Xanthomonas PROGRAMMED CELL DEATH HAS SIMILARITIES WITH EUKARYOTIC APOPTOSIS

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Summary

Xanthomonas bacterium exhibited post-exponential programmed cell death (PCD) under certain nutritional conditions especially in a protein rich medium. PCD was not displayed by Xanthomonas cells while growing in minimal medium like starch minimal medium or M9 medium. While undergoing PCD, the organism was found to transform to spherical membranous apoptotic body like structures. The transformation was confirmed by scanning electron microscopy. The membranous bodies were found to contain DNA, which was devoid of the indigenous plasmids of the organism. These bodies were also found to bind annexin V indicative of the externalization of membrane phosphatidyl serine. Nicking of DNA was also detected using a Tunnel assay in Xanthomonas campestris pv. glycines (Xcg) cultures undergoing PCD in LB medium. The PCD in Xanthomonas was found to be associated with the synthesis of an endogenous enzyme similar to human caspase-3, a known marker of apoptosis in eukaryotes. Caspase-3 like protein was detected only in the cells growing in proteinous medium and not in those growing in minimal medium. It was found that starch or its hydrolytic product acted as the repressor of biosynthesis for the Xanthomonas caspase, thereby, preventing the organism from undergoing PCD, upon its addition. Caspase negative mutants of Xcg strain AM2 did not display PCD. The importance of PCD in Xanthomonas life-cycle needs to be elucidated as the system appears to have significant similarities with the eukaryotic apoptosis.

Introduction

Xanthomonas campestris pv. glycines strain AM2 (XcgAM2), the etiological agent of the bacterial pustule disease of soybean, was isolated in this laboratory from the field grown soybean in Maharashtra and biochemically characterised (Sharma et al., 1993; 1994; Sharma, 1999). During the course of our studies on XcgAM2 it was found that the survival of the organism in protein rich medium as Luria-Bertani (LB), nutrient broth (NB), or casein medium was found to be very poor compared to that in minimal medium like starch minimal medium or M9 medium. In a protein rich medium like LB XcgAM2 was found to undergo post-exponential programmed cell death (PCD). PCD was found to be associated with the production of an endogenous enzyme similar to human caspase-3. Caspases, the cysteiny1 aspartate-specific proteases, were first implicated in programmed cell death in the nematode Caenorhabditis elegans (Yuan et al., 1993). The product of ced-3 gene in this organism was found to be related to a family of caspases in mammalian systems. These proteases are important components of the programmed cell death pathway and appear to be conserved throughout the evolution (Nicholson et al., 1995; Nicholson and Thornberry, 1997; Hengartner, 2000). Programmed cell death or apoptosis is a fundamental process in the development and maintenance of multi-cellular organisms. It may be initiated by many physiological, pathological or environmental stimuli and all cells under apoptosis are reported
to undergo similar sequence of morphological and biochemical events (Raff, 1992; Collotta et al., 1992; Hockenbery et al., 1993; Rudel and Bokoch, 1997). The existence of programmed cell death in bacteria was suggested in several studies earlier (Gerdes et al., 1986; Zambrano et al., 1993; Jensen and Gerdes, 1995; Naito et al., 1995; Snyder, 1995; Yarmolinsky, 1995; Aizenman et al., 1996; Naito et al., 1998; Nakayama and Kobayashi, 1998). However, none of these studies provided evidence for the existence of mechanisms in prokaryotes similar to eukaryotic organisms. The present study describes the identification of a caspase like protein, and other markers of apoptosis associated with a nutritionally regulated programmed cell death in *Xanthomonas*.

**Experimental Procedures**

Light microscopy. The cell suspension was spread on a glass slide, allowed to dry in air, heat fixed and stained negatively with India ink. Stained slides were visualised under a light microscope (Carl Zeiss, Germany) using 100 X oil immersion objective. The image was transferred to a TV screen using a CCD camera attached to the microscope and photographed.

**Scanning electron microscopy** : The cells and membranous apoptotic body suspension was fixed with equal volume of glutaraldehyde (25%) diluted 4 fold in sodium phosphate buffer (0.1 M, pH 7.4). Fixed sample was mounted on a metal stub, air dried, and desiccated in CaCl₂ desiccator. Gold sputtering (100 nm thick) was performed and the sample visualised under SEM (SEM-S-240, Cambridge Instruments, UK).

**Apoptotic body isolation** : For obtaining apoptotic body preparation the post-exponential phase culture of the LB medium grown XcgAM2 was centrifuged at 25,000g for 10 min twice to remove the cell debris. The resultant cell-free supernatant was re-centrifuged at 100,000g for 2 h to obtain a pellet containing membranous bodies. The pellet was suspended in sodium phosphate buffer (0.1 M, pH 7.4).

**Caspase-3 assay** : Overnight grown cells were centrifuged, washed, and lysed using 10-volume lysis buffer [Tris-HCl (10 mM), NaH₂PO₄/NaHPO₄ (10 mM, pH 7.5), NaCl (130 mM), triton X- 100 (1%) and sodium pyrophosphate (10 mM)]. A 50 µl aliquot of the cell lysate (10⁸ CFU/ ml) was reacted with 10 µl (1 µg/µl) of synthetic fluorogenic substrate, Ac-DEVD-AMC (PharMingen, USA) at 37 °C for 1h in 1 ml reaction buffer [HEPES (20 mM, pH 7.5), glycerol (10 %), and DTT (2 mM)]. In the control set the reaction was inhibited by 10 µl (0.1 µg/µl) of the synthetic inhibitor of caspase-3 (Ac-DEVD-CHO) per reaction. After incubation the fluorescence intensity was measured using a spectrofluorophotometer (EX₃₈₀ nm, EM₄₄₀ nm).

**SDS-PAGE and Western Hybridisation** : SDS-PAGE was performed as per the standard method. After completion of the electrophoresis electro-blotting was done using a Hybond–P membrane (Amersham-Pharmacia) in a transfer buffer [25 mM Tris, 192 mM glycine (pH 8.3); 20 % methanol] employing 50 mA constant current at 4°C overnight. The blotted membrane was hybridized with the affinity purified biotin-conjugated, polyclonal rabbit anti-active human caspase-3 antibody (10 µl, 0.5 mg/ml) (PharMingen, USA) in 100 ml of the antibody buffer (TBS having 0.05%Tween-20 and 1% gelatin) for 20 h and then incubated in the antibody buffer having streptavidin–horseradish peroxidase conjugate (PharMingen, USA) for 1.5 h. Lastly, the blot was detected using the colour reagent solution [4-chloro-1-naphthol (Sigma) / H₂O₂].

**Annexin V-FITC labelling assay** : An aliquot (50 µl) of the apoptotic body preparation was mixed with 900 µl Annexin V-FITC binding buffer [10 mM HEPES, pH 7.4; 140 mM NaCl and 2.5 mM CaCl₂], 5 µl Annexin V-FITC (PharMingen, USA) and incubated for 30 min at ambient temperature (26±2°C). The fluorescence intensity was measured using a spectro-
flourophotometer [EX, 488 nm, EM, 520 nm]. Similarly, Annexin V-FITC labelling was also done with the membrane preparation obtained from starch minimal medium grown XcgAM2 cells.

**TUNEL assay**: DNA from XcgAM2 cells and those undergoing PCD was isolated, purified, and subjected to nick end labelling using fluorescein isothiocyanate (FITC) labelled dUTP and terminal deoxynucleotidyl transferase (TDT) (PharMingen, USA), and incubated for 60 min at 37°C. The fluorescence intensity was measured using a spectrofluorophotometer [EX, 488 nm, EM, 520 nm].

**Mutagenesis of XcgAM2**: XcgAM2 cells were subjected to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (0.5-1 mg/ml) mutagenesis. The plates were incubated at 26±2°C for 72 h. The colonies from these plates were picked, numbered, and transferred to LB-gelatin (1%) -agar plates in duplicate and incubated at 26±2°C for 72 h. One of the two plates was treated with tichloroacetic acid (TCA, 25%) to check the level of extracellular proteolytic activity. Complete loss or significant reduction in extracellular proteolytic activity was used as a marker to screen out the putative mutants.

**Results**

Rapid cell death in *Xanthomonas campestris* pv. *glycines* AM2. *Xanthomonas campestris* pv. *glycines* AM2 (XcgAM2) cells displayed a typical exponential phase followed by a stationary phase in starch minimal medium (Fig. 1). However, in Luria-Bertani (LB) medium, instead of entering the normal stationary phase at the end of exponential phase, the culture was found to undergo rapid cell death (RCD) (Fig. 1). Other pathovars of *Xanthomonas*, *X. campestris* pv. *malvacearum* NCIM 2310 and *X. campestris* NCIM 2961 also showed RCD in stationary phase, though less.
pronounced than XcgAM2 (data not shown). RCD in LB medium was not displayed by Escherichia coli (data not shown). Incubating the LB grown post-exponential phase culture of XcgAM2 at 4°C was found to arrest RCD (data not shown). In starch minimal medium all the Xanthomonas strains tested did not display RCD (data not shown). Addition of starch to LB growing cultures of XcgAM2 could arrest RCD as shown in Fig. 2. The concentration and time of addition of starch to LB cultures determined the extent of RCD. As shown in Fig. 2 addition of starch at 5% level to LB medium was found to prevent RCD. The RCD was inhibited only if starch was added within 15 h of the start of incubation (Table 1).

Microscopy. The formation of membrane bound bodies was confirmed by the change in the cell morphology of the stationary phase cultures of LB and starch minimal medium grown Xcg under a light microscope with the help of

Table 1: Determination of the period within which addition of starch was effective in arresting RCD in LB growing cultures of XcgAM2. The RCD was inhibited only if the starch was added within 15 h of the start of incubation.

<table>
<thead>
<tr>
<th>Time (h) of incubation at which 5% starch was added to LB growing Xcg cells</th>
<th>Viable cell number at the end of incubation period (96 h) (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$7.3 \times 10^9$</td>
</tr>
<tr>
<td>3</td>
<td>$3.1 \times 10^9$</td>
</tr>
<tr>
<td>6</td>
<td>$3.3 \times 10^9$</td>
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<tr>
<td>10</td>
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</tr>
<tr>
<td>15</td>
<td>$1.3 \times 10^9$</td>
</tr>
<tr>
<td>24</td>
<td>$1.2 \times 10^6$</td>
</tr>
</tbody>
</table>

* CFU = Colony forming units.

![Fig.3. SEM photomicrograph showing image of (A) normal Xanthomonas cells, (B) morphological transformation of Xanthomonas cells to apoptotic body like structures.](image)
negative staining using India ink. The typical rod shaped Xcg cells were found to transform to spherical bodies in LB but not in starch minimal medium The membrane bodies of Xcg could be concentrated by centrifuging the cell-free culture supernatant of Xcg at 100,000g for 2 h. The normal Xcg cells and the transformed cells (membrane bodies) are seen in the scanning electron micrographs (Fig. 3, A and B). The Xcg cells generally measure between 0.4-1 µm x 1.2-3 µm. The size of the spherical membrane body of Xcg was found to range between 0.4-0.7 µm.

Presence of caspase-3 like activity:
While looking for endogenous proteases in XcgAM2, we found the presence of caspase-3 like activity in the XcgAM2 cells from LB grown cultures. The presence of endogenous caspase-3 like activity was assayed using a fluorogenic substrate of caspase-3, Ac-DEVD-AMC [N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin)] and an aldehyde inhibitor of the enzyme, Ac-DEVD-CHO [N-acetyl-Asp-Glu-Val-Asp-CHO] (Thornberry et al., 1992; Li et al., 1995a; Nicholson and Thornberry, 1997). XcgAM2 was found to possess caspase-3 like activity. This is evident from the fact that the relative fluorescence intensity produced by the action of caspase-3 like enzyme from LB grown XcgAM2 on the fluorogenic substrate was almost four fold higher (~940 units) compared to the intensity in the experimental controls, the reaction mixture containing the inhibitor of caspase-3 (~250 units). The reaction mixture with the enzyme from starch grown XcgAM2 cells showed fluorescence intensity values similar to controls (~250 units). It was therefore clear that this activity was not induced in the cells grown in the starch minimal medium.

Western blot analysis of Xanthomonas caspase:
Fig. 4 shows the western blot analysis of the Xanthomonas caspase protein using human caspase-3 antibody. As is evident from the blot a strong hybridisation signal was obtained with the Xanthomonas caspase in LB grown cultures of all the three Xanthomonas strains tested. Interestingly, the hybridisation signal was obtained only with the LB grown cells and not with the starch minimal medium grown cells of the three strains (Fig.4).The caspase protein was not detected in LB grown E. coli cells (Fig. 4).

Changes in plasma membrane:
The fluorescence intensity resulting from the binding of annexin V-FITC to the membrane preparation from the post-exponential phase XcgAM2 cultures grown in LB was very high compared to that of starch minimal medium grown cells (data not shown).

Characterisation of DNA:
The agarose gel electrophoresis of genomic DNA from LB grown cells and DNA isolated from the supernatant of post-exponential phase LB cultures of XcgAM2 showed reduced mobility compared to the genomic DNA of the starch minimal medium grown cells (data not shown). However, the DNA from the supernatant of culture under RCD was found to possess nicks as confirmed by the end labelling or TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay (Li et al., 1995b). The DNA of the post-exponential phase LB grown XcgAM2 cultures under RCD showed significantly higher fluorescence intensity.
compared to the genomic DNA of starch minimal medium grown XcgAM2 cells (data not shown).

Characterisation of caspase mutants: XcgAM2 cells were subjected to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment and screened for extracellular proteolytic activity on LB-gelatin agar. The MNNG treatment of XcgAM2 cells resulted in 2-3 log cycle reduction in cell numbers and resulted in some putative mutants showing reduced extracellular proteolytic activity. Amongst these, four mutants (M-11, M-20, M-24, M-42) were found to show poor hybridization signal with human caspase-3 antibody during western blot hybridization, indicative of lack of or reduced caspase protein presence (data not shown). All these four mutants were found to synthesize some amount of caspase-3 like protein, however no significant caspase enzyme activity was observed compared to the wild type XcgAM2 (data not shown). This showed that whatever caspase-3 like protein observed to be produced by these mutants in the western blot analysis, was largely inactive.

The caspase mutants of XcgAM2 were found to retain the other characteristics of the wild type XcgAM2, including the amylase activity, pigment production, SDS-PAGE protein profile and the two indigenous plasmids (data not shown) (Sharma et al., 1994). However, the characteristic post-exponential RCD observed in the wild type XcgAM2 was not displayed by all these four caspase mutants (Fig. 5).

Discussion

All the three Xanthomonas strains tested in this study were found to undergo post-exponential rapid cell death (RCD) in LB medium. None of the strains showed RCD in starch minimal medium. A control E. coli culture used in the study neither exhibited RCD, nor the presence of caspase protein. Addition of starch to LB culture of XcgAM2, at any point of incubation during the exponential growth, was found to arrest the onset of the RCD. LB grown post-exponential phase XcgAM2 culture was incubated at 4°C to check if the low temperature could arrest RCD. Many of the enzyme activities are reduced at low temperature, and if the process is genetically regulated, the RCD will be affected. Interestingly, at 4°C RCD was found to be inhibited indicating the possible involvement of enzymes. The onset of RCD could be correlated with the synthesis of an endogenous caspase-3 like enzyme activity in XcgAM2 cells. The protein from XcgAM2 that gave a strong hybridisation signal with the human caspase-3 antibody could be called Xanthomonas caspase (Gautam & Sharma, 2002a; 2002b). Caspase mutants of XcgAM2 obtained through MNNG mutagenesis did not display the characteristic post–exponential RCD observed in the wild type XcgAM2 (Gautam & Sharma, 2002a; 2002b). The caspase protein synthesized by these mutants was found to be enzymatically inactive. This clearly indicates the association of caspase activity with the RCD in XcgAM2. This also suggested that the observed RCD in XcgAM2 was indeed a
programmed cell death (PCD). Besides the presence of caspase activity the PCD in Xanthomonas was also found to be associated with the additional features of eukaryotic apoptosis. Annexin V binding to the membrane preparation of the post-exponential phase LB cultures of XcgAM2 indicated externalisation of phosphatidyl serine moieties in the transformed cultures. Annexin V is a 35-36kDa Ca\(^{2+}\) dependent phospholipid-binding protein that has a high affinity to phosphatidyl serine, and therefore, it binds to membranes with exposed phosphatidyl serine. Changes in plasma membrane are reported to be one of the earliest features of apoptotic transformation in eukaryotes (Raynal and Pollard, 1994; Martin et al., 1995; Vermes et al., 1995). The DNA in the post-exponential phase LB cultures of XcgAM2 was found to be nicked. However, no ladder pattern, characteristic of DNA fragmentation in eukaryotic apoptosis, was observed (Wyllie, 1980; Wyllie, 1993; Walker and Sikorska, 1994; Enari et al., 1998). DNA fragmentation has not been detected in several examples of apoptosis in higher organisms, and need not be an absolute requirement for apoptotic cell death (Barres et al., 1992; Ucker et al., 1992; Oberhammer et al., 1993).

**Conclusion**

These studies show that the RCD in Xanthomonas has similarities with the programmed cell death of eukaryotes. The possibility of existence of programmed cell death in prokaryotes has been suggested by many earlier studies (Gerdes et al., 1986; Zambrano et al., 1993; Jensen and Gerdes, 1995; Naito et al., 1995; Snyder, 1995; Yarmolinsky, 1995; Aizenman et al., 1996; Naito et al., 1998; Nakayama and Kobayashi, 1998). Programmed cell death was also reported to be an altruistic effort under the conditions of starvation, allowing survival of some cells at the expense of others, in a bacterial culture (Zambrano and Kolter, 1996). Further investigations are necessary to confirm the potential role of the observed programmed cell death in XcgAM2. These studies strongly suggested that the programmed cell death in Xanthomonas probably has the mechanism similar to eukaryotic apoptosis (Gautam & Sharma, 2002a; 2002b; Gautam, 2003; Gautam & Sharma, 2004). It is believed that apoptosis is an essential part of life for any multi-cellular organism and that the way in which most cell die is conserved from worm to mammal (Meier et al., 2000). This is the first report showing the presence of the main markers of eukaryotic apoptosis in a bacterial cell and may provide important clues to the evolution of PCD in higher life forms (Bayles, 2003; Rice & Bayles, 2003).

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**References**


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About the authors ...

**Dr Satyendra Gautam** joined the Food Technology Division in 1995 after graduating from the 38th batch of Training School as a biology-radiobiology trainee. Dr Gautam was awarded Ph.D. degree in Biochemistry by University of Mumbai under the guidance of Dr A. K. Sharma, Head, Food Technology Division. The topic of his doctoral research was “Studies on Xanthomonas campestris pv. glycines with special reference to its programmed cell death”. His current research interests include mechanism and regulation of bacterial programmed cell death, and radiation preservation of foods using gamma radiation.

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