Chapter 3: Accumulation of Proline and Associated Proline Oxidase (PutA) Activity during Metabolic Stress Induced PCD in Xanthomonas
3.1 Introduction

3.1.1 Proline metabolism

Besides serving as important building blocks for proteins, amino acids can also serve as sole source of carbon, nitrogen, sulphur and energy [1]. Proline metabolism is distinct from that of primary amino acids and plays a regulatory role in certain physiological conditions [2]. Proline catabolism has been observed to be an important source of energy in some Gram negative bacteria such as Helicobacter pylori, Bradyrhizobium japonicum, Sinorhizobium meliloti, and Mycobacterium smegmatis during stress [3–7]. PutA is significantly upregulated in Mycobacterium smegmatis and is preferentially used as an electron donor to the respiratory chain during energy limiting conditions [6]. Proline is a non-essential, secondary amino acid having cyclic structure due to bonding between amine group and the two alkyl groups (Fig 3.1).

**Figure 3.1**

![Proline Structure](http://brc.se.fju.edu.tw/protein/character/kinds.htm)


**Fig. 3.1: Structure of amino acid proline**
Proline is converted to glutamate via a four electron oxidation process (Fig 3.2). Proline dehydrogenase (PRODH or POX) performs the first oxidative step, resulting in the intermediate pyrroline-5-carboxylate (P5C). P5C is subsequently hydrolyzed to glutamic semialdehyde (GSA), which is then further oxidized by P5C dehydrogenase (P5CDH) to generate glutamate. Glutamate can be converted to α-ketoglutarate through deamination, which may be incorporated into the tricarboxylic acid (TCA) cycle. In Gram-negative bacteria, PRODH and P5CDH are fused together in a bifunctional enzyme called proline utilization A (PutA). The FADH$_2$ generated during this process directly transfers the electrons to ubiquinone of electron transport chain (ETC) [8](Fig. 3.2).

Proline anabolism begins with phosphorylation of glutamate by gamma-glutamyl kinase (GK) to generate gamma-glutamyl phosphate (gamma-GP). This is subject to feedback inhibition by proline (even at very low concentrations) in bacteria [9,10]. Gamma-GP is reduced by gamma-glutamyl phosphate reductase (GPR) to GSA, which cyclizes to form P5C. P5C is then reduced to proline via pyrroline-5-carboxylate reductase (P5CR). In higher eukaryotes such as plants and animals, GPR and GK are fused together in the bifunctional enzyme pyrroline-5-carboxylate synthase (P5CS) [11]. In bacteria (such as Escherichia coli) the three enzymes; gamma-glutamyl kinase (GK), glutamate-5-semialdehyde dehydrogenase or glutamyl phosphate reductase (GPR), and pyrroline-5-carboxylate reductase (P5CR), are encoded by genes proB, proA and proC, respectively [12].
Figure 3.2

**Fig.3.2: Metabolic pathways of proline.** PRODH – Proline dehydrogenase; P5CDH – P5C dehydrogenase; P5CR – P5C reductase; GK- gamma glutamyl kinase; GPR - gamma-glutamyl phosphate reductase; P5CS - pyrroline-5-carboxylate synthase; GSA - glutamate-5-semialdehyde; P5C - pyrroline-5-carboxylate; γ-GP - gamma-glutamyl phosphate; CoQ – ubiquinone [11].

POX and P5CDH are encoded by two different genes in eukaryotes, whereas, in bacteria it is encoded by a single gene, putA [13]. PutA is located in the bacterial membrane or inner mitochondrial membrane in higher organisms. In bacteria, it typically consists of 1000-1300 amino acid residues. In some bacteria, like Escherichia coli and Salmonella typhimurium, it also contains a DNA binding domain that auto-regulates its transcription [13].

3.1.2 Proline transport in bacteria

Proline porter I and PutA regulate the catabolism of proline in E. coli [14]. They are induced when proline is provided as a carbon or nitrogen source in the environment. E. coli PutA regulates the transcription of putA and putP (Na⁺/proline symporter) genes, and it switches its intracellular location and function by sensing the environmental proline levels. When the intracellular proline levels are low, it binds to DNA and represses the transcription of put genes. Conversely, when proline is available to the cell, PutA binds to the inner membrane and catalyzes the oxidation of proline to glutamate [15]. However, in Salmonella, Klebsiella and Vibrio the putA gene expression is regulated by c-AMP receptor protein [16–18]. Xanthomonas PutA has not been studied yet and the regulation of proline metabolism in this bacterium is not fully understood.

3.1.3 Proline metabolism and stress tolerance

Paradoxically, proline has been reported to act as an osmoprotectant in certain bacteria growing under osmotic stress [19,20]. Proline and glutamine were found to accumulate in Staphylococcus aurues grown in medium containing 5 and 10% NaCl [19]. This accumulation of proline was attributed to its preferential transport into the cell [19]. Proline pool was also found to increase in several other non halophilic bacteria as well like E. coli,
Salmonella, Bacillus, Lactobacillus and Clostridium when exposed to osmotic stress [21].

Proline has been reported to stimulate growth and respiration in some bacteria [6,21]. It has also been reported to protect E. coli from cold and heat stress [22]. Besides bacteria, proline has been shown to protect fungi, plants, and mammalian cells against oxidative stress [23]. Proline accumulation has also been reported in plants during conditions of drought, salinity, intense light, UV irradiation, heavy metals, oxidative stress, and biotic stresses [24,25].

3.1.4 Proline utilization protein A (PutA)

As mentioned above, PutA (or proline oxidase/dehydrogenase) oxidizes proline to glutamate. PutA is a flavoenzyme and requires FADH₂ as a cofactor to perform the first step of proline oxidation i.e. conversion of proline to P5C. Based on the functional domains present in the polypeptide, PutA can be classified into three types (Fig 3.3):

a) Monofunctional PutA

Monofunctional enzymes are functionally similar to eukaryotic POX as they only have PRODH domain and convert proline to P5C (Fig 3.3). Monofunctional PRODHs are typically 200–540 amino acid residues in length [11]. The best studied example of this group is the PRODH of Thermus thermophilus.
Reference: Servet et al., 2012 [26].

**Fig.3.3: Structural organization of proline dehydrogenase.** DNA indicates the DNA-binding domain of PutA. Vertical hatching indicates the mitochondrial transit peptide. Cross-hatching shows the linker that connects the two enzymatic activities. FAD: flavin adenine dinucleotide, NAD: nicotinamide adenine dinucleotide, P5CDH: P5C dehydrogenase, P5CR: P5C reductase, ProDH: proline dehydrogenase, PutA: proline utilization A [26].
b) Bifunctional PutA

Bifunctional PutA have both PRODH and P5CDH domains in a single polypeptide which is usually around 1100 amino acids long (Fig 3.3). These PutA also have a C-terminal domain (known as CTD) of unknown function. This fusion of enzymes PRODH and P5CDH catalyzing sequential steps of proline catabolism provides a kinetic advantage, because the intermediate (P5C/GSA) can be channeled between active sites without equilibrating into the bulk solvent [27]. Recently, Singh et al. provided kinetic data supporting substrate channeling for Geobacter sulfurreducens PutA [11,27]. This group reported the existence of a 75 Å long tunnel that links the two active sites. Such substrate channeling has been reported for Bradyrhizobium japonicum PutA and Salmonella typhimurium PutA as well [11]. Such fused proteins like PutA, are termed as ‘Rosetta Stone proteins’, because they decipher interactions between protein pairs [28]. Thus, the ‘Rosetta Stone hypothesis’ of protein evolution predicts that eukaryotic PRODH and P5CDH form physical and functional interactions. The best characterized PutA of this group is of Bradyrhizobium japonicum.

c) Trifunctional PutA

The trifunctional PutA have a DNA binding domain along with PRODH and P5CDH domains at the N-terminal end of the polypeptide (Fig 3.3). The length of this type of PutA is usually around 1320 amino acid residues. The protein is present in enterobacteria. The trifunctional PutA has an additional function of autoregulating transcription. It exists both as a transcriptional repressor and as a membrane-associated proline dehydrogenase (Fig 3.3; 3.4). The E. coli PutA has been well characterized and it is reported to repress transcription of put regulon (comprising of the putA and putP genes coding for PutA and a proline transporter, respectively) by binding to the control region of the put intergenic DNA [29]. The
enzymatically active, membrane-bound form of PutA is unable to bind DNA and to repress transcription [29]. Studies led to the identification of the two factors that contribute to induction of put regulon: availability of FAD and proline. The shuttle of PutA from the DNA to the membrane is accompanied by a conformational change in the protein that is triggered by the availability of proline and FAD [30]. The proline-dependent reduction of the cofactor FAD directs PutA to the membrane and subsequently causes loss of DNA-binding activity, indicating that the two locations and the two biochemical activities of the protein are mutually exclusive [13]. It has been reported that a single proline binding site is involved both in enzymatic activity and induction of the put genes. This indicated that enzymatic activity was essential for the release of PutA from DNA and for put gene induction [31].
**Figure 3.4**

**Presence of proline**

- Proline
- PutP
- PutA<sub>red</sub>
- Proline
- P5C
- Glutamate

**Absence of proline**

- PutA<sub>ox</sub>

expression of *put* genes

PutA-dependent repression

Reference: Commichau and Stülke, 2008 [29].

**Fig. 3.4: Localization of PutA determines its role in proline metabolism.** In the presence of proline, the bifunctional enzyme PutA catalyses the two-step reaction from proline to glutamate. The reduced form of PutA (PutA<sub>red</sub>) is localized in the membrane. The putP and putA genes, encoding the proline transporter PutP and the enzyme PutA, respectively, are expressed in the presence of proline. In the absence of proline, the oxidized PutA protein (PutA<sub>ox</sub>) binds to the intergenic region of the putA and putP genes and represses their transcription. P5C, Δ1-pyrroline-5-carboxylate [29].
3.1.5 Proline oxidase and reactive oxygen species (ROS)

During the oxidation of proline to glutamate, FADH$_2$ and NADH are generated (Fig 3.2). These enter the electron transport chain and can eventually lead to enhanced production of ROS. Hence, PutA activity is essential for maintaining the redox homeostasis in the cell. In eukaryotes, impairment of P5C dehydrogenase activity results in P5C-proline cycling [32]. Recently, it has been shown that proline dehydrogenase of *Arabidopsis* is involved in ROS generation during the hypersensitive response [25,33]. Similarly, human POX has been reported to contribute to apoptosis by generation of ROS (mainly superoxide) either directly by interacting with oxygen at the enzyme active site or indirectly by increasing the electron flux in the electron transport chain [34]. Interestingly, the recently discovered P5C–proline cycle explains the delivery of electrons to mitochondrial electron transport without producing glutamate and, under certain conditions, this can lead to enhanced ROS generation [24,35]. Proline catabolism is, therefore, an important regulator of cellular ROS balance and can influence numerous additional regulatory pathways. Proline metabolism has also been reported to influence cellular ATP and NADPH/NADP+ ratio during oxidative and nutrient stress in animal cell lines [36].

Similarly, structural biology studies in bacteria have revealed that proline dehydrogenase of the bacterium *Thermus thermophilus* directly interacts with oxygen to produce superoxide radical [37]. *Helicobacter* PutA has also been reported to have high reactivity with molecular oxygen leading to the formation of ROS [38]. Flavin adenine dinucleotide (FAD), the cofactor of this enzyme is accessible to dissolved oxygen allowing the direct reduction of O$_2$ to superoxide. Hence, the electrons from proline are channelized to generate ROS.
In the current study discussed in this chapter, putA gene was knocked out from one of the pathogenic strains of Xanthomonas, namely X. campestris pv. campestris strain 8004 (Xcc 8004) to understand the role of proline metabolism in PCD of Xanthomonas. The wild type (wt) and mutant strains were examined under similar growth conditions for viability, as well as PCD specific markers such as activity of caspase-3-like protease, level of phosphatidylserine (PS) externalization and the extent of DNA damage. For further understanding, the intracellular reactive oxygen species (ROS) level as well as the change in membrane potential were also analyzed. Additionally, the putA gene was cloned in an E.coli - Xanthomonas shuttle vector, and Xcc 8004 ΔputA was complemented for PutA activity by transforming it with the recombinant plasmid, and the above mentioned biochemical and molecular markers were examined.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

Xanthomonas strains were grown at 26 ± 2°C in a rotary shaker at 150 rpm in Luria-Bertani (LB) broth {PCD inducing medium (PIM)}, or starch broth (SB) {PCD non-inducing medium (PNIM); 1% starch, 0.3% K$_2$HPO4.3H$_2$O, 0.15% KH$_2$PO4, 0.2% ammonium sulphate, 0.05% L-methionine, 0.025% nicotinic acid, and 0.025% L-glutamate, pH 6.8±0.2}. All E.coli strains were grown in LB medium on a rotary shaker (150 rpm) at 37±2°C. The cell number was enumerated by the standard plate count method [39].

3.2.2 Determination of intracellular level of proline in Xanthomonas cells

Intracellular proline levels were determined in Xcc cells as mentioned before [40]. Briefly, an aliquot of overnight grown culture was washed twice with equal volume of PBS (Phosphate
buffer saline) (10 mM, pH 7.5) and resuspended in 3% sulphasalicylic acid. The cells were sonicated for 2 min (60% power) followed by heating at 95°C for 10 min. The culture was centrifuged at 12,000 x g for 10 min. To this clear supernatant 1 ml glacial acetic acid and 1 ml acidic ninhydrin (prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid) was added. This reaction mixture was kept at 100°C for 1 h after which the reaction was terminated on ice bath for 20 min. The reaction mixture was extracted with 2 ml toluene and the absorbance was read at 520 nm using UV-visible spectrophotometer (UV4, Unicam, Cambridge, UK).

3.2.3 Estimation of intracellular cysteine levels

The intracellular cysteine levels were estimated as described earlier [41]. Briefly, overnight grown cells were washed twice with PBS (10 mM, pH 7.5) and resuspended in 5% perchloric acid. Samples were boiled for 10 min followed by centrifugation at 12,000 x g for 10 minutes. The clear supernatant (100 µl) was mixed with 100 µl acetic acid and 100 µl acidic ninhydrin reagent (prepared by mixing 250 mg ninhydrin in 6 ml acetic acid and 4 ml concentrated HCl and kept at 100°C for 15 min). The reaction mixture was cooled on ice and diluted to 1 ml with 95% ethanol. The absorbance was read at 560 nm using UV–visible spectrophotometer.

3.2.4 Measurement of PutA activity in terms of proline oxidase activity

The proline oxidase activity was assayed according to Dendinger and Brill (1970) [42]. Briefly, an aliquot of 24 h culture was washed twice with PBS (10 mM, pH 7.5) and resuspended in 100 mM Tris-HCl (pH 7.4). Wherever required, inhibitors were added to the cell suspension and incubated at room temperature for 30 min. For permeabilization, 5 µl
toluene was added to the cell suspension. After 10 min, 1ml L-proline (1M) and 200 µl o-aminobenzaldehyde (50 mM in 20% ethanol) was added. The reaction mixture was kept for shaking at 26 ± 2 °C for an hour and was terminated by adding 200 µl trichloroacetic acid (20%). The cell debris was removed by centrifugation at 12,500 x g for 15 min. The absorbance of the clear supernatant was measured at 443 nm using UV-visible spectrophotometer. The millimolar extinction coefficient of the P5C (pyrroline-5-carboxylate) - o-aminobenzaldehyde complex is 2.71 [42]. PutA activity was expressed as micromoles of P5C formed min⁻¹ mg⁻¹ protein. The protein content was estimated by Lowry’s method [43].

3.2.5 Construction of putA knockout in Xanthomonas

To further verify the role of PutA in metabolic stress induced PCD of Xanthomonas, a putA knockout of Xanthomonas campestris pv. campestris strain 8004 (Xcc 8004) was constructed by insertional mutagenesis using pKNOCK-Km suicide plasmid (2 kbp) vector which has R6Kγ origin of replication [44]. Hence, the plasmid can only replicate in only those E. coli strains which can provide the replication initiator pi protein [45]. Xcc was used for this study because its genome sequence is known and it also shows post exponential cell death in LB medium similar to Xcg (Fig.3.5). An internal 600 bp region of putA gene (complete size around 3.2 kbp) was amplified using FP1 and RP1 primers (Table 3.1). Hind III restriction enzyme site was introduced at each end. The derivative pKNOCK plasmid carrying the 600 bp putA gene fragment is henceforth termed as pKNOCK-putA. This pKNOCK-putA plasmid was then used to transform competent E. coli PIR1 cells (prepared using CaCl₂ method) by heat shock and transformants were selected on LB-kanamycin (25 µg ml⁻¹) - agar plate. Competent Xcc cells (prepared by washing thrice with 10% ice-chilled glycerol) were
transformed using electroporation [46]. pKNOCK plasmid disrupts the target gene by insertional mutagenesis (Fig 3.6). This is achieved by homologous recombination between the target gene and the complimentary gene fragment cloned in the pKNOCK plasmid. The integration of pKNOCK-putA into the putA gene was confirmed by PCR amplification of full length putA gene from the transformed Xcc colony.
Fig. 3.5: Growth curve of *Xanthomonas campestris* pv. *campestris* strain 8004 in PCD inducing medium (PIM) and PCD non-inducing medium (PNIM).
Figure 3.6

Fig. 3.6: Schematic representation of insertional mutagenesis of Xcc8004 putA using pKNOCK vector.

### Table 3.1: Bacterial strains, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid/primer</th>
<th>Relevant characteristic</th>
<th>Source</th>
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<td><strong>Xanthomonas</strong></td>
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<td></td>
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<tr>
<td>Xanthomonas <em>campestris</em> campestris str. 8004 (Xcc)</td>
<td>Wild type; Rif(^r)</td>
<td>[47]</td>
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<tr>
<td>Xcc Δ<em>putA</em></td>
<td><em>putA</em> deletion mutant of Xcc 8004; Rif(^r) Kan(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>Xcc Δ<em>putA</em>/pPutA</td>
<td>Xcc Δ<em>putA</em> harboring pBBR1MCS5 containing the entire <em>putA</em> gene; ; Rif(^r) Kan(^r) Gm(^r)</td>
<td>This work</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli pir1</td>
<td>{F-- Δ<em>lac169</em> rpoS(Am) <em>robA1</em> creC510 <em>hsdR514</em> endA recA1 <em>uidA</em>ΔMluI)::*pir-116}</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>E. coli pir1/pKNOCK-<em>putA</em></td>
<td><em>E. coli</em> pir1 harboring suicide plasmid pKNOCK-<em>putA</em>; Kan(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>F-- Φ80lacZΔM15 Δ(<em>lacZYA-argF</em>) U169 recA1 endA <em>hsdR17</em> (rK–, mK+) <em>phoA</em> supE44 λ– <em>thi-1</em> <em>gyrA96</em> relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>E. coli DH5α/pPutA</td>
<td><em>E. coli</em> DH5α harboring pBBR1MCS5 containing the entire <em>putA</em> gene; Gm(^r)</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<td>pKNOCK-Km</td>
<td>Suicide vector in <em>Xanthomonas</em>; Kan(^r)</td>
<td>[44]</td>
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<tr>
<td>pKNOCK-<em>putA</em></td>
<td>pKNOCK-Km with an internal gene fragment of <em>putA</em></td>
<td>This work</td>
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<tr>
<td>pBBR1MCS-5</td>
<td>Broad host range cloning vector; Gm(^r)</td>
<td>[48]</td>
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<tr>
<td>pPutA</td>
<td>pBBR1MCS5 containing the entire <em>putA</em> gene; Gm(^r)</td>
<td>This work</td>
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<td><strong>Primers</strong></td>
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<td>FP1</td>
<td>5’ CCAGAAGCTTATGTCGCTGGCAAGCCCTTGC 3’</td>
<td>This work</td>
</tr>
<tr>
<td>RP1</td>
<td>5’ CCAGAAGCTTCTGGCAGCTGTCATCCTC 3’</td>
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<tr>
<td>FP2</td>
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<tr>
<td>RP2</td>
<td>5’ CGCGGATCCTCAGTCACCAAGGTCAG 3’</td>
<td>This work</td>
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</table>
3.2.6 Cloning of Xcc *putA* in a broad host range (bhr) shuttle vector and complementation of Xcc Δ*putA* strain

The *putA* gene in Xcc 8004 is present in single copy. It is flanked upstream by a gene for hypothetical protein (XC_3906; location: 4,610,064 - 4,610,495) and downstream by IS1478 transposase gene (XC_3908; location: 4,614,266 – 4,615,633) (Fig 3.7A). The full length *putA* gene excluding the promoter region in Xcc 8004 is 3.2 kb in size (XC_3907; location: 4,610,819 – 4,614,019). For complementing Xcc Δ*putA* strain with functional PutA, the 319 bp non-coding region present between XC_3906 gene and *putA* was amplified along with the *putA* gene (Fig 3.7B) using Pfu polymerase, FP2 and RP2 primers (Table 3.1) by colony PCR technique. This non-coding 319 bp sequence contains the *putA* promoter region which has not been characterized yet. In this study, BPROM software was used to identify the possible promoter region of *putA* and the findings have been shown in Fig S2B. The PCR product (3.52 kb in size) was cloned into a broad host range (bhr) vector pBBR1MCS5 (Fig 3.8). This vector was originally derived from pBR322 by subsequent modifications to have several advantages such as relatively smaller size (4.7 kb), extended multiple cloning site (MCS), possibility of direct selection of recombinant plasmid in *E.coli* via disruption of the LacZα peptide, mobilizable when the RK2 transfer functions are provided in *trans*, and compatible with IncP, IncQ and IncW group plasmids, as well as with ColE1- and P15a-based replicons [48]. The recombinant plasmid carrying *putA* gene is henceforth termed as pPutA and was used to transform *E.coli* DH5α cells. The transformants were selected on LB-gentamycin plate (10 µg ml⁻¹). Xcc Δ*putA* strain was further transformed with pPutA by electroporation as described above. The transformants were confirmed by PCR amplification using *putA* specific primers (Table 3.1).
Figure 3.7

**Fig.3.7:** Organization and sequence of Xcc putA gene (A) Organization of putA (NCBI gene ID: 3379526) in Xcc genome, (B) The sequence of upstream non coding promoter containing region (319 bp; source: NCBI database) of Xcc putA included in putA complementation construct. BPROM software was used for promoter prediction (-10 and -35 box). FP and RP indicate the sites for forward and reverse primers respectively, used for PCR amplification of putA along with its promoter.
Figure 3.8

Fig.3.8: pBBR1MCS5 vector map depicting cloned putA (full length) along with its promoter region.

3.2.7 Screening of recombinants

Screening of *E. coli* DH5α recombinants with pPutA was carried out by blue white screening method as mentioned earlier [46]. Briefly, to a premade LB agar plate containing the appropriate antibiotic, 40 μl X-gal (20 mg/ml in DMSO) and 4 μl of IPTG solution (200 mg/ml) were spread. These components were allowed to absorb for at least 30 min or until the plate surface appeared dry at 37°C prior to plating cells.
3.2.8 Assay of caspase-3-like activity and immunoblotting

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 4ºC 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) as described earlier [39].

Level of biosynthesis of caspase-3-like protein was analyzed using SDS-PAGE and Western hybridization as described earlier in chapter 2 [39] using affinity-purified, biotin-conjugated, polyclonal rabbit anti-active human caspase-3 antibody.

3.2.9 Analysis of active caspase-3-like protein in situ by FITC-DEVD-FMK staining

The assay was carried out using caspase-3 detection kit (Catalog no. QIA91, Calbiochem). An aliquot (250 µl) of 24 h grown cell culture containing ~10^6 cfu ml^-1 was washed twice with PBS (10 mM, pH 7.5). The cell pellet was resuspended in 300 µl PBS. To this cell suspension 1 µl of FITC-DEVD-FMK was added and incubated at room temperature for 30 min in dark. After that, the cells were centrifuged at 12,500 x g for 5 min and supernatant was discarded. The cells were washed twice with wash buffer and resuspended in 200 µl of the same. An aliquot (10 µl) was smeared on a glass slide, air dried and examined under a
fluorescent microscope (Carl Zeiss, Germany) using oil immersion objective (100x) and filter set 9 (Carl Zeiss, Germany; Excitation: 450 nm; emission: 515 nm).

### 3.2.10 Observation of cell filamentation

An aliquot (1 ml) of cells grown in PIM (24 h) or PNIM (72 h) was washed twice with PBS (10 mM, pH 7.5), resuspended in saline (0.85%). An aliquot (10 µl) was smeared on a glass slide, air dried, heat fixed, stained with crystal violet and examined under a microscope (Carl Zeiss, Germany) using oil immersion objective (100X) for observation of cell filaments.

### 3.2.11 Quantification of DNA damage by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay

TUNEL assay was performed using the APO-Direct kit, BD Pharmingen as described earlier in chapter 2. Briefly, an aliquot (1 ml) of 24 h grown cell culture containing ~10^6 cfu ml^-1 was washed twice with PBS (10 mM, pH 7.5). The cell pellet was resuspended in 50 µl DNA labeling 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 ºC in dark. After that, 1 ml rinse buffer was added and cell suspension was centrifuged at 12,000 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, and analyzed by Fluorescence Activated Cell Sorter (FACS) (10^5 cells for each sample) using flow cytometry system (Partec CyFlow space, Germany).
3.2.12 Quantification of phosphatidylserine (PS) externalization using annexin-V labeling

The assay was performed using annexinV-FITC apoptosis detection Kit (catalog no. 556547, BD Pharmingen). Briefly, an aliquot (1 ml) of 24 h cell culture containing ~10^6 cfu ml\(^{-1}\) was washed twice with PBS (10 mM, pH 7.5) and the pellet was resuspended in 250 µl of the same buffer. An aliquot (650 µl) of annexinV binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl and 2.5 mM CaCl\(_2\)) and annexinV (5 µl) were added to the cell suspension and incubated in dark for 15 min. Propidium iodide (5 µl, 50 µg ml\(^{-1}\)) was then added into the cell suspension and incubated at ambient temperature in dark for 15 min. For each analysis, 10^5 cells were analyzed by flow cytometry (Partec CyFlow space, Germany). Data was analyzed using FCS Express V4 software (demo version).

3.2.13 Analysis of reactive oxygen species (ROS) generation by dichlorohydrofluorescein staining

Dichlorohydrofluorescein (H\(_2\)DCFDA) staining was carried out as mentioned previously in chapter 2. Briefly, Xanthomonas cells were grown at 26 ± 2 °C in a rotary shaker at 150 rpm in culture medium (LB) for 18 h. A 2 ml aliquot was withdrawn and centrifuged at 12,500 x g for 2 min and the pellet was resuspended in 1 ml saline (0.85%). It was then incubated with 2 µl H\(_2\)DCFDA (5 mM, prepared in absolute ethyl alcohol) at room temperature 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 (100x) and filter set 15 (Carl Zeiss, Germany; excitation: 546 nm; emission: 590 nm).
3.2.14 Determination of membrane potential

It was carried out using BacLight bacterial membrane potential assay kit (Molecular Probes, catalog no. B34950) as per the manufacturer’s guidelines. Briefly, an aliquot (1 ml) of 24 h grown cell culture containing \(~10^6\text{ cfu ml}^{-1}\) was washed twice with PBS (10 mM, pH 7.5) and the pellet was resuspended in 1 ml of the same buffer. For preparing the depolarized control sample, 10 µl of an uncoupler CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (50 mM) was added to 1 ml of cell suspension \((\sim 10^6 \text{ cfu ml}^{-1})\) and incubated at room temperature for 30 min. CCCP is a proton ionophore which dissipates membrane potential by eliminating the proton gradient. Thereafter, cells were stained with 5 µl (50 nM final concentration) of DiOC$_2$(3) (3,3’-diethyloxacarbocyanine iodide), and 10$^5$ cells were analyzed by flow cytometry (Partec CyFlow space, Germany, and Express V4 software, demo version).

3.3 Results and Discussion

3.3.1 Effect of addition of amino acids on PCD profile of Xanthomonas campestris pv. campestris (Xcc)

The preceding chapter provided evidence of stress experienced by the cells grown in PIM. Since the PCD process in this bacterium was observed to be induced under conditions where the protein content of the medium was higher than the carbohydrate content, a fine balance of these two major nutrients was thought to be an essential factor governing survival of this microorganism. The designed conditions of growth of this organism in laboratory are quite distinct from its natural habitat on soybean leaf. Therefore in this study, starch minimal medium was used as PCD non-inducing medium (PNIM). The PNIM is composed of 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. Being rich in starch, this medium is quite similar to the *ex situ* conditions encountered by *Xanthomonas*, a plant pathogen. On the other hand, a protein rich medium like Luria Bertani (LB) broth was used as PCD –inducing medium (PIM). This medium is predominantly composed of tryptic and casein digests which provide very high levels of glutamate (15%) and proline (6%) \([49,50]\). Hence, the effect of addition of these two amino acids, either individually or in combination in PCD non-inducing medium (PNIM), on the PCD process in *Xanthomonas campestris* pv. *campestris* (Xcc) was examined. Xcc was used in this study instead of Xcg because its genome sequence is known and it also shows post exponential cell death in LB medium similar to Xcg (Fig. 3.5).

A concentration dependent decrease in viability was observed when *Xanthomonas* was grown in PNIM in the presence of varying concentrations of proline (5 - 100 mM) and the viability at 96h was lowest at 100 mM. When *Xanthomonas* cells were grown in PNIM supplemented with proline (5-100 mM), the cell count peaked at 24 h of incubation followed by a sharp decline in viable cell number during further post-exponential incubation under similar growth conditions (150 rpm, 26ºC). The viable cell count was found to be 2.1 x 10^8 and 5.3 x 10^8 for 5 mM and 50 mM respectively at 24 h followed by a decline during the course of incubation. The viable count decreased by 48% and 72% at 5 mM and 50 mM proline respectively at the end of 96 h. However, at the end of 96 h of incubation, one log cycle decrease in viable cell count was observed in case of culture where 100mM proline was added (Fig. 3.9).

Interestingly, the cell number was found to be more in the cultures supplemented with glutamate with respect to control cells (without glutamate) and it remained almost unchanged (at ~10^8 cfu/ml) at the end of 96 h of incubation (Fig. 3.9). Glutamate and glutamine are
important amino acids for bacterial metabolism [51]. Glutamate participates in both catabolism and anabolism. It can be deaminated to form α-ketoglutarate, a TCA intermediate. Glutamate accounts for ~88% of cellular nitrogen and is required for the biosynthesis of purines, pyrimidines and amino sugars [51]. Hence, when starch minimal medium (PCD non-inducing medium or PNIM) was supplemented with excess glutamate, it provided a ready source of nitrogen favoring anabolic events as it was observed to accelerate Xcc growth.

Moreover, the growth profile upon addition of both proline and glutamate together in the culture medium (PNIM) behaved similar to proline addition and the loss of viability by around one log cycle was noticed at 96 h. The observations thus indicated a regulatory role for proline in the death of Xanthomonas cells under unfavorable nutritional condition.
Figure 3.9

![Graph showing cell survival in different treatments](image)

**Fig. 3.9**: The effect of proline and glutamate supplementation on the survival of *Xanthomonas campestris* pv. *campestris* in PNIM. The different letter on the bar indicates that the means are significantly different at p<0.05.

### 3.3.2 PutA activity in Xcc cultures grown in PIM and PNIM

The above observations indicated the role of proline in induction of PCD in this bacterium. The growth medium favoring PCD during this study namely LB medium also contains higher concentration of proline compared to other amino acids [49,50]. Its concentration in casein
tryptic digest is close to 6%, next to glutamic acid (around 15%), which can also get converted to proline enzymatically inside the cell. As expected, intracellular proline level in PNIM was found to be quite low (2.4 µM mg\(^{-1}\) protein) in Xcc which increased to around 35 fold (83 µM mg\(^{-1}\) protein) in PIM growing cells (Fig 3.10A). The proline levels were even higher in Xcc ΔputA cells (151 µM mg\(^{-1}\) protein) (Fig 3.10A). Supplementing PNIM with glutamate increased the intracellular proline levels slightly (~ 5 µM mg\(^{-1}\) protein), indicating that probably only a small amount of glutamate is being converted to proline inside the cells. This is probably because the first enzyme of proline biosynthesis pathway, gamma glutamyl kinase (GK) is subject to feedback inhibition by proline (even at very low concentrations) in bacteria [9,10] thereby limiting the conversion of glutamate to proline. Intracellular proline levels were found to be around fourteen fold higher (~34.5 µM mg\(^{-1}\) protein) in Xcc cells grown in PNIM fortified with either proline or proline along with glutamate. LB medium is predominantly composed of amino acids and peptides. As a result, the microorganism has to use them both as carbon and nitrogen source. During such growth conditions certain metabolic pathways can be preferentially upregulated over others. High intracellular levels of proline in Xcc cells can be attributed to abundant free proline and peptides present in PIM.
Fig.3.10: Intracellular proline level and PutA activity in Xcc 8004. (A) Intracellular proline level, and (B) PutA activity in Xcc 8004, Xcc ΔputA and Xcc ΔputA-pPutA cells in
PIM and PNIM. The different letter on bars indicates that the means are significantly different at p<0.05.

Increased substrate concentration can affect the activity of the enzymes involved in its utilization. Hence, examining the status of proline oxidase (PutA) which is involved in metabolism of proline becomes important to further understand the regulation of PCD in these cells. It has been reported that L-proline is preferentially used as a carbon source by *E.coli* growing in LB medium and this amino acid gets depleted quite early during its growth [49]. Unlike eukaryotes, bacterial PutA has dual activity, where first proline oxidase (or dehydrogenase) activity oxidizes proline to P5C (pyrroline-5-carboxylate), which spontaneously converts to γ-glutamate semialdehyde [8,13]. This γ-glutamate semialdehyde is then eventually oxidized to glutamate by P5C dehydrogenase activity of PutA [13]. In this study the PutA activity was measured as its proline oxidase (POX) activity. PutA activity was monitored in Xcc cell lysates by measuring the adduct formed between P5C and o-aminobenzaldehyde because the formation of P5C is very specific to this pathway. PutA activity was found to be 0.06 µM min⁻¹ mg⁻¹ protein in PNIM in Xcc wt cells which increased by around 34 fold to 2.01 µM min⁻¹ mg⁻¹ protein in PIM (Fig 3.10B). This observation can be attributed to the higher intracellular proline found in Xcc cells cultured in PIM. High proline levels have been reported to induce PutA activity as well [53]. A good correlation was found between intracellular proline levels and PutA activity in Xcc wt cells cultured in PIM (Fig 3.10A, B). These results also indicated that it is not excess proline but its increased oxidation by PutA that caused cell death.

Intracellular level of cysteine was also checked as a control. Basal level of cysteine was found to be comparatively high (50 µM mg⁻¹ protein) even in PNIM indicating its constitutive
requirement for cellular metabolism. However, in PIM growing Xcc cells, cysteine level merely increased to around six fold to 301 μM mg⁻¹ protein. It is worth mentioning here that the level of cysteine in LB medium is quite lower than that of proline [49]. The findings thus indicated comparatively preferential regulation of proline metabolism in stressed Xcc cells undergoing PCD.

The above observations suggested a role of proline metabolism in PCD of this organism which was further revalidated by studying the effect of addition of tetrahydro-2-furoic acid (THFA, ≤5 mM), a competitive inhibitor of PutA (a proline analog) in PIM. Interestingly, the inhibition of PCD was observed in Xcc culture when this PutA inhibitor was added in the medium prior to inoculation, and the extent of PCD inhibition was found to be inhibitor concentration dependent (Fig.3.11). Almost two log cycle increase in the cell viability was observed at 96 h of incubation in the presence of THFA (5 mM). These findings thus confirmed the involvement of PutA activity during PCD of Xanthomonas cells in PIM.
Figure 3.11: Effect of PutA inhibitor, tetrahydro-2-furoic acid (THFA) on PCD process in Xcc 8004 cells grown in PIM

3.3.3 Effect of knocking out Xcc putA gene and its complementation

Xcc genome has been sequenced and putA gene was found to be present in single copy (NCBI accession number: XC_3907). Knocking out of putA resulted in Xcc ΔputA strain (Fig. 3.12, 3.13), which showed an increase in the cell survival in PIM by more than one log cycle (viable plate count 6.3 x10^7 cfu ml^-1) at 96 h of incubation as compared to the wild type
(wt) counterpart (2.5 x10^6 cfu ml⁻¹) (Fig 3.16A). The viability in Xcc ΔputA strain in PNIM was very close to that of wt Xcc cells growing in the same medium (Fig 3.16B) and remained unaltered even when PNIM was supplemented with proline (100 mM) unlike that observed for the wt strain (Fig 3.16B and 3.9 respectively). An increase in cell number was observed when Xcc ΔputA strain was grown in PNIM supplemented with glutamate (100 mM) (Fig 3.16B). This observation was similar to that found in the case of Xcc wt strain (Fig 3.9). The increase in growth of Xcc ΔputA cells in the presence of glutamate could be attributed to the fact that since 88% of the nitrogen in the cell is derived from glutamate, it provided a ready source of nitrogen favoring anabolic events [51].

The findings thus indicated of inhibition PCD upon abolition of PutA activity in Xcc ΔputA strain which could be attributed to the loss of PutA activity (Fig 3.10B). As expected, this activity was found to be completely abolished in Xcc ΔputA strain indicating that it is precisely regulated by the putA gene, which is present in single copy in the Xcc genome. Moreover, the intracellular proline levels were almost double (151 µM mg⁻¹ protein) in Xcc ΔputA strain compared to Xcc wt grown in PIM (Fig 3.16A). Thus, these observations confirmed that high levels of proline alone do not cause death, rather its enhanced oxidation by PutA plays a role in inducing PCD (Fig 3.16).
**Fig. 3.12:** Construction of XccΔputA strain

(A) Screening of recombinant plasmid (pKNOCK-Km; 2kbp) carrying the desired insert: lane 1 - 500 bp DNA ladder, lane 2 - recombinant plasmid with insert, lane 3 - native plasmid; (B) Confirmation of recombination by restriction digestion of the plasmid: lane 1 - restriction digestion profile of recombinant plasmid, lane 2 - 500 bp DNA ladder.
Fig. 3.13: Confirmation of Xcc ΔputA strain (A) PCR amplification of disrupted full length putA (with pPutA inserted) increasing its size from 3 kbp to 5 kbp: lane 1 - 500 bp DNA ladder; lane 2 - 5kbp PCR product; (B) PCR amplification of putA gene using internal primers: lane 1 - 500 bp DNA ladder, lane 2 – 1 kbp PCR product.

Further, Xcc ΔputA strain was complemented with putA gene cloned in a plasmid shuttle vector pBBR1MCS-5 (Fig 3.14 and 3.15) and its viability was monitored in PIM. The PCD phenotype was found to be restored upon complementation with functional PutA. The cell death was 15 fold higher in Xcc ΔputA strain carrying pPutA vector (2.75 x 10^6 cfu ml^-1) at
96 h of incubation in PIM compared to the strain carrying the vector without \textit{putA} \((4.14 \times 10^7\text{cfu ml}^{-1})\) (Fig 3.16A). PutA activity was also found to be restored in Xcc \textit{ΔputA} strain upon complementation with functional Xcc PutA and was around three fold higher than the Xcc \textit{wt} strain growing in PIM (Fig 3.10B and 3.15C). However, negligible enzyme activity was detected when this strain was cultured in PNIM (Fig 3.10B and 3.15C).

\textbf{Figure 3.14}

(A) \hspace{1cm} (B)

\textbf{Fig.3.14: Screening of recombinants by blue white screening method:} (A) \textit{E. coli} harboring native pBBR1MCS5 plasmid spread on IPTG-X-gal plate; (B) \textit{E. coli} transformants spread on IPTG-X-gal plate
Fig. 3.15: Construction of Xcc ΔputA-pPutA strain: (A) Screening of recombinant plasmid: lane 1 – native plasmid DNA, lane 2 – recombinant plasmid with putA gene, lane 3 – 1 kbp DNA ladder; (B) Confirmation of recombination by restriction digestion of the plasmid: lane 1 - 1 kbp DNA ladder, lane 2 - restriction digestion profile of recombinant plasmid; (C) PutA activity analysis to confirm the complementation of XccΔputAstrain. The yellow colored OBA-P5C adduct measured at 520 nm in (a) Xcc ΔputA and (b) Xcc ΔputA-pPutA strain.
Fig. 3.16: PCD in Xanthomonas cells: (A) Effect of knocking out of putA and its complementation on the PCD profile of Xcc 8004 strains grown in PIM; (B) Effect of supplementation of proline and glutamate in PNIM on the growth of Xcc ΔputA cells.
3.3.4 Status of PCD specific markers in Xcc (wild type), Xcc ΔputA and Xcc ΔputA-pPutA strains

In our earlier studies Xanthomonas cells were found to undergo PCD in PIM and displayed certain PCD specific markers such as activation of caspase-3-like protease activity (analyzed by enzyme assay as well as Western blot using polyclonal human caspase-3 antibody), externalization of membrane phosphatidylserine (PS) (assayed using annexinV-FITC labeling), and DNA damage (determined by TUNEL- Terminal deoxynucleotidyl transferase dUTP nick end labeling assay) [39,54–57]. The status of these PCD markers was also examined in this study in wild type Xcc, Xcc ΔputA and Xcc ΔputA- pPutA strains.

3.3.5 Caspase-3-like protease activity

Caspase-3-like activity which was quantified in terms of fluorescence level of AMC (amino methyl coumarin) released from a synthetic tetrapeptide substrate (Ac-DEVD-AMC) due to protease activity of caspase-3 [58]. Caspase-3-like activity in Xcc ΔputA strain while growing in PIM was found to be around 40% less than Xcc wt strain at 24 h of growth in PIM (Fig 3.17A), however no change in its levels were detected by western blotting in Xcc ΔputA strain (Fig 3.17B). FITC-DEVD-FMK, a fluorescent dye tagged with an irreversible caspase-3 inhibitor (DEVD-FMK) was also used for in situ labeling of Xanthomonas cells having active caspase-3-like enzyme. Interestingly, Xcc wt and XccΔputA-pPutA cells grown in PIM fluoresced brightly when treated with this dye (Fig 3.17 C and E). A negligible number of Xcc ΔputA cells fluoresced when treated with FITC-DEVD-FMK (Fig 3.17D). Notably, cells showing caspase activity were mostly found to be filaments. Cell filamentation was also observed in PIM growing cells by monochrome (crystal violet) staining. Some hypochromic cell filaments were also observed indicating loss of membrane integrity (Fig 3.17G).
contrary, *Xanthomonas* cells grown in PNIM did not display any significant morphological change even after 72 h of incubation (Fig 3.17F). Among different possible explanations for cell filamentation, one could be due to ROS mediated DNA damage leading to upregulation of error prone repair pathway like SOS response in different bacteria. The induction of SOS response depends upon the extent and nature of DNA damage.
Figure 3.17

Fig. 3.17: Caspase-3-like protein activity and cell morphology of Xcc strains grown in PIM (A) Caspase-3-like activity in Xcc wt, Xcc ΔputA, and Xcc ΔputA-pPutA cells in PIM.
The different letter on bars indicates that the means are significantly different at p<0.05. (B) Western blot indicating the status of caspase-3-like protein: lane 1: Xcc wt cells, lane 2: Xcc ΔputA cells, lane 3: ccoloured protein molecular weight marker. In situ labeling with FITC-DEVD-FMK indicating active caspase-3-like protein in (C) Xcc wt, (D) Xcc ΔputA and, (E) Xcc ΔputA-pPutA cells grown in PIM. Cell morphology of: (F) Xcc wt cells in PNIM, (G) Xcc wt cells in PIM, (H) Xcc ΔputA and, (I) Xcc ΔputA-pPutA cells in PIM.

3.3.6 Extent of DNA fragmentation in Xcc strains in PIM as monitored by TUNEL assay

Activation of caspase-3 enzyme has been reported to activate CAD (caspase activated DNase) resulting in DNA fragmentation prior to cell death [59]. With this analogy in this study too, DNA fragmentation was measured in Xcc strains growing in PIM by TUNEL assay. DNA breaks are labeled in situ with dUTPs tagged with a fluorophore, fluorescein isothiocyanate (FITC) with the help of an enzyme, terminal deoxynucleotidyl transferase (TdT) and the extent of labeling which obviously depends upon the extent of DNA fragmentation, is monitored by flow cytometry. This assay has also been used earlier to detect DNA fragmentation in bacteria [60]. Only 4% Xcc ΔputA cells were found to be TUNEL positive compared to 24% of Xcc wt and 19% in Xcc ΔputA complemented with PutA (Fig 3.18).
Fig. 3.18: Status of DNA fragmentation in Xcc 8004, Xcc ΔputA and Xcc ΔputA-pPutA cells in PIM as detected by TUNEL assay. The gated region (represented by the arrow) depicts the area under the histogram and indicates the percentage of cells labeled by FITC-dUTP which implies DNA damage.

3.3.7 Level of phosphatidylserine externalization in Xcc strains in PIM

Phosphatidylserine (PS) externalization has been reported as an important marker of PCD in various organisms, however, its exact implication remains to be elucidated, particularly in the case of microorganisms [61]. It has been reported as the hallmark of PCD in various systems including *E.coli*, *Saccharomyces* and *Aspergillus* [61–63]. PS externalization is detected by
flow cytometry using AnnexinV-FITC fluorophore. AnnexinV is a 36 kDa Ca\(^{2+}\) dependent phospholipid binding protein having high affinity for PS. Only 7% Xcc ΔputA cells were found to be AnnexinV-FITC positive as compared to 50% of Xcc wt cells growing in PIM (Fig.3.19).

**Figure 3.19**

![Graph showing PS externalization in Xcc 8004, Xcc ΔputA and Xcc ΔputA-pPutA cells in PIM as detected by AnnexinV-FITC assay. The gated region (represented by the arrow) depicts the area under the histogram and indicates the percentage of cells labeled by AnnexinV-FITC.](image)

Fig.3.19: Status of PS externalization in Xcc 8004, Xcc ΔputA and Xcc ΔputA-pPutA cells in PIM as detected by AnnexinV-FITC assay. The gated region (represented by the arrow) depicts the area under the histogram and indicates the percentage of cells labeled by AnnexinV-FITC.
3.3.8 Status of membrane depolarization in *Xanthomonas* strains grown in PIM

Recently, membrane depolarization has also been used to monitor PCD in *E.coli* [60]. Membrane depolarization can be monitored by using the membrane potential sensitive carbocyanine dye, DiOC<sub>2</sub>(3) (3, 3’- diethyloxa-carbocyanine iodide). The proportion of depolarized cells was found to be greater in Xcc wt culture (29%) compared to Xcc Δ*putA* (5%) (Fig.3.20). In eukaryotes too, mitochondrial membrane depolarization leading to cytochrome c release has been reported as one of the early events occurring during apoptosis [64].

**Figure 3.20**

![Figure 3.20: Membrane depolarization in *Xanthomonas* strains in PIM analyzed by flow cytometry.](image)

The gated region (represented by the arrow) indicates the percentage of depolarized cells.
3.3.9 Inhibition of PutA activity by electron transport chain (ETC) inhibitors

Involvement of proline metabolism in ETC was confirmed by using ETC inhibitors like rotenone and antimycin. Both these inhibitors were found to inhibit the activity of Xcc PutA by 65% (Fig.3.21A). Rotenone inhibits the electron flow from the Fe-S centres of complex I to ubiquinone, whereas, antimycin A inhibits the transfer of electrons from cytochrome b to c1 (complex III). Similar inhibitor of complex I (amytal) has been reported as a non-competitive inhibitor of PutA in E.coli [65]. Xanthomonas PutA activity was found to be linked to ETC (Fig.3.21A) suggesting that PutA is also involved in regulating the redox homeostasis of the cell.

3.3.10 Status of ROS in Xcc strains

Proline oxidase (POX) in higher organisms, which is functionally similar to PutA in prokaryotes, has been reported to be an inner mitochondrial membrane protein that generates electrons during oxidation of proline to glutamate, and subsequent coupled reduction of FAD to FADH2. This FADH2 transfers electron to ubiquinone (UQ), an electron carrier in ETC. Thus proline can eventually get oxidized to generate ATP as well as superoxide. Though bacteria lack mitochondria, a similar process of electron transfer exists in membrane. The possibility of increased ROS generation upon activation of PutA was examined by monitoring the level of ROS in the Xcc wt, Xcc ΔputA and Xcc ΔputA-pPutA strains using H2DCFDA staining (Fig.3.21B, C and D). The ROS level in putA knockout strain was 30% lower than the wt Xcc strain. This observation indicated that PutA was partly involved in generating ROS in Xanthomonas cells growing in PIM. Since the extent of DNA damage and the level of ROS were found to be lower in Xcc ΔputA compared to the wt strain, it indicated that PutA is possibly involved in ROS generation
leading to DNA damage and eventually cell death (Fig.3.18, 3.21B and C). There are previous reports regarding the role of this enzyme in ROS generation in other organisms including *Helicobacter* and *Thermus thermophilus*. It has been demonstrated recently that proline dehydrogenase of *Arabidopsis* is involved in ROS formation during the hypersensitive response [25,33]. Similarly, human POX also contributes to generation of ROS (mainly superoxide) either directly by interacting with oxygen at the enzyme active site or indirectly by increasing the electron flux in the electron transport chain eventually leading to apoptosis [24,34]. Interestingly, the recently discovered P5C–proline cycle can deliver electrons to mitochondrial electron transport without producing glutamate and, under certain conditions, can generate more ROS in the mitochondria [24,35]. Proline catabolism is, therefore, an important regulator of cellular ROS balance and can influence numerous additional regulatory pathways.
**Fig.3.21: PutA activity and status of ROS in Xcc cells**

(A) PutA activity in Xcc 8004 cells in the presence of different inhibitors. The different letter on bars indicates that the means are significantly different at p<0.05. (B-D) Reactive oxygen species (ROS) generation observed by 2’, 7’-dichlorohydrofluorescein-diaceate (H$_2$DCFDA) stain in (B) Xcc wt, (C) Xcc ΔputA and, (D) Xcc ΔputA-pPutA cells in PIM.

POX is reported to play an important role in cancer, apoptosis and schizophrenia in humans [66]. It has been found to be one of the 14 genes to be induced more than 10 fold by p53 and
has been termed as p53-induced gene 6 (PIG6) [53]. POX is regarded as a tumor suppressor protein and any anomaly in its functioning results in cancer [67]. POX activation in higher systems has been reported to induce both intrinsic and extrinsic pathways of apoptosis by regulating the redox homeostasis of the cell and has been observed to activate caspase-3, 8 and 9 [68]. Overexpression of POX leads to apoptotic cell death in several cancer cell types [68–70]. Its role has been established in eukaryotic apoptosis and is considered as an important protein for preventing initiation of cancer.

This study provides evidence of involvement of proline oxidase (PutA) in the observed PCD of *Xanthomonas*. This could be due to proline oxidase linked leakage of electrons from electron transport chain causing ROS generation and the resultant activation of caspase-3-like protein leading to cell death (Fig. 3.22). The findings are quite similar to the events observed in higher organisms indicating an evolutionarily conserved role of this protein in PCD.
Fig. 3.22: Proposed mechanism of PCD in *Xanthomonas* due to enhanced PutA activity
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