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Original Research Article

Bacteria undergo programmed cell death upon low dose gamma radiation exposure

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A B S T R A C T

A subpopulation of *Salmonella enterica* sv. *Typhimurium*, *Bordetella bronchiseptica*, *Xanthomonas campestris* and *Bacillus subtilis* was found to undergo caspase dependent programmed cell death (PCD) upon exposure to gamma radiation. Irradiating these bacteria at their respective D_{10} dose in the presence of inhibitor of caspase-3 or poly (ADP ribose) polymerase (PARP) increased the cell survival significantly. FACS analysis indicated phosphatidylserine externalization (PS) in these bacteria upon radiation exposure. A PCD negative mutant of *Xanthomonas* lacking caspase-3-like activity and displaying reduced PS externalization was found to be comparatively less susceptible to radiation than its wild type counterpart. Together these findings established the involvement of PCD in a subpopulation of radiation exposed bacteria.

Introduction

Microbial contamination is a menace causing food spoilage, food borne illnesses and other human and animal diseases. Controlling microbial contamination is quite challenging. Food contamination can be overcome by various physical and chemical antimicrobial treatments. Radiation processing is one of the physical treatments used to sanitize food and has been approved by various international bodies such as WHO, FAO and IAEA (Farkas, 2006). In general, radiation treatment kills the microrganisms by damaging its DNA and inhibiting their reproductive capability. Cells either succumb to DNA damage or respond in other ways which suppress their growth and induces the process of damage repair.

Recently, bacterial cultures have been found to display population behavior such as altruism, quorum sensing and biofilm formation. In the current scenario, it is known that bacteria die due to radiation exposure, but how does the cell respond to lethal or sub-lethal doses of genotoxic treatments like gamma radiation still

remains to be resolved. To address this issue, in the current study different bacterial cultures namely, *Salmonella enterica* sv. *Typhimurium*, *Bordetella bronchiseptica*, *Xanthomonas campestris* pv. *glycines* and *Bacillus subtilis* were exposed to various doses of gamma radiation and their radiation sensitivity was determined. Also, the nature of death (programmed or necrotic) was ascertained by exposing these cells to radiation in the presence of cell permeable inhibitor of caspase-3 or poly (ADP ribose) polymerase (PARP), the key enzymes reported to be involved in the process of PCD in other systems.

Additionally, the level of caspase-3-like activity, status of phosphatidylserine (PS) externalization (an important marker of PCD) and intracellular level of ROS were also evaluated in radiation treated bacterial cultures. Also, a PCD and caspase negative mutant of *Xanthomonas campestris* pv. *glycines*, XcgM42, was used during the course of this study .

Results and Discussion

Radiation treatment caused cell death in bacteria

With an increase in radiation dose enhanced cell killing was observed in different bacteria, however, the extent of death varied among different genera. *Salmonella enterica* sv. *Typhimurium* was found to be comparatively moderately radiation sensitive and its D_{10} was found to be 148 Gy (Fig. 1C). D_{10} indicates decimal reduction dose, i.e. the dose required to kill 90% of the cell population (Vincent *et al.*, 1990). Many isolates of *S. Typhimurium* have been found to be pathogenic to humans. *Bordetella bronchiseptica* and *Bacillus subtilis* were found to be comparatively more resistant with a D_{10} of 318 and 330 Gy respectively (Fig. 1A and B). Some strains of *Bordetella* have been found to be opportunistic pathogens in humans and animals. *Bacillus subtilis* is a soil saprophytic bacterium but also causes food spoilage. A pathogen of soybean plant, *Xanthomonas campestris* pv. g*lycines*, was found to be radiation sensitive with D_{10} of 66 Gy whereas the D_{10} for XcgM42, a caspase and PCD negative mutant of *X. campestris* (Gautam and Sharma, 2002a) was found to be 77 Gy, 17% higher than its wild type counterpart (Fig. 1D). Increase in D_{10} or in other words radioresistance of this mutant indicated that possibly caspase mediated PCD plays a significant role in radiation induced cell death (RICD) in this organism. Although the possible existence of caspase/metacaspase-like domain containing proteins has been reported from different bacterial species by many authors (Koonin and Aravind, 2002; Ning *et al.,* 2002; Sahoo *et al.,* 2006; Jimenez *et al.,* 2009), still the exact sequence of its gene and protein has not been resolved.

Radiation treatment resulted in activation of caspase-3-like protein in bacteria

Activation of caspase-3 enzyme is an important event in case of eukaryotic cells undergoing PCD (Michelin *et al.,* 2004; Yuan and Horvitz, 2004; Elmore, 2007). Here too, radiation exposure was found to significantly induce caspase-3-like activity in these bacteria (Fig. 2A). The activity of this enzyme was found to be negligible in all the non-irradiated bacterial cultures. Upon radiation treatment it was found to increase by 3.5 fold in *S. enterica* sv. *Typhimurium* cells, 2.7 fold in *B. subtilis* and *B. bronchiseptica* and 2.3 fold in *X. campestris.* However, the caspase-3-like activity dropped significantly in the cells pre-incubated with Ac-DEVD-CMK, a cell permeable caspase-3 inhibitor. This strongly

correlated with the increase in cell survival found in these bacteria when exposed to radiation in the presence of caspase-3 inhibitor (as discussed later in Fig. 2C). This clearly implies that radiation induced cell death (RICD) in a subpopulation of bacteria was caspase dependent. However, after gamma radiation exposure no significant increase in caspase-3-like protein (CLP) biosynthesis was observed in these bacteria as analyzed by immunoblotting using polyclonal anti-caspase-3 antibody (Fig. 2B). The molecular weight of CLP detected by the caspase-3 antibody varied in different bacterial strains. CLP was found to be 15 kDa in *S. enterica* sv. *Typhimurium* (Fig. 2B, lanes 8 and 9). The detected molecular weight is quite smaller than the reported molecular weight of caspase in different organisms. In case of *B. subtilis* and *B. bronchiseptica* a protein band of ~150 kDa (Fig. 2B, lanes 2–5) and in *X. campestris* ~90 kDa protein band was detected by anti-caspase-3 antibody (Fig. 2B, lanes 6 and 7). The presence of caspase-3-like protein has been reported earlier from this laboratory in *X. campestris* (Gautam and Sharma 2002b; Gautam and Sharma, 2005; Gautam *et al.,* 2005; Raju *et al.,* 2006; Wadhawan *et al.,* 2010; Wadhawan *et al.,* 2014; Bayles, 2014) and in *B. subtilis* by other authors (Sahoo *et al.,* 2006).

Inhibition of radiation induced cell death (RICD) by cell permeable caspase-3 inhibitor

Several small peptide inhibitors mimicking the recognition sequence of caspases alkylate the cysteine residue in the active site of caspase and irreversibly inactivate it (Schotte *et al.,* 1999). Based on this, the effect of an irreversible caspase-3 inhibitor on the survival of radiation treated bacterial population was investigated. Interestingly, the cell survival in all these bacteria was found to improve remarkably when cells were incubated with the caspase-3 inhibitor 30 min prior to radiation treatment at their respective D_{10} (Fig. 2C). This resulted in two fold increase in survival in the case of *S. enterica* sv. *Typhimurium* and *B. subtilis* cells and a threefold increase in survival of *B. bronchiseptica* and *X. campestris* cells. Unlike its wild type counterpart, no increase in cell survival of radiation treated XcgM42 cultures (PCD and caspase negative mutant) was observed even after pre-incubation with caspase-3 inhibitor (Fig. 2C). There are similar reports of reversal of PCD induced by radiation or other stress in eukaryotic cells and thymocytes in the presence of peptide based caspase inhibitors (Toyooka *et al.,* 1998). Caspase has also been reported to be activated upon radiation exposure in other systems like neural cell precursors and HeLa cells (Michelin *et al.,* 2004; Kim *et al.,* 2003). Probably, a fraction of the dying population undergoes caspase dependent cell death (i.e. those which were rescued in the presence of caspase-3 inhibitor) and the rest of the cells die by some other mechanism like necrosis due to acute cellular damage.

PARP inhibitor also protects bacteria from RICD

Poly (ADP-ribose) polymerase (PARP) has also been reported to be involved in DNA repair and PCD in different systems (Giansanti *et al.,* 2010). It catalyzes the cleavage of NAD^+ into ADP and ADP-ribose and attaches several molecules of the latter to the target protein in a process called poly ADP-ribosylation. PARP is activated by DNA breaks, and can deplete NAD^+ and ATP of a cell in an attempt to repair the damaged DNA. ATP depletion in a cell could lead to necrosis. Presence of PARP inhibitor, 3-aminobenzamide (3-ABA), was found to offer varying degrees of protection against RICD in these bacteria (Fig. 2C).

Fig.1 Effect of radiation treatment at different doses (upto 1 kGy) on the viability of bacteria (A) *Bacillus subtilis*, (B) *Bordetella bronchiseptica*, (C) *Salmonella enterica* sv. *Typhimurium*, (D) *Xanthomonas campestris* pv. *glycines* (wild type) and a PCD and caspase negative mutant (Xcg M42) (earlier obtained by a random mutagenesis using 1-methyl-2-nitro-1-nitrosoguanidine (MNNG) mutagenesis, Gautam and Sharma, 2002a). An aliquot (1 ml) of log phase grown culture of above mentioned bacteria was withdrawn and serially diluted using saline (0.85%) to achieve the cell density of ~10^6 cfu/ml. The cell suspension was irradiated at different doses in a Gamma Chamber (Co^{60} source, dose rate - 5 Gy/min). The viable plate count was determined ~1h after irradiation.

Figure1

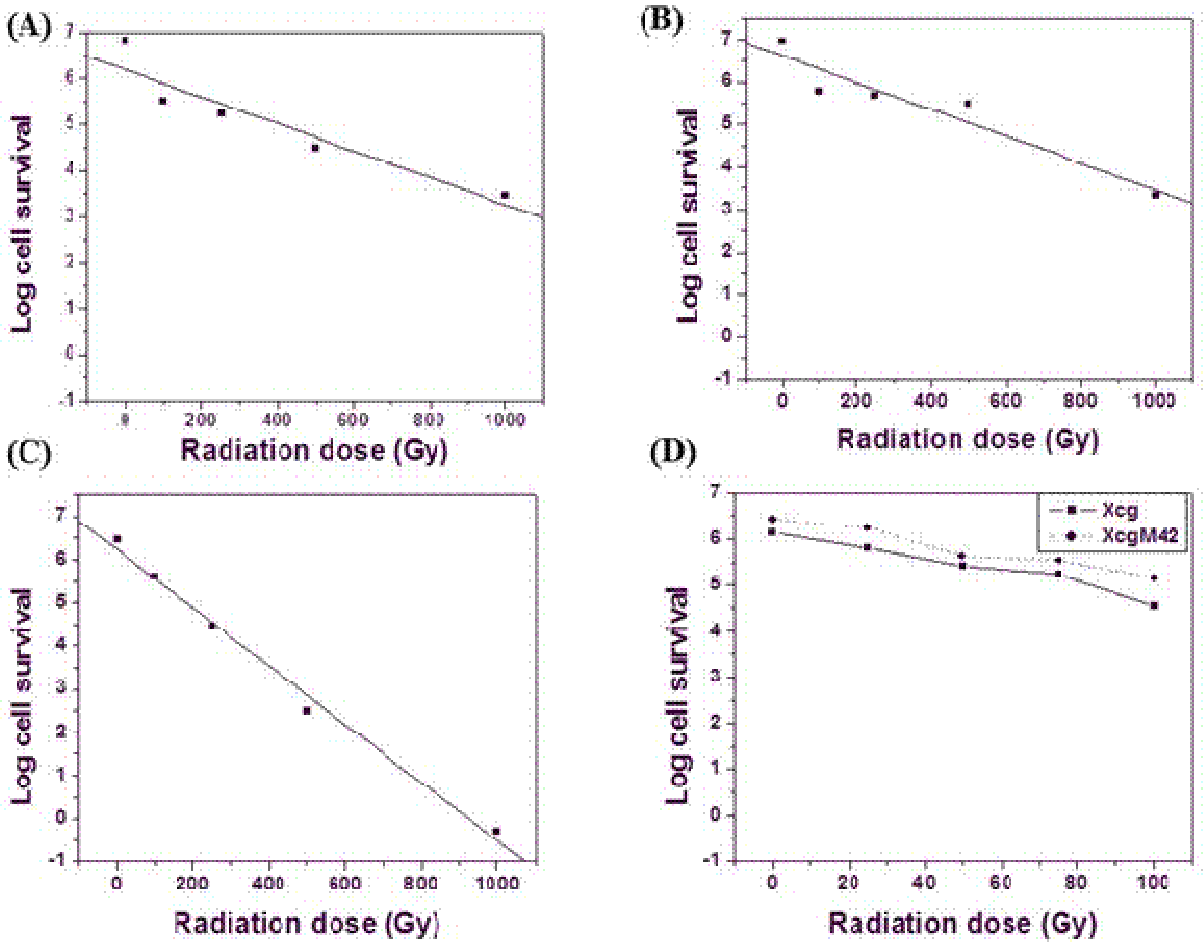


Fig.2 Effect of radiation on markers of PCD (A) Caspase-3-like enzyme activity in radiation treated bacterial cells (at their respective D_{10}) in absence or presence of caspase-3 inhibitor assayed using a caspase-3 assay kit (BD Pharmingen, USA) using Ac-DEVD-AMC, a synthetic tertrapeptide substrate as described earlier (Gautam and Sharma, 2002a); (B) Western blot hybridization indicating caspase-3-like protein expression in different bacteria using affinity-purified, biotin-conjugated, polyclonal rabbit anti-active human caspase-3 antibody (BD Pharmingen, USA) as described earlier (Gautam and Sharma, 2002a): (lane 1: colour protein molecular weight marker; lane 2: *B. bronchiseptica* cells – non-irradiated; lane 3: *B. bronchiseptica* cells – irradiated at respective D_{10}; lane 4: *B. subtilis* – non-irradiated; lane 5: *B. subtilis* – irradiated at respective D_{10}; lane 6: *X. campestris* – non-irradiated; lane 7: *X. campestris* – irradiated at respective D_{10}; lane 8: *S. Typhimurium* – non-irradiated; lane 9: *S. Typhimurium* – irradiated at respective D_{10}); (C) the culture was pre-incubated with either caspase-3 inhibitor (Ac-DEVD-CMK which is a water soluble chloromethylketone derivative, 10 µM), or PARP inhibitor (3-aminobezamide, 500 µM) for 30 min prior to irradiation treatment to determine the rescue potential of these inhibitors from radiation induced death of bacterial cells; (D) Status of reactive oxygen species in different bacterial cells upon gamma radiation treatment was examined by dichlorohydrofluorescein (H_2DCFDA) staining and was carried out as mentioned previously (Wadhawan *et al.*, 2014). Different letters on the bars indicate that the means are significantly different at $p<0.05$

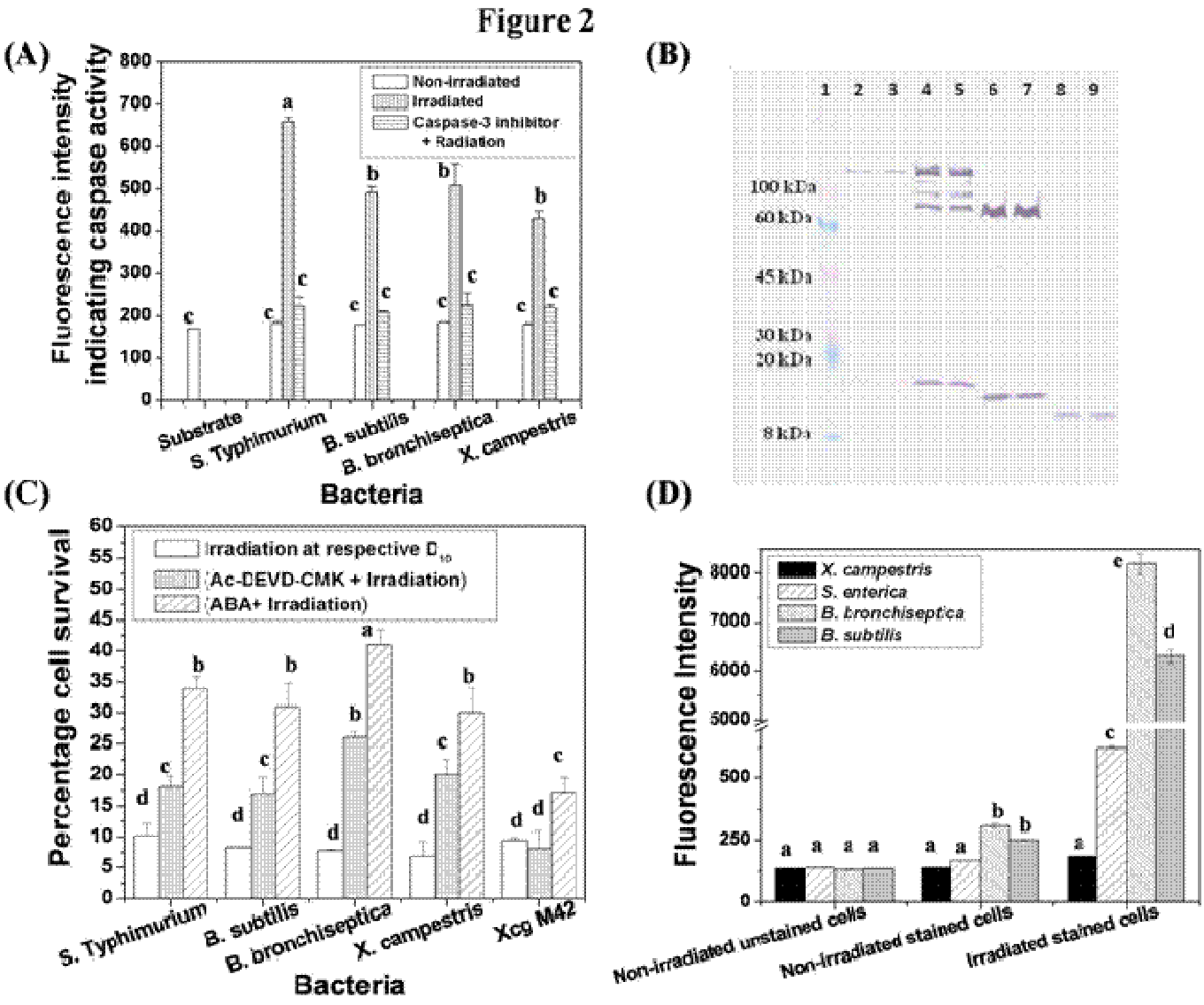
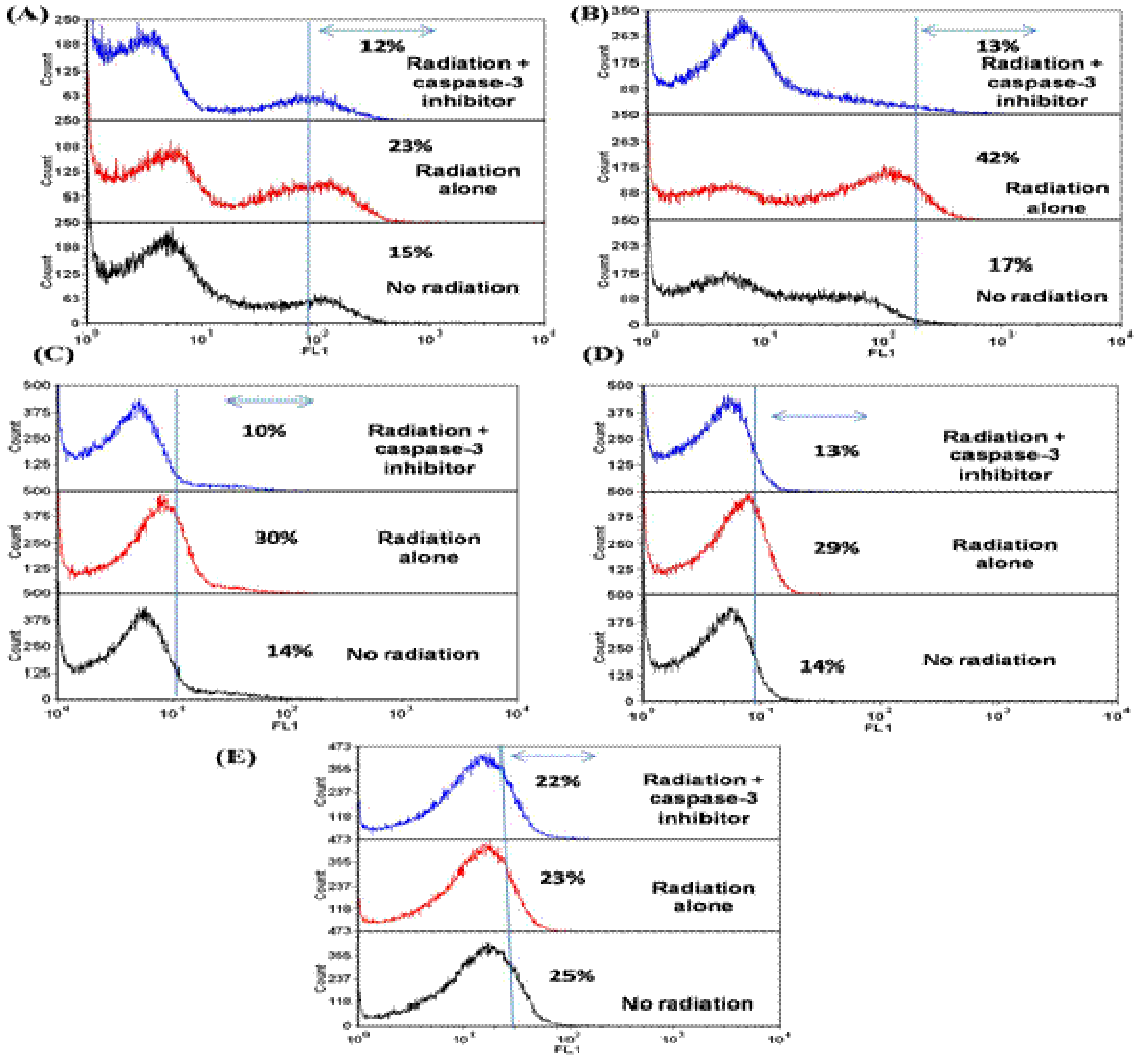


Fig.3 Level of PS externalization in radiation treated bacteria (at their respective D_{10}) was determined by AnnexinV-FITC labeling and was carried out using the AnnexinV-FITC apoptosis detection kit (BD Pharmingen) as described earlier (Wadhawan et al, 2013). Level of PS externalization in (A) *B. subtilis*; (B) *B. bronchiseptica*; (C) *S. enterica Typhimurium*; (D) *X. campestris* and (E) XcgM42



Cell survival increased by three fold in *S. enterica* sv. *Typhimurium*, four fold in *B. subtilis* and *X. campestris*, five fold in *B. bronchiseptica* cells as well as by two fold in XcgM42 when these cells were incubated with PARP inhibitor for 30 min prior to radiation treatment.

Phosphatidylserine externalization in radiation treated cells

Phosphatidylserine (PS) belongs to a class of acidic phospholipids normally found on the internal leaflet of plasma membrane (Naito *et al.*, 1997). PS externalization has been reported to be a distinct event during PCD in many systems including bacteria (Gautam and Sharma, 2002a; Raju *et al.*, 2006; Sahoo *et al.*, 2006; Elmore, 2007; Dwyer *et al.*, 2012; Wadhawan *et al.*, 2013, 2014). It is a downstream event and occurs after caspase activation. PS externalization was assayed by annexinV labeling and subsequent FACS analysis.

The extent of PS externalization increased to 30, 42, 23 and 29% in radiation treated cultures of *S. enterica* sv. *Typhimurium*, *B. bronchiseptica*, *B. subtilis* and *X. campestris*, respectively (Fig. 3A–D).

PS externalization reduced significantly in cells incubated for 30 min with caspase-3 inhibitor prior to radiation exposure and was similar to that found in control non-irradiated cells (Fig. 3A–D). Also, no change in PS externalization level was detected in radiation treated culture of XcgM42 (Fig. 3E), a PCD negative mutant of *Xanthomonas* indicating the requirement of functional caspase-3 protein for this event to occur.

Gamma radiation exposure resulted in enhanced reactive oxygen species (ROS) generation

An increase in ROS level was observed when these bacterial cells (*Xanthomonas campestris*, *Bacillus subtilis*, *Bordetella bronchiseptica* and *Salmonella enterica* sv. *Typhimurium*) were exposed to radiation (at their half D_{10} dose). The ROS level was determined by H_2DCFDA (2, 7' dichlorohydrofluorescein) staining. This increase in intracellular level of ROS in radiation exposed bacterial cells was found to be least in the case of *Xanthomonas* (~1.5 fold), four fold in *Salmonella*, and highest in *Bordetella* (27 fold) followed by *Bacillus* (25 fold) (Fig. 2D). The observation indicates that *Xanthomonas* is comparatively more sensitive to oxidative stress. This observation also explains the probable reason of difference in D_{10} values of these bacteria. Unlike *Bacillus* and *Bordetella* which have higher D_{10}, *Xanthomonas* succumbs to death even at lower ROS level making it comparatively more radiosensitive. This could be due to the differences in the antioxidant defense mechanisms of these bacteria.

The findings of the current study indicated the activation of inherent caspase-3-like activity in different bacteria upon radiation treatment resulting in induction of programmed cell death. This also indicated the evolutionary conserved nature of PCD among different organisms which serves as one of the mechanisms of cell death upon radiation treatment.

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