



Homi Bhabha National Institute

SYNOPSIS OF Ph. D. THESIS

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3. Enrolment no.:	LIFE01200904005
4. Title of the thesis:	Mechanism of programmed cell death in <i>Xanthomonas</i>
5. Board of studies:	Life Sciences

Introduction

In the past couple of decades, several studies have demonstrated existence of programmed cell death (PCD) in a number of unicellular eukaryotes including *Trypanosoma*, *Leishmania*, *Plasmodium* and *Sacharromyces*, as well as, in some prokaryotic organisms (Gautam et al., 2005; Gautam and Sharma, 2005; Bayles, 2014). Among prokaryotes, PCD is known to occur during sporulation and mother cell lysis of *Bacillus*, myxobacterial fruiting body formation, under salt stress in *Anabaena*, and metabolic stress in *Xanthomonas campestris* (Gautam and Sharma, 2002a,b; Gautam and Sharma, 2005; Gautam et al., 2005; Raju et al., 2006; Bayles, 2014). PCD has been reported to play a vital role in bacterial stress response (Bayles, 2014).

Xanthomonas is a Gram negative, aerobic plant pathogenic bacterium having a capsular envelop of xanthan, composed of pentasaccharide repeat units of glucose, mannose and

glucuronic acid (García-Ochoa et al., 2000). Xanthan has wide industrial applications in food and oil industry. In an earlier study from this laboratory, *Xanthomonas* displayed atypical growth in protein rich Luria Bertani (LB) medium where it showed rapid cell death just after log phase without any noticeable stationary phase, whereas, in a carbohydrate rich starch medium it showed the usual bacterial growth pattern with lag, log, and a prolonged stationary phase followed by death (Gautam and Sharma, 2002a,b). Later, while studying this medium dependent differential survival, *Xanthomonas* was found to undergo PCD in LB medium, where it displayed many important markers of apoptosis as reported in eukaryotic cells (Gautam and Sharma, 2002a,b; Gautam and Sharma, 2005). In the due course of study, some metabolites like alanine, glycine, pyruvate, citrate and malate were found to induce PCD in non-inducing conditions, whereas, others like glucose, starch and cAMP inhibited this process (Raju et al., 2006).

The objectives of the study were:

- a) To investigate the mechanism of PCD induction in *Xanthomonas* under certain nutrient conditions.
- b) Study the impact of different physico-chemical stresses on the PCD profile of *Xanthomonas* and other bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Xanthomonas strains were grown at $26 \pm 2^\circ$ 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)}. All *E. coli* strains, *Bacillus subtilis* (ATCC6633), *Bordetella*

bronchiseptica (NCIM 2267) and *Salmonella enterica* sv. Typhimurium were grown in LB medium on a rotary shaker (150 rpm) at 37 ± 2 °C for 3-5 h. The cell number was enumerated by the standard plate count method.

Quantification of intracellular NADH, ADP and ATP

Alkaline extraction of NADH was carried out using the protocol of Caruso *et al.*, 2004. Acid extraction of ATP and ADP was carried out based on the method described previously (Giannattasio *et al.*, 2003). The samples in both the cases were filtered through 0.22 µm filter (Millipore, USA) and analyzed using HPLC.

Analysis of reactive oxygen species (ROS) generation

(A) 2',7'-Dichlorohydrofluorescein diacetate (H₂DCFDA) staining of cells: This dye gets oxidized inside the cells upon ROS generation, forming an intracellular fluorescent DCF (dichlorofluorescein) molecule and the brightly fluorescent cells were observed under a fluorescent microscope.

(B) Electron Spin Resonance (ESR) spectroscopy: Hydroxyl radical (OH·) formation inside the cells during the course of PCD was detected with an ESR based spin trapping system, which contained 10 mM α-(4-pyridyl-1-oxide)-N-tert-butyl-nitron (POBN). The POBN adduct displays a spectrum consisting of triplet of a doublet.

(C) Scopoletin assay: Intracellular H₂O₂ level was measured using fluorogenic substrate scopoletin (2.5 µM) and horseradish peroxidase (5 U/ml) as detected by the decrease in fluorescence intensity.

Measurement of intracellular proline levels and proline oxidase (PutA) activity

Intracellular proline levels were determined in Xcc cells as mentioned before (Bates et al., 1973) using acidic ninhydrin and glacial acetic acid. The reaction mixture was extracted with 2 ml toluene and the absorbance was read at 520 nm using UV–visible spectrophotometer. The proline oxidase activity was assayed in 24 h culture of Xcc according to the method described earlier (Dendinger and Brill, 1970). Cells were permeabilized using toluene and the reaction was performed in the presence of L-proline (1M) and 200 μ l o-aminobenzaldehyde (50 mM in 20% ethanol) at 26 ± 2 °C for 1h and terminated with trichloroacetic acid (20%). The absorbance of the clear supernatant was measured at 443 nm. PutA activity was also analyzed in the presence of its inhibitor, tetrahydro-2-furoic acid (THFA), and electron transport chain inhibitors rotenone and antimycin.

Construction of *putA* knockout in *Xanthomonas*

A *putA* knockout was constructed by insertional mutagenesis using pKNOCK-Km suicide plasmid (2 kbp) vector (Alexeyev, 1999) in Xcc8004 which was preferred over Xcg because complete genome sequence of Xcc is available. An internal 600 bp region of *putA* gene (complete size ~ 3 kbp) was amplified and cloned in pKNOCK plasmid. This pKNOCK-putA plasmid was used to transform competent *E. coli* PIR1 and later Xcc cells using electroporation. The integration of pKNOCK-putA into the Xcc genome was confirmed by PCR amplification of full length *putA* from the transformed Xcc colony.

Cloning of Xcc *putA* in a broad host range (bhr) shuttle vector and complementation of Xcc Δ *putA* strain

The full length *putA* gene from Xcc was PCR amplified by colony PCR and the PCR product was cloned in pBBR1MCS5-Gm plasmid (Kovach et al., 1995) which was used to transform

E. coli DH5 α cells. The transformants were selected on LB-gentamycin plate. Xcc Δ putA strain was further complemented with pPutA purified from transformed *E. coli* cells.

Analysis of PCD markers

- a) **TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay:** It was performed according to the manufacturer's guidelines (APO-Direct Kit, BD Pharmingen) to detect DNA nicks.
- b) **Phosphatidylserine (PS) externalization:** PS externalization was detected by AnnexinV-FITC labeling followed by Fluorescence Activated Cell Sorting (FACS) analysis. AnnexinV is a 36 kDa Ca²⁺ dependent phospholipid binding protein having high affinity for PS.
- c) **Caspase-3 activity assay:** It was performed using synthetic fluorogenic substrate Ac-DEVD-AMC as per the method described earlier (Gautam and Sharma, 2002b). The effect of ROS scavengers and an ETC (electron transport chain) uncoupler (2, 4-dinitrophenol) on PCD profile and caspase-3 activity was also monitored.
- d) **Analysis of *in situ* active caspase 3-like protein by FITC-DEVD-FMK staining**
The assay was carried out using Caspase-3 detection kit (Catalog no. QIA91, Calbiochem) where cells were labeled with cell permeable FITC-DEVD-FMK stain and visualized by fluorescence microscopy.

Cell filamentation assay

Gamma radiation exposed *E. coli* cells were 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). The cells having a length of more than 1 μ M were considered as filaments.

Immunoblotting

Protein samples for detection of LexA and caspase-3-like protein were subjected to SDS-PAGE followed by Western blotting as described earlier (Gautam and Sharma, 2002b).

RESULTS AND DISCUSSION

Enhanced level of NADH in *Xanthomonas* cells undergoing PCD exhibited metabolic stress

Xanthomonas campestris pv. *glycines* (Xcg) grown in LB medium, hence after referred to as PCD inducing medium or PIM, accumulated intracellular NADH and ATP. This was revealed by comparative HPLC analysis of intracellular NADH and ATP levels in PIM and PNIM grown cells. High intracellular NADH resulted in enhanced reactive oxygen species (ROS) generation in cells grown in PIM as confirmed by 2', 7'- dichlorohydrofluorescein diacetate (H₂DCFDA) labeling and ESR spectroscopy. This eventually resulted in the activation of caspase-3-like protein in Xcg leading to PCD. ROS scavengers like dimethylsulfoxide, glutathione, n-propyl gallate and catalase significantly inhibited PCD, caspase biosynthesis as well as caspase-like enzyme activity in this organism. Enhanced ROS level was conferred as one of the possible reasons contributing to caspase activation. This was confirmed by the addition of an electron transport chain (ETC) uncoupler, 2, 4-dinitrophenol, that reduced ROS generation and increased the cell survival. Thus, these results indicated that Xcg cells grown in PIM experience metabolic stress leading to electron leakage during electron transfer in ETC which leads to generation of ROS and subsequent activation of caspase-3-like protein, resulting in PCD.

Role of proline oxidase (PutA) in regulating PCD in *Xanthomonas*

LB medium has glutamate (15%) and proline (6%) in abundance (BD Bionutrients technical manual, 2006; Sezonov et al., 2007). PCD was induced in *Xanthomonas* when it was grown

in PNIM supplemented with proline. However, this was not the case when *Xanthomonas* was grown in PNIM in the presence of glutamate. Proline being a secondary amino acid is not metabolized by transaminases and carboxylases but is oxidized by PutA {also called as proline oxidase (POX) or proline dehydrogenase (PRODH)} (Liu and Phang, 2012). PutA converts proline to glutamate through an intermediate P5C. Notably, *Xanthomonas* cells grown in PIM were found to accumulate proline and have higher proline oxidase activity. Moreover, cells grown in PNIM in the presence of higher levels of proline were also found to undergo PCD. Tetrahydro-2-furoic acid (5mM), an inhibitor of PutA, was found to prevent PCD in PIM growing Xcc cells. ETC inhibitors rotenone and antimycin were also found to inhibit PutA activity in Xcc. To further confirm the role of PutA in PCD, a *putA* knockout was constructed. Interestingly, PCD was abolished in Xcc $\Delta putA$ cells, and the phenotype could be further restored upon complementation with a plasmid vector carrying wild type PutA (pPutA). Contrary to Xcc wt cells, Xcc $\Delta putA$ cells showed diminished ROS generation and reduced caspase-3-like activity as well as PCD inhibition. Xcc wt cells also displayed cell filamentation and *in situ* caspase-3-like activity when treated with a fluorophore tagged caspase-3 inhibitor (FITC-DEVD-FMK). PCD markers like DNA damage (determined by TUNEL assay), phosphatidylserine (PS) externalization and membrane depolarization were significantly less in Xcc $\Delta putA$ cells with respect to wt cells. The findings indicate that the oxidation of proline by PutA is one of the contributing factors leading to an increase in ROS levels and PCD of stressed *Xanthomonas* cells.

PCD in other bacteria and involvement of ROS

To evaluate the conserved existence of PCD-like process in other bacteria besides *Xanthomonas*, the study was performed in *Bacillus subtilis*, *Bordetella bronchiseptica*, *Escherichia coli* and *Salmonella enterica* sv. Typhimurium. Radiation was used as a means to

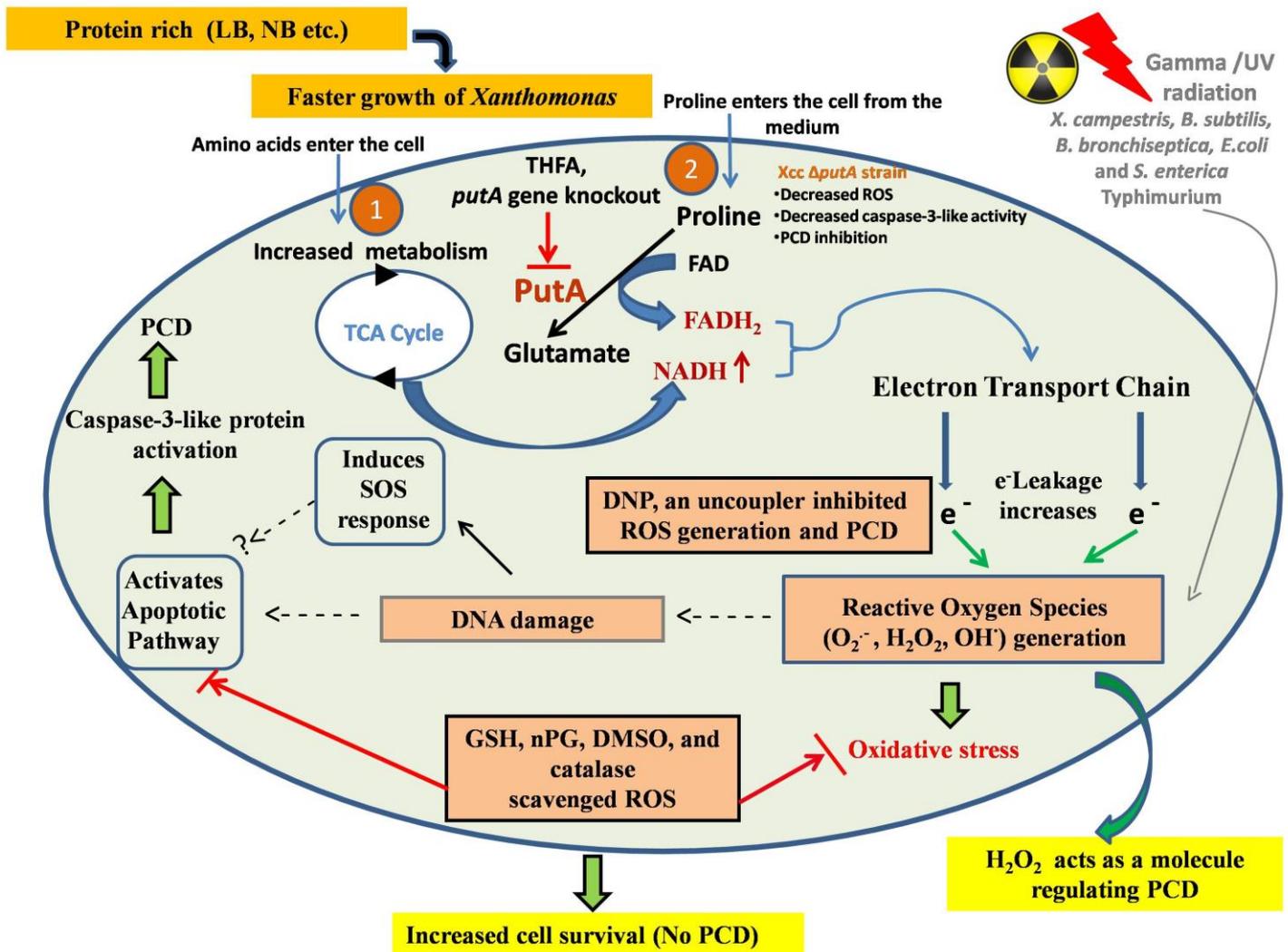
generate ROS. Irradiating these bacteria at their respective D_{10} in the presence of Ac-DEVD-CMK and 3-aminobenzamide, the cell permeable inhibitors of caspase-3 and poly (ADP ribose) polymerase (PARP) respectively, increased the cell survival significantly. FACS analysis indicated an increase in phosphatidylserine (PS) externalization in the radiation exposed bacteria and was found to be reduced in the cells pre-incubated with the cell permeable caspase-3 inhibitor. Radiation-induced SOS response in *E.coli* was also alleviated in the presence of caspase-3 inhibitor as indicated by decrease in LexA degradation and reduced cell filamentation frequency. This might indicate a probable linkage between SOS response and PCD in radiation exposed *E. coli* cells and needs to be confirmed by additional evidences.

SUMMARY

Xanthomonas campestris was observed to experience metabolic stress when grown in a medium (like Luria Bertani broth) where amino acids are the predominant source of carbon and nitrogen. Reactive oxygen species (ROS) were found to be produced in *Xanthomonas* cells undergoing PCD in PIM (PCD inducing medium) and leakage of electrons from electron transport chain (ETC) was found to be a possible source of ROS generation. PutA was also found to be involved in PCD of *Xanthomonas* growing in PIM. PutA enzyme activity was found to be linked to ETC resulting in ROS generation during oxidation of proline and induction of PCD in *Xanthomonas*. Besides *Xanthomonas*, PCD was also observed in other bacteria like, *B.subtilis*, *B. bronchiseptica*, *E.coli* and *S. Typhimurium* when they were exposed to oxidative stress caused by γ -radiation. Bacteria undergoing radiation induced cell death displayed PS externalization and activation of caspase-3-like protein. The presence of cell permeable caspase-3 inhibitor inhibited these processes resulting

in PCD inhibition. Cell filamentation and LexA degradation were observed in radiation exposed *E.coli* cells indicating activation of SOS pathway.

The present understanding of the mechanism of PCD in *Xanthomonas* is depicted in the figure below:



Mechanism of PCD in *Xanthomonas* cells under metabolic stress or *E.coli* and other bacteria upon oxidative stress

CONCLUSION

Metabolism associated stress in *Xanthomonas* cells was found to lead to ROS generation and caspase dependent PCD during which involvement of PutA activity was observed as well. Moreover, other triggers of oxidative stress like radiation treatment also elicited a similar response in different bacteria.

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a. Published**(i) Journals**

- **Wadhawan S**, Gautam S, Sharma A. Involvement of Proline Oxidase (PutA) in Programmed Cell Death of *Xanthomonas*. PLoS ONE, 2014, 9(5):e96423.
- **Wadhawan S**, Gautam S, Sharma A. Bacteria undergo programmed cell death upon low dose gamma radiation exposure. 2014, Int.J.Curr.Microbiol.App.Sci. 3(12):276-283.
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- **Wadhawan S**, Gautam S, and Sharma A. Metabolic stress induced programmed cell death in *Xanthomonas*. FEMS Microbiol. Letters., 2010, 312 (2):176-183.

(ii) BARC Newsletter

- **Wadhawan S**, Gautam S., and Sharma, A. Radiation induced cell death in bacteria is partially programmed. BARC Newsletter. Oct. 2013, 348-354.

b. Accepted: Four (published in journals)

c. Communicated: Nil

d. Other Publications: Four (Symposia and Newsletter)

Symposium/ Conference

- **Wadhawan S**, Gautam S., and Sharma, A. Radiation induced cell death in bacteria is partially programmed. XXXVI All India Cell Biology Conference and International Symposium on “Stress Adaptive Response and Genome Integrity (SARGI)”, Mumbai, Oct., 2012, 130 (*Best poster award*).

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