Chapter 2: Metabolic Stress
Induced and ROS Mediated PCD
in Xanthomonas
2.1 Introduction

2.1.1 Metabolic stress response in bacteria

The term metabolism indicates the sum total of all the cellular chemical processes involving both catabolic and anabolic processes (Fig 2.1). These processes are very fine tuned in a healthy cell and any imbalance can lead to metabolic stress. Bacteria are metabolically much simpler but diverse organisms and therefore thrive in various harsh environments. Most bacteria are exposed to a constantly changing physical and chemical environment and respond to these changes through modifications in their metabolic activities and alterations in the levels of cellular constituents such as structural proteins, transport proteins, toxins and enzymes, which help them adapt to these changes. If required they conserve energy by genetic regulation and restricting gene expression depending upon their growth condition. For example, if simple metabolites such as amino acids and sugars are readily available in the environment then the enzymes involved in their biosynthesis are not expressed. Similarly, bacteria do not synthesize catabolic enzymes unless the respective substrate is present in the environment. Moreover, in case of lactose utilizing bacteria such as \textit{E. coli} if glucose and lactose are both present then glucose is first utilized followed by lactose because catabolism of glucose (a monosaccharide) requires two less enzymes, β-galactoside permease and β-galactosidase than lactose catabolism. Similarly, the \textit{trp} operon regulates the biosynthesis of the amino acid tryptophan such that if it is available in the environment, these genes are not transcribed.
Bacterial stress response is an interesting field of study. A condition which is stressful for one bacterial species might not be so for others and their stress response can also vary to a certain extent. *Bacillus subtilis* undergo sporulation in response to nutritional stress [2]. These spores are sturdy and can survive in various environmental conditions and germinate once they encounter favourable environmental state. The mother cell undergoes programmed death during this process. Other bacteria like *E. coli*, exhibit a stress response termed as “stringent response” upon encountering stress like nutrient deprivation, heat shock and iron limitation.
This is mediated by an alarmone (p)ppGpp (GDP 3’-diphosphate or GTP 3’-diphosphate) which affects the central dogma in the cell [3].

2.1.2 Stress Response and Toxin-antitoxin (TA) modules in bacteria

The role of toxin-antitoxin module has been well established in bacterial PCD. The TA systems are not essential for cell growth but are considered to play important roles in survival under stress conditions [4]. This module comprises of a pair of closely linked genes that encode a toxin and an antitoxin. The toxin is always a protein, whereas, antitoxin can either be a protein or anti-sense RNA. These were first observed in E. coli on low copy number plasmids and were reported to be responsible for post-segregational killing. When the cells lose these plasmids, the cured cells are selectively killed by the toxin because the antitoxin is relatively less stable and is degraded faster [5–7]. Thus the cells are ‘addicted’ to the short-lived product called antitoxin, because its de novo synthesis is essential for cell survival and for maintaining the stability of extrachromosomal elements.

The toxin targets one of the following cellular processes: DNA replication, mRNA stability, protein synthesis, ATP synthesis or cell wall synthesis [4]. The toxin-antitoxin modules are classified into five types depending on the mechanism of their gene regulation and the nature of antitoxin [4]. The antitoxin can prevent the lethal action of toxin in various ways as elaborated in chapter 1.

2.1.3 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are highly reactive forms of oxygen and are formed by its incomplete reduction [8]. It includes O₂ derived radicals such as superoxide anion (O₂⁻), hydroxyl (OH⁻), and alkoxyl (RO⁻) radicals, as well as O₂ derived non radical species such as
hydrogen peroxide ($H_2O_2$) [9] (Fig 2.2). ROS differ in their intrinsic chemical properties, lipid solubility and stability (half life) which govern its reactivity and preferred biological targets [8]. The chemical reactivity of ROS has a direct relationship with its stability i.e. longer the half life of a species greater the damaging effects produced by it. In *Escherichia coli*, the steady-state concentration of $O_2^-$ is very low ($\sim 10^{-11}$ M) mostly due to its instability and high reactivity. $O_2^-$ is unable to diffuse through membranes because of its negative charge [8]. Due to high chemical reactivity, $O_2^-$ oxidizes iron–sulphur ([Fe–S]) clusters at a rate that is almost diffusion-limited, and releases iron. The shorter half life ($10^{-9}$ s) of $HO^-$ limits its diffusion to sites of production. $HO^-$ causes oxidation of lipids, proteins and DNA due to its indiscriminate reactivity resulting in damage to the cell. $H_2O_2$ is highly reactive towards thiols as well as cysteine residues and has a longer half life ($\sim 1$ ms) than $O_2^-$. The selective reactivity and diffusibility of $H_2O_2$ through the membrane make it an ideal intercellular signaling molecule.

**Fig. 2.2 Origin of reactive oxygen species (ROS) and their cellular targets:** ROS are generated from molecular O$_2$ by its subsequent reduction. The targets of ROS have been shown in colored boxes. Since they differ in their intrinsic properties, each ROS reacts with preferred biological targets. O$_2^•$ is a by-product of respiration and is produced by NADPH oxidases. O$_2^•$ oxidizes iron–sulphur ([Fe–S]) clusters at a rate that is almost diffusion-limited, and releases iron. O$_2^•$ can react with thiols in vitro, but the slow reaction rates mean that this
cannot occur in vivo. In *Escherichia coli*, the steady-state concentration of $O_2^-$ is very low (approx $10^{-11} \text{ M}$), which reflects its instability; this is not only due to its reaction with the [Fe–S] cluster, but also to spontaneous and superoxide-dismutase-mediated $O_2^-$ dismutation to H$_2$O$_2$. The instability of $O_2^-$ and its inability to diffuse through membranes because of its negative charge make this ROS a poor signalling molecule. H$_2$O$_2$ toxicity is essentially the consequence of its reduction to HO· by metal-catalysed Fenton reaction. H$_2$O$_2$ is a poor oxidant and reacts mildly with [Fe–S] and loosely bound metals, and very slowly with glutathione and free cysteine and with methionine residues. By contrast, its reactivity towards cysteine residues can significantly increase depending on the protein environment. H$_2$O$_2$ is relatively stable (cellular half-life approx $1 \text{ ms}$). Its selective reactivity and diffusibility makes H$_2$O$_2$ fit for signalling [8].

### 2.1.4 Electron Transport Chain (ETC)

The final stage of aerobic respiration occurs through a series of oxidation-reduction electron transfer reactions that yield the energy to drive oxidative phosphorylation; this in turn produces ATP. Oxidative phosphorylation is thus the culmination of energy yielding metabolism in aerobic organisms. All oxidative steps in the degradation of carbohydrates, fats, and amino acids converge at this final stage of cellular respiration, in which the energy of oxidation drives the synthesis of ATP. In eukaryotes the flow of electrons is:

NADH dehydrogenase (Complex I) $\rightarrow$ ubiquinone $\rightarrow$ Cytochrome bc$_1$ complex (Complex III) $\rightarrow$ cytochrome $\rightarrow$ Cytochrome c oxidase (Complex IV) $\rightarrow$ O$_2$

The electron transport chain in bacteria is much more diverse than eukaryotes because it can use a number of electron donors, dehydrogenases, oxidases and reductases depending on its environment (Fig. 2.3 and 2.4). The enzymes involved in electron transport and oxidative
phosphorylation are located on the bacterial inner (cytoplasmic) membrane. This membrane is invaginated to form structures called respiratory vesicles, lamellar vesicles, or mesosomes, which function as the bacterial equivalent of the eukaryotic mitochondrial membrane.

**Figure 2.3**

![Figure 2.3: Schematic representation of electron transport chain (ETC) in bacterial membrane.](image)

Reference: Schaetzle et al., 2008 [10]

**Fig. 2.3**: Schematic representation of electron transport chain (ETC) in bacterial membrane. The number of redox intermediaries i.e. Int 1, 2, ..., N) of the ETC varies with species.

Fig.2.4. Respiratory flexibility in *Escherichia coli*. MQ, menaquinone; UQ, ubiquinone; DMQ, demethylmenoquinone [11].

### 2.1.5 Electron Donors

Microbes can use either organic molecules like carbohydrates, proteins, or inorganic molecules like nitrate, sulphide, H₂S, ferrous ions, as electron donors. In the case where organic molecules mainly donate electrons, the organism is called an organotroph, whereas, if inorganic matter is used to derive energy, the organism is called chemolithotroph.
2.1.6 Dehydrogenases

Succinate dehydrogenase, NADH dehydrogenase and lactate dehydrogenase are a few dehydrogenases used by bacteria.

2.1.7 Terminal oxidase

In most bacteria, there is more than one terminal oxidase in the cytoplasmic membrane. Hence, the respiratory chain is branched both at the dehydrogenase and oxidase sites. There are two types of terminal oxidases. Class I or the cytochrome c oxidase receive electrons from ferrocytochrome c and reduce molecular O$_2$ to water. Class II or quinol oxidases are unique to bacteria and receive electrons from ubiquinols and/or menaquinols and transfer them to molecular O$_2$ [11].

2.1.8 Terminal electron acceptors

Unlike eukaryotes, where O$_2$ serves as the terminal electron acceptor, bacteria can use a diverse range of electron acceptors like, elemental sulphur and sulphur oxyanions, organic sulphoxides and sulphonates, nitrogen oxy-anions and nitrogen oxides, organic N-oxides, halogenated organics, metalloid oxy-anions such as selenate and arsenate, oxides of transition metals (such as iron and manganese) and radionuclides (such as uranium and technetium) [11]. This respiratory flexibility has enabled bacteria to thrive even in the harshest environments.

2.1.9 Uncouplers

Uncouplers are hydrophobic molecules that uncouple respiration (i.e. electron flow through ETC) from oxidative phosphorylation [12]. Some examples of uncouplers are: 2, 4
dinitrophenol (DNP), carbonyl cyanide-p-trifluorocarbonyl-cyanide methoxyphenyl hydrazone (FCCP), and Dicumarol. Being weak acids, these bind to protons on the acidic side of the membrane and carry them to the alkaline side thereby dissipating the proton gradient (Fig. 2.5). Uncouplers are known to reduce ROS generation through ETC by preventing one electron reduction of O\textsubscript{2}. Leakage of electrons during ETC is considered as one of the important routes of free radical generation in obligate aerobic organisms [13,14]. At high proton motive force, respiration slows, so electron would accumulate on Q instead of passing down the ETC to oxygen. This would increase the steady-state concentration of semiquinone radical (QH·) which can directly transfer the electron to O\textsubscript{2} leading to increase in the rate of ROS production [12] (Fig. 2.5 and 2.6). DNP being a lipid soluble weak acid can cross the membrane barrier in both protonated or unprotonated state, and sets up a catalytic cycle that dissipates the protonmotive force leading to more oxidized ubiquinone (Q) and lesser semiquinone radical (QH\textsuperscript{-}) (Fig.2.5). Uncouplers abolish link between oxidation and phosphorylation, allowing electron transport to proceed without coupled ATP synthesis [12]. Since the formation of superoxide radical depends on the level of QH, the presence of an uncoupler results in the decrease in reactive oxygen species (ROS) generation from ETC [12].
Fig 2.5: Mechanism of action of 2, 4 dinitrophenol (DNP)

Reference: https://www.tamu.edu/faculty/bmiles/lectures/uncoupler.pdf
2.1.10 Mechanism of intracellular ROS generation in bacteria

The leakage of electrons from the bacterial respiratory chain has been observed at the NADH dehydrogenase and ubiquinone sites, and was similar to that observed in eukaryotic mitochondria [14]. Almost 87% of H$_2$O$_2$ generation in the bacterial cell has been found to be
associated with electron leakage from ETC [14]. The second possible source of ROS in the bacterial cells is the auto-oxidation of flavoproteins [16]. A number of flavoproteins such as fumarate reductase, aspartate oxidase, glutathione reductase, lipoamide dehydrogenase and glutamate synthase produce ROS \textit{in vitro}. The flavins of dehydrogenases were subsequently identified as the primary sources of the O$_2^-$ and H$_2$O$_2$. Flavoprotein autoxidation occurs when molecular oxygen adventitiously collides with the dihydroflavin of the reduced enzyme. Resultant electron transfer generates O$_2^-$ and a flavosemiquinone species. Sometimes the O$_2^-$ immediately diffuses away, but most of the time a second electron transfer occurs before O$_2^-$ escapes the active site eventually producing H$_2$O$_2$. Thus these enzymes are probably responsible for both O$_2^-$ and H$_2$O$_2$ generation. The autoxidation rates of flavoenzymes vary, depending upon the degree of flavin exposure, the flavin midpoint potential, and the residence time of electrons on it [16].

2.1.11 Metabolic rate, ROS generation and its implications

The “free radical theory of aging” was proposed by Harman more than fifty years ago [17] which suggested a correlation between metabolic rate and life span. According to this theory, a higher metabolic rate and energy turnover accelerates aging in organisms leading to early mortality [18]. This is due to the generation of free radicals or ROS which are the byproducts of metabolism in the cell. Several experiments on mice demonstrated that slowing down the metabolism by means of caloric restriction helps increase their longevity [19] [20]. In the case of fruit flies, their life span was reported to be increased by boosting the antioxidant defense mechanism like superoxide dismutase. This theory has been extended to unicellular eukaryotes like \textit{Saccharomyces} and prokaryotes as well [21]. The rate of senescence in \textit{E. coli} cells was reported to decrease significantly by reducing oxygen levels in the culture [22].
The levels of oxidized proteins were found to increase in the early stages of stasis in *E. coli* cultures thereby decreasing ribosome fidelity leading to mistranslation. The aberrant proteins synthesized were found to be susceptible to oxidative damage.

Rea et al (2003) [23] have proposed an ‘Energy Switch’ hypothesis to describe the longevity of mutants in *C. elegans*. They suggested that the relative balance between TCA-based mitochondrial dependent metabolism and alternative pathways (like glycolysis) that do not involve the entire ETC are independent of the mitochondria, may determine the overall redox homeostasis of the cell. In *C. elegans*, alternate energy pathways include malate dismutation. Such alternate pathways (like glycolysis in mammals) do not contribute much to the oxidative burden of the cell. On the contrary, metabolic pathways involving ETC tend to generate ROS leading to oxidative stress in the cell.

### 2.1.12 Role of Reactive oxygen species (ROS) in PCD

ROS are sensed by certain transcription factors in the bacterial cell [8]. Transcription factors SoxR and PerR sense superoxide and peroxide, respectively. These sensors help in keeping the levels of ROS from reaching toxic limits by inducing the expression of antioxidant and repair proteins. Additionally, the antioxidant enzymes (superoxide dismutase, peroxidase and catalase) play important roles in mitigating ROS. However, when ROS reach an unmanageable level, they can create havoc for the cell. These free radicals (mainly OH·) inflict significant damage on cellular macromolecules like lipids, proteins, and DNA. Cell death is imminent when the damage is irreparable. It has been demonstrated that certain antibiotics like kanamycin, tetracyclin execute their bactericidal action by generating ROS in the cells (the mechanism of which still remains unknown) [24]. However, two recent reports contradict these findings and indicate that ROS do not mediate antibiotic induced cell death.
ROS seem to be associated with PCD. Its generation can either precede PCD process, leading to DNA damage and induction of PCD by different mechanism. On the other hand, PCD can also inflict ROS generation in neighboring cells leading to their death. Certain toxin-antitoxin modules in bacteria such as mazEF are known to cause ROS mediated cell death in bacteria [4,27].

Marchetti et al. (2006) [28] reported that replication stress leads to cell death due to ROS production in Schizisacharomyces pombe. In both budding and fission yeasts, conditional mutants of genes encoding replication initiation factors die with elevated levels of intracellular ROS at the non-permissive temperature. In S. pombe, there seems to be checkpoint-dependent and independent pathways leading to ROS generation and cell death.

Pyruvate oxidase in Streptococcus pneumoniae and Staphylococcus aureus plays an important role in cell death during stationary phase [29]. It was observed that S. pneumoniae cells containing a mutation in the spxB gene encoding this pyruvate oxidase exhibited increased viability in stationary phase due to the absence of the reactive oxygen species (ROS), generated as a product of this enzyme’s activity [29].

2.1.13 ROS scavengers

ROS scavengers or antioxidants eliminate ROS by converting them to non toxic or less toxic molecules. They can be classified into two categories:

(a) **Enzymes as ROS scavengers:** They form the main framework of antioxidant defense system of the cell. Some of them include catalase, peroxidase and superoxide dismutase. Catalase is commonly found in all organisms. It converts hydrogen peroxide to water through the following reaction:
$2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$

On the other hand, peroxidases act on hydrogen peroxide as well as other organic peroxides (e.g. lipid peroxides) and catalyzes the following reaction:

$$\text{ROOR'} + \text{electron donor (2 e') + 2H}^+ \rightarrow \text{ROH} + \text{R'OH}$$

Majority of the $\text{H}_2\text{O}_2$ in the cell is a byproduct of the activity of a metalloenzyme, superoxide dismutase (SOD) which converts superoxide radical to $\text{H}_2\text{O}_2$ in the following way:

$$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$$

Depending on the type of co-factor present in the enzyme, SOD can be classified into three types:-

(i) Cooper (Cu)/Zinc (Zn): These SODs bind both copper and zinc and are most commonly found in the cytosol of eukaryotes.

(ii) Iron (Fe)/Manganese (Mn): These SODs bind either Fe or Mn and are mostly found in prokaryotes, mitochondria and peroxisomes.

(iii) Nickel (Ni): This SOD is commonly found in prokaryotes.

(b) Non-enzymatic ROS scavengers: These are small molecules which eliminate ROS by reacting with them to generate a non-toxic product and thus prevent the oxidation of other cellular components. Some of these include glutathione (GSH), dimethylsulfoxide (DMSO), ascorbic acid (vitamin C) and tocopherol (vitamin E). GSH undergoes oxidation to convert into GSSG which can be recycled back to GSH in the cell by the enzyme GSSG reductase (Fig. 2.7).
Figure 2.7

**Fig. 2.7: Maintenance of cellular reduced GSH status during oxidative challenge.** During peroxide elimination, the regeneration of GSH from GSSG is maintained by the GSH peroxidase and GSSG reductase system, the GSH redox cycle. The continued function of the redox cycle activity is dependent on the availability of NADPH, of which the pentose phosphate pathway is a major source. The cellular rate of NADPH supply is regulated by glucose flux in the pathway and the activity of glucose-6-phosphate dehydrogenase [30].

This chapter addresses the question whether nutritionally regulated PCD in *Xanthomonas* is an outcome of metabolic stress generated due to varied nutritional condition. To investigate this hypothesis, the status of intracellular molecules like NADH, ATP, and reactive oxygen species (ROS) under PCD inducing and non-inducing conditions was examined. Further,
impact of ROS scavengers on caspase-3 biosynthesis as well as activity, and PCD profile of Xcg was investigated.

2.2 Materials and Methods

2.2.1 Bacterial culture and growth conditions

*Xanthomonas campestris* pv. *glycines* cells were grown at 26 ± 2 °C in a rotary shaker at 150 rpm in Luria-Bertani (LB) broth {PCD inducing medium (PIM)}, or raw starch broth (RSB) {PCD non-inducing medium (PNIM); 1% starch, 0.3% K$_2$HPO$_4$·3H$_2$O, 0.15% KH$_2$PO$_4$, 0.2% ammonium sulphate, 0.05% L-methionine, 0.025% nicotinic acid, and 0.025% L-glutamate, pH 6.8±0.2}. The cell number was enumerated using the standard plate count method by serially diluting the cells in sterile saline (0.85%) and spreading an aliquot (100 µl) of the appropriate dilution on Luria Agar (LA) plates. The plates were incubated at 26 ± 2 °C for 48 h for the colonies to appear for counting [31].

2.2.2 Quantification of intracellular NADH

Alkaline extraction of NADH was carried out using protocol of Caruso *et al.* 2004, with some modifications [32]. Briefly, an aliquot (20 ml) from 18 h LB or RSB grown Xcg culture was centrifuged at 12500g for 10 min at 4°C. The cell pellet was washed once with 20 ml PBS (10 mM, pH 7.5) and suspended in 2 ml of chilled KOH (0.5 M). To this alkaline suspension, 2 volumes of cold milliQ water was added and vortexed for 2 min. The mixture was centrifuged at 12500g for 40 min at 4°C. The supernatant was collected and neutralized by adding 10% volume of KH$_2$PO$_4$ (1 M, pH 6.5). The sample was filtered through 0.22 µm filter (Millipore, USA) and analyzed using HPLC (Waters, USA). C18 column (dimension 150 mm x 4 mm) was used for analysis. Sample was loaded into a vial of the autosampler.
The mobile phase consisted of buffers A and B [A: 0.1 M KH$_2$PO$_4$, pH 6.0; and B: 0.1 M KH$_2$PO$_4$ (pH 6.0) having 10% (v/v) methanol)]. Buffers were filtered through 0.22 μm filter (Millipore, USA) and degassed. Before beginning the analysis of samples, the HPLC system was equilibrated with 50% buffer A / 50% buffer B for 30 min. The flow rate was adjusted to 1 ml min$^{-1}$. The analysis of each sample was performed using the binary gradient [32](Caruso et al., 2004): 100% buffer A for 2 min followed by sample injection, 100% buffer A for 5 min, 0-25% buffer B for 6 min, 25-60% buffer B for 2.5 min, 60 - 100% buffer B for 5 min, 100% buffer B for 7.5 min, and lastly 100% buffer A for 2 min to equilibrate the system for the next analysis. Detection was performed by measuring the absorbance at 254 nm (Waters 996 Photodiode array detector).

2.2.3 Quantification of intracellular ATP and ADP

Acid extraction of ATP and ADP was carried out based on the method described previously [33]. Briefly, an aliquot (20 ml) from 18 h grown LB or RSB grown Xcg cultures was centrifuged at 12, 500 x g for 10 min at 4°C. The cells were washed once with 20 ml PBS (10mM, pH 7.5) and the pellet suspended in 4 ml chilled perchloric acid (PCA) (0.5 M). The cell suspension was sonicated for 3 min and further incubated for 45 min with vigorous shaking at 10 min interval. The acid extract was neutralized by 0.8 x of 0.5 M KOH and 0.2x of 1 M KH$_2$PO$_4$ (pH 7.5) and kept on ice for 15 min. The potassium perchlorate precipitate was finally removed by centrifugation (12500g for 30 min at 4°C). The supernatant was filtered through 0.22 μm filter (Millipore, USA) and was subjected to HPLC analysis (Waters, USA) using C18 column (dimension 150 mm x 4 mm). Samples were loaded into a vial of the autosampler. The mobile phase consisted of buffers A (0.1 M KH$_2$PO$_4$, pH 6.0; and 8 mM tetrabutylammonium hydrogen sulphate (TBA)) and B (0.1 M KH$_2$PO$_4$, pH 6.0; 8
mM TBA, and 30% (v/v) acetonitrile). The buffers were filtered through 0.22 μm filter (Millipore, USA) and degassed. Before starting the analysis, HPLC system was equilibrated with 50% buffer A / 50% buffer B for 30 min. The flow rate was adjusted to 1ml min$^{-1}$. The analysis of each sample was performed by the following binary gradient (13): 100% buffer A for 2 min followed by sample injection, 100% buffer A for 2.5 min, 0 - 10% buffer B for 1.5 min, 10% buffer B for 2 min, 10 - 20% buffer B for 1 min, 20 - 40% buffer B for 5 min, 40 - 100% buffer B for 3 min, 100% buffer B for 5 min, 100 - 0% buffer B for 1 min, and 100% buffer A for 9 min to equilibrate the system for the next analysis. Absorbance at 254 nm was measured for detection using a Waters 996 Photodiode array detector.

2.2.4 Dichlorohydrofluorescein staining

Xcg cells were grown at 26 ± 2 °C in a rotary shaker at 150 rpm in culture media (LB or RSB) for 16 h. A 2 ml culture ($10^8$ cfu/ml) was withdrawn and centrifuged at 12500 x g for 2 min and the pellet was resuspended in 1ml saline (0.85%). It was then incubated with 2 μl H$_2$DCFDA (5 mM, prepared in absolute ethyl alcohol) at 37ºC for 30 min. An aliquot was smeared on a glass slide, air dried, and examined under a fluorescent microscope (Carl Zeiss, Germany) using oil immersion objective (1000x) and filter set 15 (Carl Zeiss, Germany; Excitation: 546 nm; emission: 590 nm).

2.2.5 Electron spin resonance (ESR) spectroscopy

Hydroxyl radical (OH$^\cdot$) formation inside the cells during the course of PCD was detected with an ESR based spin trapping system, which contained 50 mM α-(4-pyridyl-1-oxide)-N-tert-butyl-nitrone (POBN) and 250 mM DMSO. A 2 ml aliquot of 20 h grown culture containing around $10^8$cells/ml was mixed with POBN. Analysis was then performed using ESR
spectrometer (Bruker, Germany). The spin trapping spectra are the result of four signal averaged scans and were obtained at ambient temperature (26 ± 2°C). Instrument settings were as follows: Power, 15.94 mW; receiver gain, $7.96 \times 10^4$; modulation frequency, 100 kHz; modulation amplitude, 0.920 G; sweep width, 100G; and sweep time, 83.886 sec.

2.2.6 Scopoletin assay

Intracellular H$_2$O$_2$ level was measured by scopoletin assay. An aliquot of Xcg culture was withdrawn and centrifuged at 12500 x g for 5 min. In a fresh tube, one ml supernatant was mixed with fluorogenic substrate scopoletin (2.5 μM) and horseradish peroxidase (5 U/ml), and incubated for 5 min at ambient temperature (26 ± 2°C). The fluorescence intensity was measured (Excitation: 360 nm, emission: 465 nm) using spectrofluorometer (FP-6500, Jasco, Japan)

2.2.7 Assay of caspase-3-like activity

Caspase-3-like activity was assayed according to the manufacturer’s guidelines (caspase-3 assay kit, BD Pharmingen, USA). Briefly, a 1 ml aliquot of 24 h grown culture was washed twice with phosphate buffered saline (PBS) (10 mM, pH 7.5) and resuspended in saline (0.85%). The cell suspension was centrifuged at 12,500 x g for 10 min. The pellet was resuspended in 100 μl of sodium phosphate buffer (10 mM, pH 7.5), mixed with 1 ml cell lysis buffer {Tris-HCl (10 mM), sodium phosphate buffer (10 mM, pH 7.5), NaCl (130 mM), triton X-100 (1%) and sodium pyrophosphate (10 mM)} and kept at 4°C for 4 h for lysis. The cell lysate was then centrifuged at 12,500 x g for 15 min and an aliquot (50 μl) of the above supernatant was used for caspase-3 assay using synthetic fluorogenic substrate Ac-DEVD-AMC (BD Pharmingen, USA). In the control set the reaction was inhibited by 10 μl (0.1mg
ml⁻¹) of the synthetic inhibitor of caspase-3 (Ac-DEVD-CHO) per reaction. After incubation the fluorescence intensity was measured using a spectrofluorophotometer (Ex 380 nm, Em 440 nm, and bandwidth 10 nm).

2.2.8 Analysis of caspase-3-like protein biosynthesis by SDS PAGE and immunoblotting

Level of biosynthesis of caspase-3 was analysed using SDS-PAGE and Western hybridization using affinity-purified, biotin-conjugated, polyclonal rabbit anti-active human caspase-3 antibody. The cells were grown for 24 h and harvested by centrifuging at 10,000 x g for 10 min. The pellet was washed twice with PBS (10mM, pH 7.5) and suspended in sterile Milli-Q water.

The washed cell suspension was mixed with an equal volume of 2X gel loading buffer (100 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 0.002% bromophenol blue, and 200mM b-mercaptoethanol). The mixture was heated at 95°C for 10 min and centrifuged at 12,000 x g for 10 min. A 50 µl aliquot of the supernatant was loaded on a 12% (w/v) SDS-polyacrylamide gel, which was run vertically at 25 mA constant current on a PAGE system (Techno Source). Electroblotting was performed after the completion of the electrophoresis, using a Hybond–P membrane (Amersham-Pharmacia) in a chilled transfer buffer [25 mM Tris, 192 mM glycine (pH 8.3), 20% methanol] using 50 mA constant current at 4°C overnight. The blotted membrane was wetted with methanol and then equilibrated with Tris-buffered saline (TBS) (20 mM Tris, pH 7.6; 500 mM NaCl) for 30 min. This was followed by incubation with the blocking reagent (TBS containing 0.05% Tween-20 and 3% gelatin) for 1 h. The blot was washed twice with TBS–Tween-20 (0.05%) for 5 min and incubated with 10 ml (0.5 mg ml⁻¹) of affinity-purified biotin-conjugated, polyclonal rabbit anti-active human caspase-3 antibody (BD Pharmingen, USA) in 100 ml antibody buffer (TBS containing
0.05% Tween-20 and 1% gelatin) for 20 h. After incubation, the blot was washed twice with TBS–Tween-20 (0.05%) for 10 min followed by incubation in the antibody buffer containing streptavidin–horseradish peroxidase conjugate (BD Pharmingen, USA) for 1.5 h. The blot was washed once with TBS–Tween-20 (0.05%) and once with TBS for 5 min. Finally, the blot was dipped in the colour reagent solution [4-chloro-1-naphthol (Sigma)/H₂O₂] and kept static for 5–10 min or until the colour appeared. The band intensity (optical density /mm) of caspase-3-like protein from the blot was determined using a TLsee 2.0 software (demo version).

2.2.9 TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay

TUNEL assay was performed according to the manufacturer’s guidelines (APO-Direct Kit, BD Pharmingen). Briefly, 1 ml aliquot of 24 h grown culture containing ~10⁶ cfu/ml was washed twice with PBS and resuspended in saline (0.85%). The cell suspension was mixed with 50 µl DNA labelling solution [reaction buffer (10 µl), Terminal deoxynucleotidyl transferase (TdT) enzyme (0.75 µl), FITC-dUTP (8 µl) and distilled water (32.25 µl)] and incubated for 60 min at 37 °C in dark. After that 1ml rinse buffer was added and the suspension was centrifuged at 12,500 x g for 10 min. This rinsing step was repeated once more. PI/RNase staining buffer (500 µl) was added to the samples which were further incubated in dark for 30 min followed by FACS (10⁵ cells for each sample) using flow cytometry system (Partec CyFlow space, Germany) and analyzed using FCS Express V4 software (demo version).
2.2.10 Statistical analysis

The experiments were repeated in three independent sets, each set comprising of three replicates. The mean and standard deviations (SD) were calculated taking all the data points in consideration. The mean values were further compared using one-way ANOVA (analysis of variance) test for establishing the significance of variation among the means (p < 0.05). With respect to flow cytometry analysis and microscopic examinations, a representative data is presented.

2.3 Results and Discussion

2.3.1 Increased accumulation of NADH and ATP

The levels of NADH, ATP, and ADP were quantified using high performance liquid chromatography (HPLC) in Xcg cells growing in PCD inducing (LB) and non-inducing (RSB) media as shown in Fig. 2.8. Henceforth, PCD inducing medium and PCD non-inducing medium have been abbreviated as PIM, and PNIM, respectively. NADH level was found to be around 40 times higher in PIM grown cells than those grown in PNIM. This indicated a hyper active TCA cycle and metabolism in these cells since NADH is the by-product of the TCA cycle. A protein rich medium contains many freely available amino acids. Some of these amino acids readily convert to tricarboxylic acid cycle (TCA) intermediates through transamination reaction [34].

ATP levels in PIM grown cells were found to be around 1.6 times higher than the cells grown in PNIM at similar cell density. This increase was found to be statistically significant (p<0.05). Conversely, ADP levels were found to be lower in PIM and higher in PNIM. ATP/ADP ratio in PIM growing cells was found to be as high as 14 as compared to 1.2 in
PNIM grown cells. This indicated faster conversion of NADH to ATP through electron transport chain (ETC) in PIM growing cells. However, ATP level did not increase in proportion to NADH level noticed during PCD probably due to simultaneous electron leakage.
2.3.2 Estimation of free radical status in Xcg cells undergoing PCD

NADH is known to act as a pro-oxidant in bacteria as well as eukaryotic cells [35–39]. NADH is oxidized by complex I (NADH dehydrogenase) and the electrons (e-) are subsequently transferred to other respiratory complexes to eventually reduce O₂ to H₂O [38]. The proton motive force created by the movement of electrons is harnessed to produce ATP by ATP synthase. Although this process is universal among all aerobic organisms, it is inherently dangerous due to its ability to create a highly oxidative intracellular environment. The inefficient transfer of e- via the respiratory complexes results in the one electron reduction of oxygen leading to toxic ROS formation [35,38]. The status of ROS was checked
by 2’, 7’-dichlorofluorescein diacetate (H$_2$DCFDA) staining and electron spin resonance (ESR) spectroscopy. H$_2$DCFDA is a unique fluorescence precursor that rapidly diffuses inside the cells where cellular esterases cleave the acetate moiety, allowing accumulation of the membrane impermeable form H$_2$DCF [40]. Further, H$_2$DCF is usually oxidized by peroxides (e.g. H$_2$O$_2$) in the presence of peroxidase, cytochrome c, or Fe$^{2+}$ to form 2’, 7’, dichlorofluorescein (DCF) which can then be visualized using a fluorescent microscope. The assay provides a semi-quantitative measure of general intracellular reactive oxygen species (ROS) activity. The intensity of fluorescence is proportional to the levels of ROS generated within the cell. Cells from PIM culture when stained with H$_2$DCFDA fluoresced brightly under the fluorescence microscope (Fig.2.9A), whereas, negligible number of fluorescent cells was found in PNIM culture (Fig.2.9B).

Figure 2.9

Fig. 2.9: Evaluation of ROS generation in Xcg cells: H$_2$DCFDA stained- (A) PIM grown cells, (B) PNIM grown cells. An aliquot was smeared on a glass slide, air dried, and examined under a fluorescent microscope (Carl Zeiss, Germany) using oil immersion.
objective (1000x) and filter set 15 (Carl Zeiss, Germany; Excitation: 546 nm; emission: 590 nm).

The presence of free radicals was further investigated by electron spin resonance (ESR) spectroscopy using a spin trap system containing α-(4-pyridyl 1-oxide)-N-tert-butyl nitronitrone (POBN) and DMSO, which showed the presence of hydroxyl radical (OH·). In the spin trap system employed here, dimethyl sulfoxide (DMSO) reacted with OH· and converted it into methyl radical (CH₃). In addition, CH₃ is converted to methoxy radical (OCH₃) in the presence of O₂. The CH₃ and OCH₃ then reacted with POBN to form adducts [41]. These POBN adducts were detected using ESR spectroscopy. ESR studies of PCD exhibiting Xcg cells confirmed the presence of hydroxyl radical (Fig.2.10A). The triplet of POBN adducts was observed in Xcg cells undergoing PCD, but was found to be absent in PCD inhibiting condition (Fig.2.10A and B). Source of hydroxyl radical (OH·) in PCD exhibiting Xcg cells could be intracellularly generated hydrogen peroxide. The most important mechanism of OH· generation from H₂O₂ inside cells is via Fenton reaction [H₂O₂ + Fe(II) or Cu(I)→ OH· + OH· + Fe(III) or Cu(II)] [42].
2.3.3 Effect of ETC uncoupler 2, 4-dinitrophenol

It has been reported that ~87% ROS in the *E. coli* cells origins from ETC [14]. In the bacterial and mitochondrial electron transport chain (ETC), there are two sites of electron leakage which lead to ROS formation [14,40]: site 1 on complex I (NADH-Q oxidoreductase) and site 2 at the interface between mobile lipid-soluble carrier, ubiquinone (Q) and complex III (Q-cytochrome c oxidoreductase). To explore the possibility of ETC as a source of ROS in the cells, 2, 4- dinitrophenol (DNP), an uncoupler was used in this study. Uncouplers are known to reduce ROS generation through ETC by preventing one electron reduction of O$_2$ as explained earlier (Fig.2.5). When Xcg cells were grown in PIM with DNP, the cell survival was found to be increased by one log cycle (Fig. 2.11A). Hydrogen peroxide levels were significantly lower in Xcg cells grown in PIM in the presence of 500 µM DNP as detected by scopoletin assay (Fig.2.11B)
Figure 2.11

Fig. 2.11: Effect of ETC uncoupler 2, 4 dinitrophenol (DNP): (A) Xcg was grown in PIM in the presence of varying concentrations of DNP. Inhibition of PCD by DNP was observed.
in PIM growing Xcg cells in a concentration dependent manner; (B) Inhibition of intracellular H$_2$O$_2$ generation (Scopoletin assay) in PIM growing Xcg cells in the presence of DNP. Scopoletin is a fluorescent dye. Its oxidation by hydrogen peroxide (H$_2$O$_2$) leads to a decrease in fluorescence which is proportional to the levels of H$_2$O$_2$ in the medium. The different letter on bars indicates that the means are significantly different at p<0.05.

2.3.4 Effect of ROS scavengers on PCD profile of Xanthomonas

Although above studies ascertained the formation of free radicals during PCD in Xcg, it was not clear if these radicals are the cause or the effect of PCD. To answer this question, ROS scavengers dimethysulfoxide (DMSO), glutathione (GSH), n-propyl gallate (nPG), and catalase were tested for their effect on PCD. The cell survival almost doubled in the presence of DMSO (0.25 – 0.5%) compared to control at the end of 96 h incubation period and the increase was found to be statistically significant (p<0.05) (Fig.2.12A). However, the increase observed in survival was not found to be significantly affected by increase in DMSO concentration (p≤ 0.05). When GSH was added to PIM, a concentration dependent increase in cell survival was observed when assayed at 96 h of incubation and PCD was completely inhibited with 10 mM GSH (Fig.2.12B). Similar to GSH, PCD was significantly abolished with 100 μM nPG (Fig.2.12C), and 500 U/ml of catalase (Fig.2.12D). No growth was observed at higher concentrations of GSH or nPG and both were found to be more effective than DMSO in inhibiting the PCD. Caspase-3 biosynthesis was also found to be lower in cells grown in the presence of these ROS scavengers (Fig 2.13A). In comparison to PIM grown Xcg cells, the caspase-3 band intensity was 14, 25, 53, and 57% in cells grown in PIM in the presence of GSH (10 mM), DMSO (0.5%), nPG (100 μM), and catalase (500 U/ml), respectively. The inhibition of caspase-3 expression by DMSO (0.5%), or GSH (10 mM) was
quite prominent compared to npG, or catalase. This effect could be possibly due to difference in the mechanism of action of different ROS scavengers. Caspase-3 activity decreased by 15, 10, and 20% in Xcg cells grown in PIM in the presence of GSH (10 mM), nPG (100 μM), and catalase (100 U/ml), respectively as compared to Xcg cells grown in PIM alone (Fig. 2.13B). Caspase-3 activity in Xcg cells grown in PIM in the presence of DMSO (0.5%) was negligible (data not shown).
Fig. 2.12: Inhibition of PCD by ROS scavengers. PCD inhibition by: (A) DMSO, (B) GSH, (C) nPG, and (D) catalase. The different letter on bars indicates that the means are significantly different at p<0.05.
Figure 2.13

(A)

![Ponceau S stained blot](image)

(B)

![Bar chart indicating caspase-3 activity](image)
Fig. 2.13: Inhibition caspase-3 biosynthesis and activity in PIM growing Xcg cells by ROS scavengers: (A) Caspase biosynthesis inhibition (western blot): lane1: Coloured molecular weight marker, lane 2: PIM grown cells, lanes 3 - 8: PIM grown cells in presence of different ROS scavengers; 3- DMSO, 4- nPG (50 µM), 5- nPG (100µM), 6- catalase (500 units/ml), 7- GSH (5 mM), 8- GSH (10 mM). Ponceau S staining was carried out to check protein transfer and confirm equal protein amount in each lane.; (B) Inhibition of caspase-3 activity. The different letter on bars indicates that the means are significantly different at p<0.05.

To monitor the level of DNA damage, TUNEL assay was performed in the presence of above ROS scavengers. The population of TUNEL positive cells increased to 23% in PIM. It decreased significantly to 2, 4, and 6% in cultures grown independently in the presence of ROS scavengers, n-propyl gallate, catalase, and glutathione respectively (Fig 2.14).
Fig. 2.14: Inhibition of DNA damage in Xcg cells grown in PIM in the presence of ROS scavengers as assessed by TUNEL assay: (A) Xcg cells grown in PIM; (B) Xcg cells grown in the presence of GSH (10 mM); (C) Xcg cells grown in the presence of nPG (100 µM); (D) Xcg cells grown in the presence of catalase (500 units/ml).

2.3.5 Effect of catalase on survival of Xcg

Addition of catalase in PIM increased the Xcg cell survival by two log cycles (Fig 2.15 A). On the contrary, addition of Proteinase K to the same didn’t prevent PCD, hence ruling out
the possibility of protein or peptide to act as signaling molecule which has been reported earlier in many cases to have this role. This indicated that H$_2$O$_2$ plays an important role in accelerating PCD in Xcg (Fig 2.15 A and B).

The cell survival improved significantly in the presence of ROS scavengers DMSO, GSH, n-propyl gallate (nPG), and catalase. DMSO scavenges OH$^-$, whereas, nPG scavenges superoxide radical. GSH and catalase can degrade hydrogen peroxide. Maximum protection was seen in the presence of GSH, indicating a significant role of H$_2$O$_2$ during PCD in Xcg. Catalase increased the cell survival by two log cycles indicating a possible role of H$_2$O$_2$ in cell-cell signaling during PCD in Xcg, as catalase being a large molecule (250 kDa) cannot enter the cell.

Among the various types of ROS produced in a cell, due to chemical stability, and membrane permeability, H$_2$O$_2$ is considered to be one of the good candidates for involvement in intercellular signaling during the process of PCD [43–45]. H$_2$O$_2$ produced by lysine oxidase has been reported to help in biofilm differentiation and dispersal in several Gram negative bacteria [46]. Besides microbes, ROS was found to play a vital role in eukaryotic (both in plants and animals) apoptosis where it has been reported to act as a long distance cell messenger [45–47]. In animals H$_2$O$_2$ could mediate induction of apoptosis during self-elimination of organs in ontogenesis such as the disappearance of the tadpole tail [45]. Similarly, H$_2$O$_2$ production was found to be enhanced in human cervical carcinoma (HeLa) cells undergoing apoptosis [43,45].
Fig. 2.15: Growth profile of Xcg in the presence of catalase or proteinase K: (A) Inhibition of PCD in PIM growing Xcg cells by catalase; (B) Xcg growth profile in the
2.3.6 H$_2$O$_2$ accumulation

Intracellular concentration of H$_2$O$_2$ was compared using scopoletin assay [48]. The amount of H$_2$O$_2$ was measured by horseradish peroxidase catalyzed oxidation of the fluorescent dye scopoletin (7-hydroxy-6-methoxycoumarin). The fluorescence intensity was proportional to the amount of H$_2$O$_2$ present in the cell. In PNIM culture, H$_2$O$_2$ was below detectable level. H$_2$O$_2$ concentration in PIM growing cells steadily increased till 24 h and further remained stable till 48 h of incubation (Fig 2.16A). As reported earlier [31,49], PCD started at this time point only.

2.3.7 In vitro activation of Xcg caspase by H$_2$O$_2$

When PNIM grown Xcg cell lysate was exposed to H$_2$O$_2$, the level of caspase activity increased in a concentration dependent manner as evidenced by the observed increase in the fluorescence intensity (Fig 2.16B). Together these findings indicate that hydrogen peroxide is involved in induction of PCD signal in Xcg. Recently it has been shown that oxidative modification of Cys-403 of caspase-9 facilitates its activation via disulfide mediated interaction with Apaf-1 [50].

Caspase has been reported to be activated by direct oxidative modification of its cysteine residue in higher organisms [50]. H$_2$O$_2$, a mild oxidant, can oxidize specific protein sulphydryl groups, producing proteins with cysteine sulfenic acid (CysS-OH) or disulfide residues, both of which can be reduced back to Cys-SH by various cellular reductants. Very few proteins are expected to have a Cys-SH that is susceptible to oxidation by H$_2$O$_2$ in cells because this
oxidation requires that the target Cys-SH have a pKa below 7.0, whereas, the pKa values of most protein Cys-SH residues are higher than 8.0. Proteins like cysteine proteases (e.g. caspases) are known to contain an essential Cys-SH with a low pKa at their active sites [51], and is thus a potential candidate for reversible oxidation by intracellularly generated H₂O₂ [52]. Interestingly, H₂O₂ exposure activated Xcg caspase-3 activity \textit{in vitro}.
Fig. 2.16: Level of H$_2$O$_2$ in Xcg cultures and in vitro activation of Xcg caspase by H$_2$O$_2$

(A) H$_2$O$_2$ level measured in PIM and PNIM Xcg cultures by scopoletin assay; (B) In vitro activation of Xcg caspase-3 by hydrogen peroxide. The different letter on bars indicates that the means are significantly different at p<0.05.
References


