides such as LOOH. The second question is whether a high molecular weight and hydrophobic fatty acid hydroperoxide like LOOH could get

inside the bacterial cells to oxidize OhrR. To clarify this point, a strategy previously used to test role of phytanic acid in the release of the Bm3R1 repressor from its DNA binding site (159) was applied. The crude lysates prepared from uninduced and LOOH induced cultures were added to binding reactions containing OhrR and the P1 fragment. Addition of a lysate prepared from LOOH induced cells lead to release of OhrR from the P1 promoter fragment while a control lysate prepared from an uninduced culture had no effect (Fig. 37). This data indicates that the highly hydrophobic molecule, LOOH, can find its way to enter the bacterial cells and proceeds the inactivation process. At present, the mechanism responsible for uptake of LOOH is not known. At high concentration of LOOH, a simple diffusion is thought to contribute to the uptake process while at low concentrations of LOOH, the energy-dependent fatty acid uptake system could be involved (160).

The inducible adaptive response against peroxide killing could play important roles in plant-microbe interactions. During initial interactions, bacteria are exposed to low-concentration of peroxides (40). These could induce protection against subsequent exposure to higher concentration of peroxides that extend bacterial survival in the plant and may affect disease progression. The adaptive response to lipid hydroperoxide, LOOH, was investigated in *Xanthomonas*. The data show that there are adaptive responses in the parental and the *Xp ahpC* mutant strains that have been pretreated with sublethal concentration of LOOH (50 µM) and subsequently exposed to lethal doses (1, 2 and 3 mM) (Fig. 38, 39). By contrast, a similar pre-exposure of the *ohr* mutant to LOOH fails to induce increased protection, relative to uninduced cells, against subsequent LOOH killing treatments (Fig. 40). Clearly, *ohr*, but not *ahpC,* is required for the LOOH adaptive response in *X. campestris* pv. phaseoli. To determine whether proper regulation of *ohr* or simply the presence of functional *ohr* is required for the LOOH adaptive response, the LOOH adaptive response experiment has been repeated using the *ohrR* mutant. As expected, pre-treatment of the *ohrR* mutant with LOOH does not induce adaptive protection against subsequent LOOH killing (Fig. 41) indicating that proper regulation of the *ohrR-ohr* operon is required for the LOOH induced adaptive response. Loss of the induced adaptive protection in *ohrR* and *ohr*, mutants, but not in *ahpC1* mutant is consistent with the data from the

physiological, and gene regulation analyses indicating that the *ohrR-ohr* system plays the major role in protecting *X. campestris* pv. phaseoli from LOOH.

An important physiological question is whether *Xanthomonas* is likely to be exposed to LOOH in its natural environment. *Xanthomonas* spp. are important bacterial phytopathogens. During plant microbe interactions, bacteria are likely to be exposed to lipid hydroperoxide produced by plants as part of an active defense response against microbial invasion. It has been shown that increased lipoxygenase, an enzyme involved in lipid hydroperoxide synthesis, is associated with the plant defense response and fatty acid precursors such as linoleic or linolenic acids are abundant in plants (162, 163). Thus, *Xanthomonas* is likely to encounter LOOH during its interaction with host plants. Interestingly, *ohr* homologues have been found in all genomes of bacterial plant pathogens thus far sequenced. This conservation of *ohr* implies its important physiological role in the protection against lipid hydroperoxide exposure during plant-microbe interactions.

CHAPTER VI
CONCLUSION

This study describes the roles of *ohr* and *ahpC* in organic hydroperoxide detoxification in *Xanthomonas campestris* pv. *phaseoli*. The data obtained can be concluded as follows:

1. *Xanthomonas campestris* pv. *phaseoli ahpC-ohr* double mutant (*Xp ahpC-ohr*) was constructed by the insertional inactivation and confirmed by the Southern blot analyses.

2. Inactivation of both *ahpC-ohr* and either of these genes caused non-detectable defect on aerobic growth rate in rich medium. The growth of *Xp ahpC-ohr* markedly retarded in the presence of sub-inhibitory concentration of tBOOH and CuOOH (100 µM) comparing with *Xp ahpC* and *Xp ohr*.

3. *Xp ahpC-ohr* mutant was highly sensitive to killing treatment with organic hydroperoxides, tBOOH (500 µM), CuOOH (500 µM) and LOOH (50 mM) than the single *Xp ahpC* or *Xp ohr* mutant. The *Xp ohr* mutant was slightly more sensitive to all organic hydroperoxides than the *Xp ahpC* mutant.

4. In complementation experiments, high level expression of *ohr* (from pOhr) in the *ahpC-ohr* double mutant could better protect the cells from tBOOH and CuOOH than *ahpC* (from pAhpC). However, full protections require expression of both genes.

5. The *in vivo* functions of *ahpC* and *ohr* in degradation of organic hydroperoxide were evaluated in various *Xp* mutant strains. AhpC and Ohr could degrade both synthetic organic (tBOOH and CuOOH) and lipid hydroperoxides (LOOH), but through different pathways. Ohr was more efficiently detoxified CuOOH and particularly LOOH than AhpC, while both proteins efficiently removed simple organic hydroperoxide (tBOOH).

6. Northern blot analyses showed that the expression levels of *ahpC* expression in the *ohr* mutant induced by CuOOH (10, 50 and 100 µM) were higher than in the parental strain, while *ohr* expressions did not show the different degree of inductions in both the parental strain and the *ahpC* mutant.

7. The ability of LOOH and tBOOH to induce *ohr* and *ahpC* expression was evaluated using Northern blot and promoter fusion analyses. It was found that LOOH, a complex lipid hydroperoxide, is a potent inducer for *ohr* expression while tBOOH, a simple organic hydroperoxide, is a strong inducer of *ahpC*.

8. Induction of *ohrR-ohr* P1 promoter by LOOH mediated by OhrR required the presence of peroxide sensing cysteine residue (Cys-22).

9. In gel shift assay demonstrated that OhrR tightly binds to the P1 promoter but the binding reaction was completely negated after addition of low concentration of LOOH (3 μM). Mutation of Cys-22 in OhrR to Ser cause no change on binding affinity of the protein to P1 promoter.

10. Crude lysates prepared from LOOH induced cultures could release OhrR from the P1 promoter fragment. The results suggest that LOOH or its metabolites could enter into the bacterial cells.

11. Pretreatment of *X. campestris* pv. *phaseoli* with sublethal concentration of LOOH induced adaptive protection against subsequent killing treatment with LOOH. This induced adaptation was absence in the *ohr* and the *ohrR* mutants indicated the importance of *ohrR-ohr* system in this adaptive response.

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APPENDIX

APPENDIX

MEDIUM

SB medium

Peptone	10	g
Yeast extrac	5	g
Sucrose	5	g
Glutamic acid	5	g

950 ml of deionized water are added and mixed until the solutes have dissolved. Adjust the pH to 7.0 with 5 M NaOH. Adjust the volume of the solution to 1 liter with deionized water and sterile by autoclaving.

LB medium

Tryptone	10	g
Yeast extract	5	g
NaCl	5	g

950 ml of deionized water are added and mixed until the solutes have dissolved. Adjust the pH to 7.0 with 5 M NaOH. Adjust the volume of the solution to 1 liter with deionized water and sterile by autoclaving.

GENERAL REAGENTS

0.5 M EDTA (pH 8.0)

EDTA ($C_{10}H_{14}N_2O_8Na_2.2H_2O$) (Sigma)	18.61	g

The chemical is dissolved in distilled water, adjusted to pH 8.0 with NaOH, and adjusted to 100 ml final volume with distilled water. The solution is sterilized by autoclaving for 10 minutes at 121°C, 15 Ib/square inches.

1.0 M Tris-Cl (pH 7.5)

Tris base (Sigma)	121.14	g

The chemical is dissolved in distilled water, adjusted to pH 7.5 with HCl, and adjusted to 1000 ml final volume with distilled water. The solution is sterilized by autoclaving for 10 minutes at 121°C, 15 Ib/square inches.

0.5 M NaOH

NaOH (Fluka)	20.0	g

The chemical is dissolved in distilled water and adjusted to 500 ml final volume with distilled water.

5.0 M NaCl

NaCl (Fluka)	29.22	g

The chemical is dissolved in distilled water and adjusted to 100 ml final volume with distilled water.

20X SSC

NaCl (Fluka)	175.3	g
Sodium citrated (Fluka)	88.2	g
Distilled water adjusted to	1000	g

The ingredient is dissolved with distilled water and adjusted to pH 7.0 with a few drops of s 10 M NaOH. Then, final volume is adjusted to 1000 ml with distilled water and sterilized.

REGENTS FOR MOLECULAR CLONING

1XTE pH 8.0 (10 mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0)

1 M Tris-Cl pH 8.0	1.0	ml
0.5 M EDTA pH 8.0	0.2	ml

These ingredients are mixed and adjusted the final volume to 100 ml with distilled water. Then, the buffer is sterilized by autoclaving for 15 minutes at 121°C, 15 Ib/square inches.

1.0 M IPTG (Isopropylthio-β-D-galactoside)

Isopropylthio-β-D-galactoside (Sigma)	2.38	g

The chemical is dissolved in distilled water and adjusted to 10 ml final volume with distilled water. Then, the buffer is sterilized by filtration through a 0.2 μm disposable filter and dispensed the solution into 1 ml aliquoted and stored at -20°C.

10X TAE buffer

Tris-base	24.2	g
Glacial acetic acid	5.7	ml
0.5 M EDTA	10	ml

Adjust to pH 7.2 and bring the final volume to 1000 ml with distilled water.

6X Gel-loading buffer

Bromophenol blue (Sigma)	25	g

The dye chemical is dissolved and adjusted the final volume to 10 ml with 40% sucrose in water.

RNAase (10 mg /ml)

RNAase (Sigma)	10.0	mg

The RNAase is dissolved in 10 mM Tris-HCl (pH7.5), 15mM NaCl and stored at -20°C.

Ethydium bromide (10 mg/ml)

Ethydium bromide (Sigma) 1.0 g

The chemical is dissolved in distilled water and adjusted to 100 ml final volume with distilled water. Then, the mixture solution should be stored in dark bottle at room temperature.

2X Binding buffer

10 mM Tris pH 8.0

25 mM $MgCl_2$

40% glycerol

2 mM EDTA

2 mM Nonidet P-40

200 mM KCl

Reagent for polycrylamide gel electrophoresis

1. Native-polycrylamide gel electrophoresis

Acrylamide solution (30%)

acrylamide 6 g

The chemical is dissolved in distilled water and adjusted to 20 ml final volume with deionized water. Then, the mixture solution should be stored in dark bottle at room temperature.

2% bis-acrylamide

bis-acrylamide 0.1 g

The chemical is dissolved in distilled water and adjusted to 5 ml final volume with deionized water.

30% ammonium persulfate

ammonium persulfate 3 g

The chemical is dissolved in distilled water and adjusted to 1 ml final volume with deionized water.

Gel mixed

30% acrylamide	5.5	ml
2% bis-acrylamide	1	ml
50% glycerol	2	ml
10X TAE	4	ml

Adjust to 1 ml final volume with deionized water.

5 % Native gel recipes

Gel mixed	20	ml
30% ammonium persulfate	75	µl
TEMED	20	µl

Running buffer

1X TAE buffer (0.04 M Tris, 0.01 M EDTA)

Table 6. Restriction enzymes with their recognition sequence

Buffer	Salt	Incubation Temperature (^{o}C)	Recognition Sequence
*Bam*HI	E	37	G'GATCC
*Eco*RI	H	37	G'AATTC
*Sac*II	C	37	G'TCGAC
*Sac*I	A	37	GAGCT'C

Table7. Restriction enzyme buffer composition

Buffer	pH	Tris-HCl (mM)	$MgCl_2$ (mM)	NaCl (mM)	KCl (mM)	DTT (mM)
A	7.5	6	6	6	-	1
C	7.9	10	10	50	-	1
E	7.5	6	6	100	-	1
H	7.5	90	10	50	-	-

BIOGRAPHY

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