CHAPTER 1: INTRODUCTION

1.1 Programmed cell death or apoptosis

The term programmed cell death (PCD) refers to the form of cell death which is regulated by intracellular factors. Apoptosis (greek: falling off), considered to be the physiological cell death, is different from accidental or pathological cell death termed necrosis. The process of programmed cell death, or apoptosis, is usually characterized by distinct morphological characteristics (cell membrane blebbing, externalization of phosphatidylserine, and DNA fragmentation) and energy-dependent biochemical mechanisms (activation of caspases).

Programmed cell death (PCD) was first discovered in *C. elegans* by Carl Vogt around the nineteenth century. It was again investigated by Kerr, Wyllie and Currie in 1972 [1]. The Nobel Prize in Physiology or Medicine 2002 was awarded jointly to Sydney Brenner, H. Robert Horvitz and John E. Sulston "for their discoveries concerning genetic regulation of organ development and programmed cell death" in *Caenorhabditis elegans*. Apoptosis is essential for proper development and functioning of the body. It is involved in the embryonic development, functioning of the immune system, and hormone-dependent atrophy. Inappropriate apoptosis (either too little or too much) is a factor in many human ailments including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer [2] (Elmore, 2007).

In the past couple of decades, several studies have demonstrated the existence of PCD like phenomenon in several unicellular eukaryotes including *Dictyostelium*, *Trypanosoma*, *Leishmania*, *Tetrahymena*, and *Peridinium* as well as in some prokaryotic cells [1,3–5]. Apoptosis associated changes have also been reported in certain groups of fungi like *Aspergillus fumigatus* and yeast, like *Candida albicans* upon oxidative and amphotericin B-mediated stresses [6,7].

PCD has been experimentally proven and established to play vital roles in bacterial developmental processes [3,8]. Some of these include mother cell lysis during sporulation in *Bacillus* [3], vegetative cell lysis during myxobacterial fruiting body formation [3], and salt stress induced PCD in *Anabaena* [9], a cyanobacterium. PCD in bacteria has been proposed to play an altruistic role where defective cells are removed and the nutrients are made accessible to the remaining healthy population.

1.2 Programmed cell death in Archaebacteria

Archaebacteria are the most primitive of all lifeforms and are usually found in extreme environments. They mainly comprise of halophiles, methanogens and thermophiles. Archaebacteria share certain characteristic features with both eubacteria and eukaryotes. They lack a nucleus but their majority proteins involved in replication, transcription and translation have homology to eukaryotic counterparts [10]. Until recently they were considered immortal unless they succumb to death by predators. PCD in archaebacteria like *Haloferax volcanii* under salt stress has recently been reported [11,12]. This archaebacteria exhibits caspase-8 and caspase-4 like activity under salt stress which was inhibited by caspase inhibitor, zVAD-FMK. This activity was also found to be inhibited by EDTA, a metalloprotease inhibitor.

1.3 Programmed cell death in cyanobacteria

Cyanobacteria are photosynthetic microbes, commonly known as 'blue green algae'. They are predominantly present in soil and water bodies as phytoplankton. Cyanobacteria can fix nitrogen as well. Some species (e.g. *Microcystis*) form harmful algal blooms. It has been reported that cyanobacteria like *Microcystis aeruginosa, Trichodesmium,* and *Anabaena*

undergo metacaspase dependent PCD during environmental stress conditions [9,12–15]. *Trichodesmium* forms extensive blooms that may disappear abruptly within 1 to 2 days. Earlier it was thought to be caused by bacteriophage infection, but the work by Berman-Frank et al. [14] has shown that it undergoes autocatalytic PCD due to nutrient deprivation. The deficiency of iron (Fe) and phosphorous (P) was found to initiate PCD in this organism.

1.4 Programmed Cell Death in Eubacteria

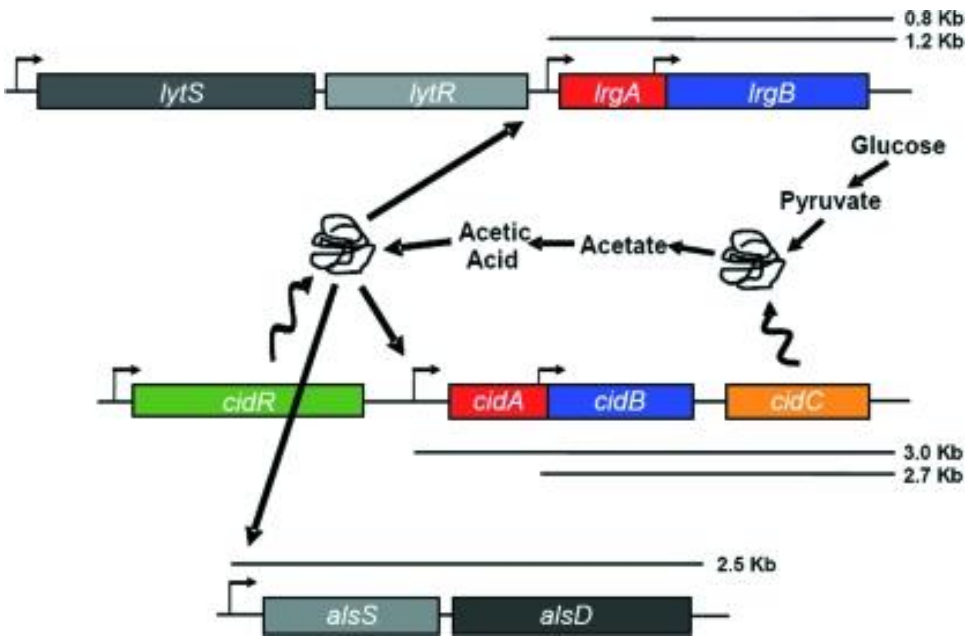
Regulation of cell death is essential for living organisms. In eukaryotic cells, apoptotic cell death is essential for embryonic development, for maintenance of normal cell homeostasis, and for elimination of cells damaged by stress or pathogen infection. In bacteria, regulation of cell growth and cell death is also important under various stress conditions [16].

1.5 PCD in *Staphylococcus aureus*: Cid/Lrg Regulatory System

Staphylococcus aureus is a Gram positive cocci commonly found on the skin and in the respiratory tract of humans. The discovery and characterization of the *cid* and *lrg* operons evolved from the initial identification in 1996 of a novel two-component regulatory system from *S. aureus*, termed LytSR, that affected murein hydrolase activity and autolysis. Although the CidA and LrgA proteins are present in most bacteria, their role in PCD of *S. aureus* has been well characterized [17]. They have been recently reported to be integral membrane proteins in this bacterium [18]. *cidA* and *lrgA* encode for holin and anti-holin, respectively [17,18]. One biological function of the *cid* and *lrg* genes is the coordination of cell death and lysis during biofilm development, causing release of genomic DNA (termed as extracellular DNA or eDNA), which eventually becomes a structural component of the biofilm matrix [18]. Rice et al. (2007) reported that the *S. aureus cidA* mutant exhibited

decreased lysis during biofilm formation [19], while the *lrgAB* mutant, as well as the *lytSR* mutant (which exhibits reduced *lrgAB* expression), exhibited increased lysis [20,21]. The consequence of decreased lysis was a decrease in genomic DNA release and biofilm adherence [19]. In contrast, increased cell lysis during biofilm development resulted in increased biofilm adherence [20,21]. Based on their roles in controlling cell death and lysis during biofilm development, it was proposed that these proteins form the regulatory elements of bacterial programmed cell death (PCD). These genes were found to be induced when *S. aureus* was grown in a glucose rich medium that caused production of acetic acid [22]. The murein hydrolase activity of CidA triggers cell lysis. LrgA prevents cell lysis by inhibiting the activity of CidA. It has been reported that the transcription of both *cidABC* and *lrgAB* was induced by growth in the presence of excess glucose, an effect that was shown to be a result of the metabolism of glucose and the subsequent generation of acetic acid (Fig. 1.1 and 1.2) [22]. Interestingly, the *cidC* gene was found to encode a pyruvate oxidase that could contribute to the acetate (and acetic acid) accumulation in the culture medium during growth in excess glucose [22]. Furthermore, cells containing a *cidC* mutation maintained a much higher level of cell viability in stationary phase than did the parental strain when grown in the presence of excess glucose [23]. Recently, it was reported that the drop in pH due to acetic acid production resulted in PCD which was accompanied by ROS generation and DNA damage [22].

Figure 1.1



Reference: Rice and Bayles, 2008 [23]

Fig. 1.1: CidR-mediated regulation of holin, antiholin and carbohydrate metabolism. The *cidA* and *lrgA* genes encode homologous hydrophobic proteins believed to function as a holin (toxin) and an antiholin (or antititoxin), respectively. The *cidB* and *lrgB* genes also encode homologous hydrophobic proteins whose functions are unknown. The CidR protein, a LysR-type transcription regulator, enhances the expression of *cidABC*, *lrgAB*, and *alsSD* (encoding acetolactate synthase and acetolactate decarboxylase) in response to carbohydrate metabolism. The *cidC* gene encodes pyruvate oxidase. The transcripts associated with each operon are indicated by black bars [23].

1.6 Analogy of Cid/Lrg Regulatory System with Other Reported Systems

Bax/Bcl-2-mediated control of apoptosis leading to the disruption of mitochondria during the initial stages of apoptosis and the holin/antiholin-mediated control of bacterial death and lysis

have recently led to the hypothesis that these events are analogous, both biochemically and physically, to the events. The Bcl-2 proteins are a large family of proteins that are well conserved in eukaryotic organisms. Similar to holins, Bax can cause mitochondrial membrane permeabilization probably by pore formation involving lipid destabilization (Fig. 1.3). This results in the release of cytochrome c which acts as a trigger to activate the caspase cascade. Like antiholins, Bcl-2 (and related antiapoptotic proteins) can interact with Bax to inhibit the induction of cell death, though the mechanism by which this occurs is still being unravelled (Fig. 1.3) [17,23].

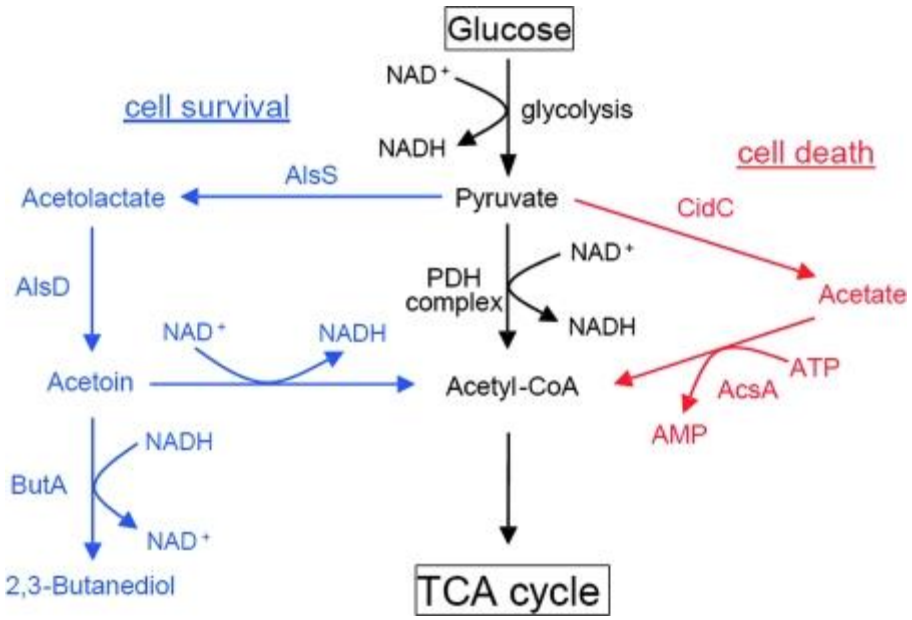
Like many other reported systems of PCD, the Cid/Lrg system has been found to be associated with carbohydrate metabolism. The metabolism of glucose appears to play a central role in the control of cid/lrg expression and in cell death [22,23]. *S. aureus* metabolizes glucose in the presence of oxygen via glycolysis and inhibits the tricarboxylic acid (TCA) cycle, resulting in the secretion of large amounts of acetate into the growth medium (Fig. 1.2) [17,22,23]. The bacteria succumb to acidic milieu. The fate of pyruvate in bacteria (whether it is converted to acetic acid or acetoin) appears to be a key determinant in the decision between life and death (Fig. 1.2), similar to the role that the pyruvate dehydrogenase complex has in controlling the commitment to apoptosis [24–26].

Similar observation has been reported in the case of tumour cells which preferentially metabolize glucose at a higher rate by glycolysis. This observation led to the hypothesis that tumour cells alter metabolism, leading to "aerobic glycolysis" or the "Warburg effect" [25,26]. Recent studies have indicated that the mitochondria in tumour cells undergo a physiological or metabolic "remodelling" that promotes glycolysis rather than mitochondrial glucose oxidation (involving TCA cycle and ETC) [26]. This phenotype of preferential glucose metabolism is used as a marker for detection of cancer cells by PET scan. It is

proposed that the functioning of glycolysis is essential early in the transformation of a cell, which typically occurs in a hypoxic environment prior to vascularisation [27]. Interestingly, it was observed that this glycolytic phenotype is associated with the suppression of apoptosis and resistance to the acidosis produced as a consequence of increased lactic acid generation [28]. Indeed, it is thought that during carcinogenesis, tumour cells "evolve" phenotypic adaptations to the toxic effects of acidosis, culminating in resistance to apoptosis [26].

Thus, in both prokaryotes and eukaryotes, evidence suggests that rapid growth is fuelled by glycolysis and that pyruvate metabolism plays a critical role in the control of cell death (Fig. 1.3) [23].

Figure 1.2

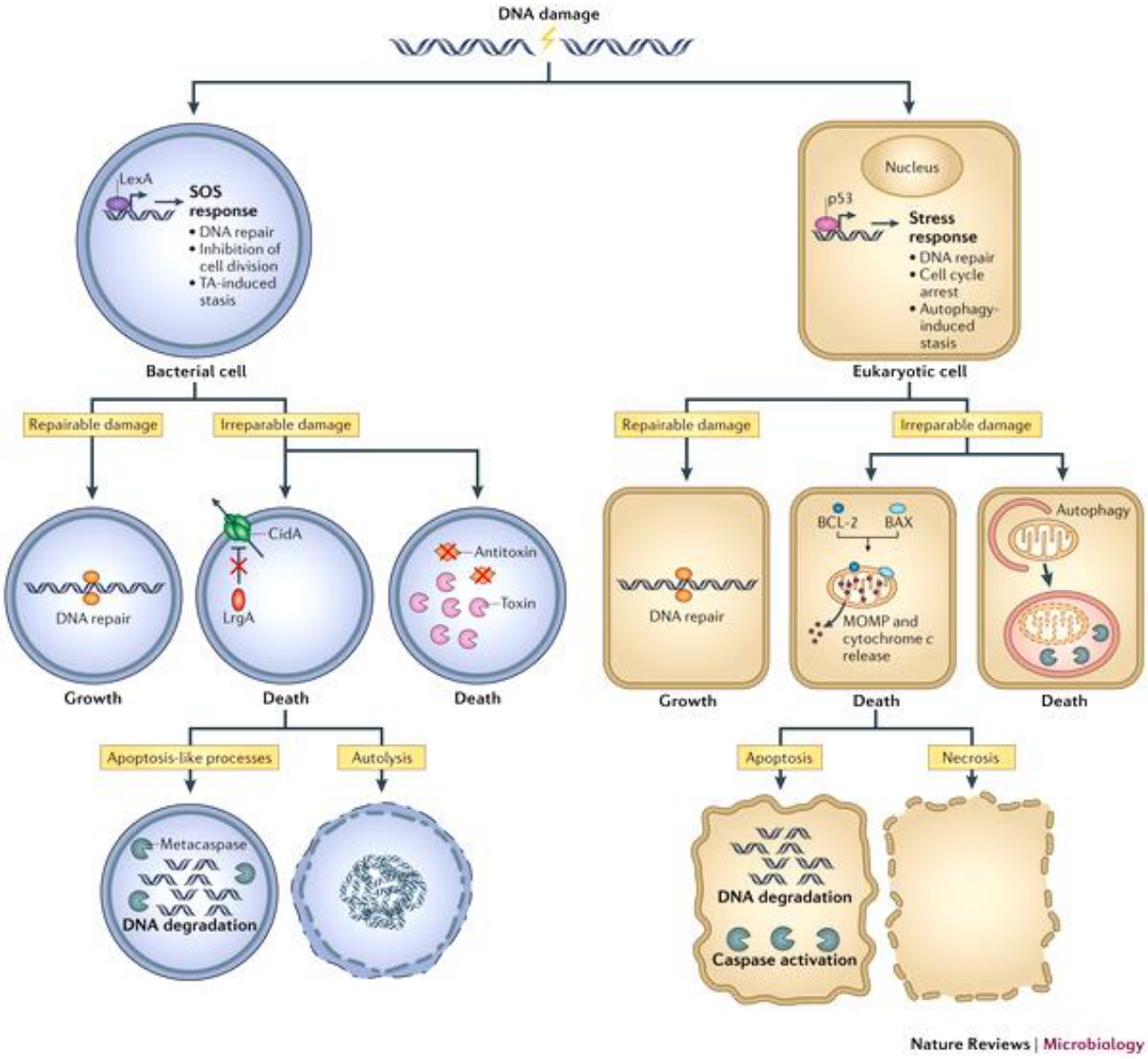


Reference: Rice and Bayles, 2008 [23]

Fig. 1.2: Conversion of pyruvate to acetyl-CoA in *S. aureus*. A major pathway involved in the conversion of pyruvate to acetyl-CoA (shown in black) in bacteria requires the pyruvate dehydrogenase (PDH) complex. Other pathways involved include the AlsSD pathway (blue)

and the CidC pathway (red), which appear to promote cell survival and death, respectively. Enzymes contributing to these pathways include acetolactate synthase (AlsS), acetolactate decarboxylase (AlsD), pyruvate oxidase (CidC), and acetyl-CoA synthetase (AcsA). Also shown is the conversion of acetoin to 2,3-butanediol, requiring the enzyme acetoin reductase (ButA) [23].

Figure 1.3



Reference: Bayles, 2014 [17]

Fig. 1.3: Similarities of PCD mechanism in bacteria and eukaryotes: Cell stress, such as that elicited by DNA-damaging agents, induces a response programme that includes DNA-repair mechanisms and cell death pathways. This response includes mechanisms to inhibit cell division, which directs all available resources to repair the damage. If the levels of damage are minimal, the repair mechanisms will be sufficient to restore the cell to working

order. Similar to the role of p53 in assessing the extent of damage in eukaryotic cells and then coordinating an appropriate response, it was thought that the LexA regulator of the SOS response has a role in coordinating the response to DNA damage in bacteria. In both cases, the processes that result in the recycling of cytoplasmic components (such as toxin–antitoxin (TA) systems in bacteria and autophagy in eukaryotes) are supposed to promote DNA repair. If the damage is irreparable, the repair processes will be nullified leading to programmed cell death. CidA–LrgA-induced cell permeabilization and lysis occurs in bacteria and B cell lymphoma 2 (BCL-2) protein family-induced death (including mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release) is induced in eukaryotes. Alternatively, TA system-induced death (in bacteria) or autophagic death (in eukaryotes) can also be triggered. Finally, post-mortem events are activated, such as those that are associated with apoptosis and necrosis in eukaryotes, and those that are associated with apoptosis-like processes and autolysis in bacteria [17].

1.7 Toxin-antitoxin Modules in Bacteria

The role of toxin-antitoxin module has been well established in bacterial PCD. The TA systems are not essential for cell growth but are considered to play important roles in survival under stress conditions. This module comprises of a pair of closely linked genes that encode a toxin and an antitoxin. The toxin is always a protein, whereas, antitoxin can either be a protein or anti-sense RNA. These were first observed in *E. coli* on low copy number plasmids found to be responsible for post-segregational killing. When the cells lose these plasmids, the cured cells are selectively killed by the toxin because the antitoxin is relatively less stable and is degraded faster [29–31]. Thus the cells harbouring such systems were ‘addicted’ to the short-lived antitoxin and their *de novo* synthesis was found to be essential for cell survival

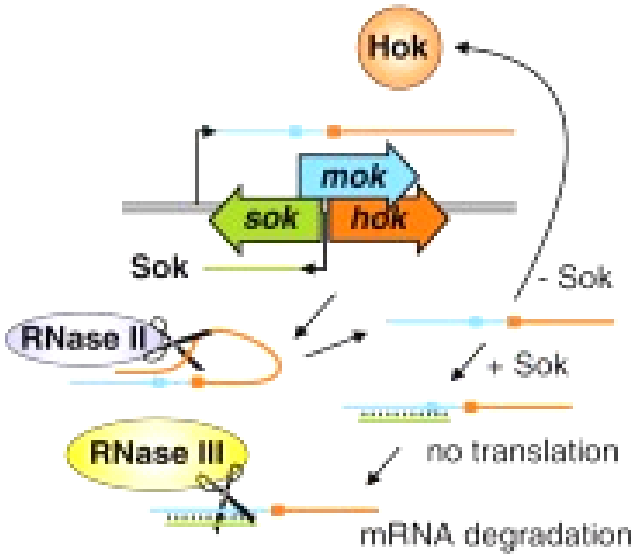
resulting in development of the concept of 'addiction modules' which were implicated in maintaining the stability of extrachromosomal elements. The toxin targets any one of the following cellular processes: DNA replication, mRNA stability, protein synthesis, ATP synthesis or cell wall synthesis [16]. The toxin-antitoxin modules are classified into five types depending on the mechanism of their genetic regulation and also on the nature of antitoxin [32,33]. The antitoxin can prevent the lethal action of toxin in following ways:

(a) Type I

Type I antitoxins are antisense RNAs with short half-life. They work by base pairing with the stable toxin RNA thereby preventing toxin's expression. Examples include *symR/symE, tisB/istR-111, ibs/sib* and hok (host killing)/sok (suppression of killing). The hok/sok genes are encoded by plasmid R1 (Fig. 1.4). In this case regulation is slightly more complex. The RNA antitoxin sok is expressed from a strong promoter but the transcript has a very short half-life of around 30 sec. In contrast, the hok mRNA is expressed from a very weak promoter but has a half-life of ~20 min. The hok transcript shows extensive secondary structure and the 3′ end folds back to the 5′ end. The folded full-length hok mRNA is neither accessible for the ribosome nor the Sok-RNA antitoxin. Processing by RNase II removes a part of the 3′ terminus causing a major structural rearrangement including the 5′ part of the hok mRNA. This allows translation but also binding of the Sok-RNA. However, the Sok antisense-RNA does not show complementarity to the Shine-Dalgarno sequence of hok. Interestingly, the *hok*/*sok* locus contains in addition to the *hok* toxin and *sok* antitoxin a third gene called *mok* for modulator of killing, which overlaps almost the entire *hok* gene. Analysis of point mutations revealed that prevention of *mok* translation abolished efficient expression of *hok*, indicating that the *hok* and *mok* open reading frames are translationally coupled and that the Sok-RNA regulates *hok* translation indirectly by preventing translation of *mok*.

Finally, the hok mRNA/Sok-RNA hybrids are cleaved by RNase III, which is the initial step for decay of the hok mRNA [32]. However, for some type I modules like txpA/ratA18, bsrG/sr419, and yonT/as-yonT18 of *Bacillus subtilis* cleavage of double-stranded RNA regions created by binding of the antitoxin RNA to the toxin mRNA by RNase III is crucial for regulation of toxin expression.

Figure 1.4



Reference: Unterholzner et al, 2013 [32].

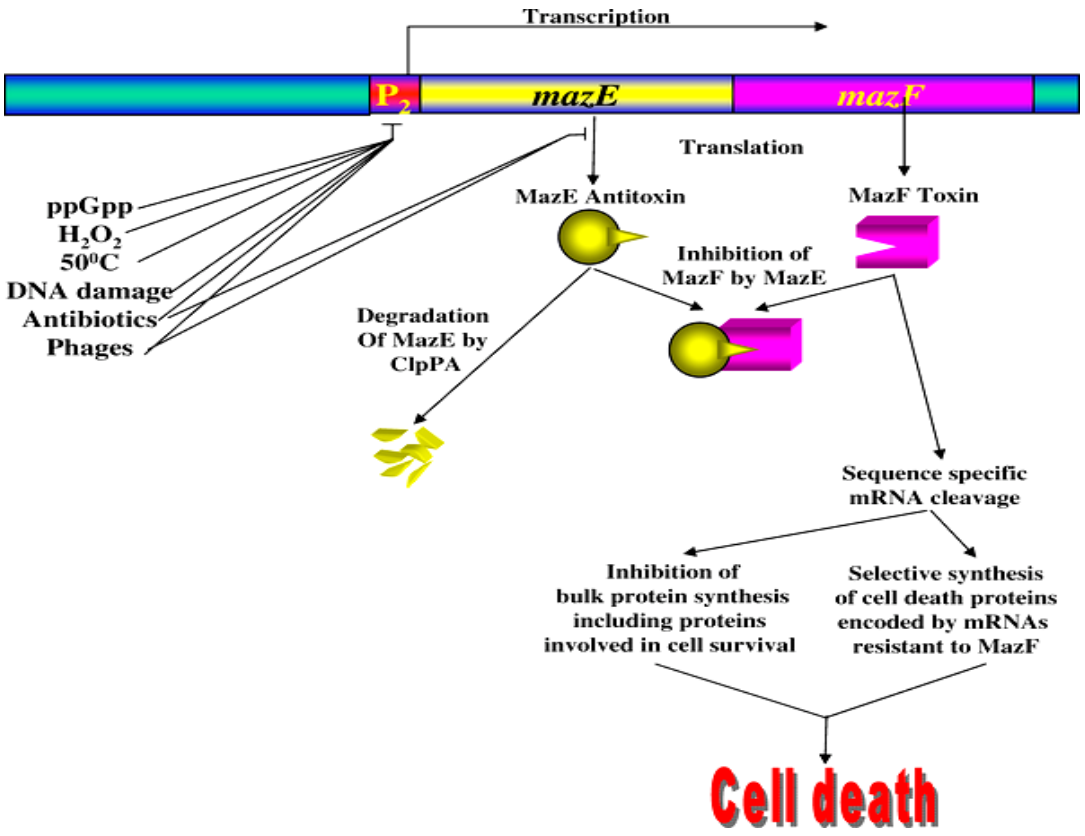
Fig. 1.4: Regulation of the type I system hok/sok of plasmid R1. The toxin and its encoding gene are shown in orange while the antitoxin and its encoding gene are shown in green.

(b) Type II

Unlike Type I, here both toxin as well as antitoxin is protein. This is the best studied type of all TA systems. The antitoxin forms a protein-protein complex with the toxin and inhibits its action. The antitoxin is comparatively labile and is degraded by cellular proteases [Clp (caseinolytic protease) or Lon] under stress conditions, thereby, releasing the toxin leading to either bacteriostasis or cell death. Some examples of type II include *mazE/F, relB/E,*

parD/E, mqsR/A, vapB/C, higA/higB and *yefM/*yoeB [16,32,34,35]. The operon of type II TA modules typically comprises two open reading frames where the upstream gene usually encodes the antitoxin. However, exceptions of this conserved gene organization are known, for instance the *higB/higA* TA module, where the toxin gene higB is located upstream of the antitoxin gene higA. Another TA pair, MazEF has been studied in great detail in *E. coli*. It mediates cell death under various stress conditions like amino acid starvation, antibiotic treatment, inhibition of transcription or translation, DNA damage (by mitomycin C or UV irradiation) and oxidative stress. Under these conditions, MazE (antitoxin) is degraded by cellular proteases releasing MazF to exert its endoribonucleolytic activity on mRNAs (Fig 1.5) [30]. MazF endoribonuclease preferentially cleaves single-stranded mRNAs at ACA sequences [30]. Recently, it was shown that a pentapeptide (Asn-Asn-Trp-Asn-Asn), also known as extracellular death factor (EDF) was required for *mazEF* mediated cell death [36–38]. This pentapeptide was found to induce the endoribonucleolytic activities of two toxins: MazF and ChpBK [39]. It has been reported that the genes specifying MazEF, the glucose-6-phosphate dehydrogenase, and ClpXP protease are critical in EDF production [37].

Figure 1.5



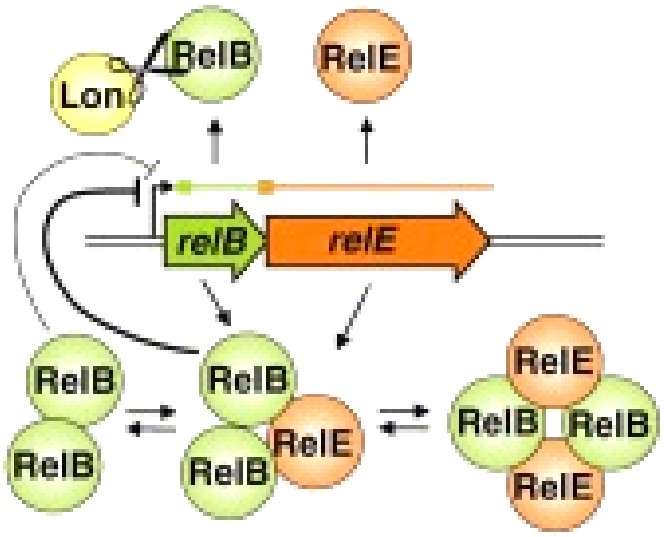
Reference: Engelberg-Kulka et al., 2006 [30]

Fig. 1.5: MazEF toxin antitoxin system in *E. coli*

Typically, transcription of the TA operon is autoregulated by binding of the antitoxin or by the toxin–antitoxin complex to the promoter. Depending on the stoichiometric ratio of the antitoxin to the toxin several types of complexes may be formed with distinct affinities to the promoter. For example, in an excess of RelB over RelE dimers of RelB (RelB2) and the 2:1 complex RelB2RelE are formed, both of which inhibit the *relB/relE* promoter (Fig. 1.6). The RelB2RelE has a stronger inhibitory effect on the *relB/relE* promoter than RelB2 and thus RelE acts as a transcriptional co-repressor [32]. On the contrary, in an excess of RelE the 2:2

complex (RelB2RelE2) is formed, which cannot bind the promoter and, consequently, transcription is activated. This mode of regulation is frequently called conditional cooperativity and is believed to be important for stabilization of the antitoxin level in rapidly growing cells to minimise random induction of relB/relE [32]. Also other TA systems including *phd-doc* from the *E.coli* bacteriophage P137 and *vapB/vapC* of *Salmonella enterica* are regulated by conditional cooperativity.

Figure 1.6



Reference: Unterholzner et al, 2013 [32].

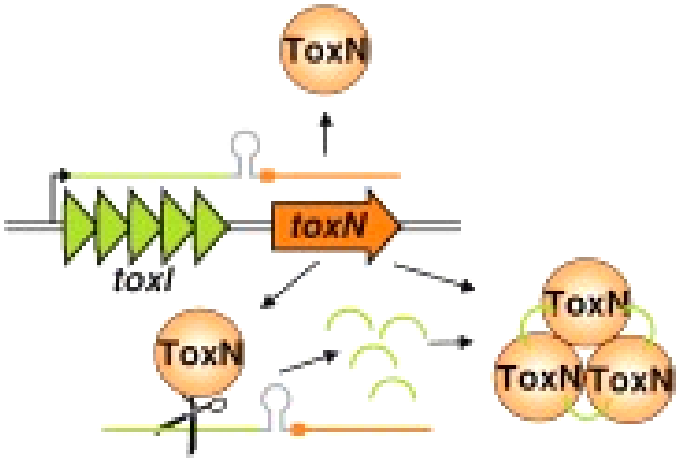
Fig. 1.6: The *relB/E* TA module type II system from *E. coli.* The toxin and its encoding gene are shown in orange while the antitoxin and its encoding gene are shown in green.

(c) Type III

Similar to type I systems, the antitoxin of type III modules is RNA. Type III toxin-antitoxin systems encode protein toxins that are inhibited by pseudoknots of antitoxin RNA [40]. Type III TA loci were first isolated and defined as abortive infection (Abi) systems, protecting

bacterial populations from bacteriophage assault [41]. Within each Type III locus, a toxin gene is preceded by a short palindromic repeat, which is itself preceded by a tandem array of nucleotide repeats. The short palindromic repeat acts as a transcriptional terminator, regulating the relative levels of antitoxic RNA and toxin transcript [41]. The first reported Type III TA system, ToxIN, was encoded on plasmid pECA1039 of the Gram-negative phytopathogen, *Pectobacterium atrosepticum* [40,41]. This locus encodes a 19.7-kDa toxic protein, ToxN, and upstream of ToxN is a repetitive array containing 5.5 tandem repeats of a 36 nt sequence, collectively known as the ToxI antitoxin (Fig. 1.7). Through genetic studies, it was predicted that each 36 nt ToxI RNA repeat was able to inhibit the activity of ToxN [41]. The crystal structure of the ToxI/N complex revealed a heterohexameric triangular assembly of three ToxN proteins interspersed by three, 36 nt, ToxI RNA pseudoknots (Fig. 1.7). ToxN was demonstrated to be an endoribonuclease, related in structure to the endoribonucleases Kid and MazF [40].

Figure 1.7



Reference: Unterholzner et al., 2013 [32].

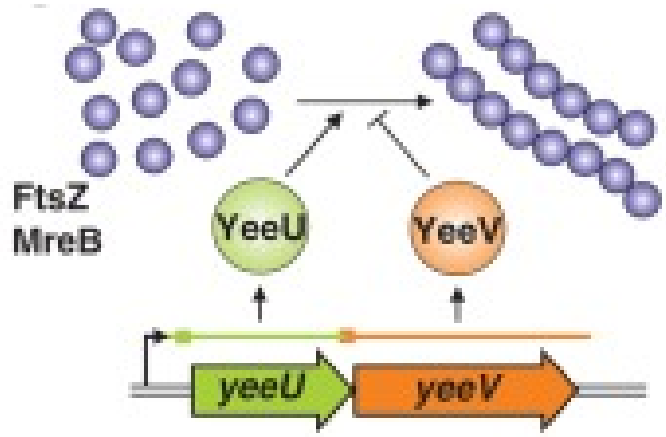
Fig. 1.7: The *toxI/N* type III system from the *Erwinia carotovora* plasmid pECA1039. The toxin and its encoding gene are shown in orange while the antitoxin and its encoding gene are shown in green.

Recently, Samson and co-workers reported AbiQ system of *Lactococcus lactis* to behave as type III TA module [42,43]. AbiQ is a phage resistance mechanism found on a native plasmid pSRQ900 of *Lactococcus lactis* that abort virulent phage infections. The two components of the AbiQ system are *antiQ* repeats (antitoxin) and *abiQ* gene (encoding ABIQ endoribonuclease or toxin) [42]. The AbiQ system was found to be active against members of the common 936 and c2 phage groups as well as rare lactococcal phage groups [42,43]. It has also been demonstrated that the free toxin can cleave, through its endoribonuclease activity, the cognate antitoxins [42,43] as well as housekeeping bacterial RNA molecules [42,43] leading to cell death. During the phage infection process, this TA interaction is likely to be disrupted, leading to cell death and abortion of the phage infection.

(d) Type IV

A type IV TA system designation was proposed for the *yeeU/yeeV* (also named *cbtA/cbeA*) TA module of *E. coli* [44]. The functional analysis of this TA module revealed that the toxin YeeV interacts with cytoskeletal proteins MreB and FtsZ and thereby interferes with their polymerization (Fig. 1.8). The YeeU antitoxin protein counteracts YeeV by stabilizing MreB and FtsZ polymers (Fig. 1.8). A similar mode of action was also reported for cptA/cptB (ygfX/ygfY), another TA module of *E. coli* [45]. YgfX is the first membrane associating toxin in bacterial TA systems [45].While the toxin and antitoxin of all other TA classes interact either at the RNA or the protein level, the toxin and antitoxin of this TA class do not directly interact.

Figure 1.8



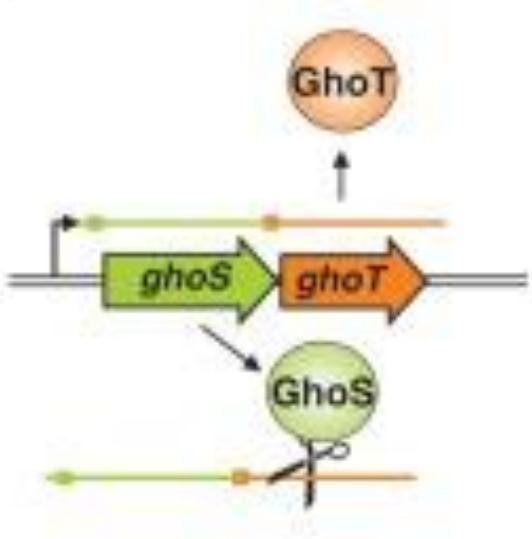
Reference: Unterholzner et al., 2013 [32]

Fig. 1.8: Type IV YeeU/V TA module of *E. coli*. The toxin and its encoding gene are shown in orange while the antitoxin and its encoding gene are shown in green.

(e) Type V

Recently, the *ghoS/ghoT* TA module of *E. coli* was designated as a type V TA system wherein the protein antitoxin (GhoS) inhibits the toxin by cleaving specifically its mRNA. (Fig. 1.9) [46]. GhoT (toxin) is a membrane lytic peptide that causes ghost cell formation (lysed cells with damaged membranes) and increases the population of persister cells (i.e. cells that are tolerant to antibiotics without undergoing genetic change) [46].The antitoxin protein GhoS has a sequence specific endoribonuclease activity for the cleavage of the GhoT toxin mRNA, and thereby prevents the translation of the toxin. The authors further revealed the NMR structure of GhoS indicating it to be related to the CRISPR-associated-2 RNase.

Figure 1.9



Reference: Unterholzner et al., 2013 [32].

Fig. 1.9: The type V GhoS/T TA module of *E. coli.* The toxin (GhoT) and its encoding gene are shown in orange while the antitoxin (GhoS) and its encoding gene are shown in green.

Toxin-antitoxin system has been implicated in the generation of "persisters," i.e. a subfraction of the population that is characterized by low growth and high resistance to antibiotics [47]. By adapting a mixed strategy in which some cells are specialized for growth while others are specialized for persistence, the culture can insure itself against a sudden loss [32].

1.8 Programmed Cell Death in *Streptococcus pneumoniae*: Fratricide

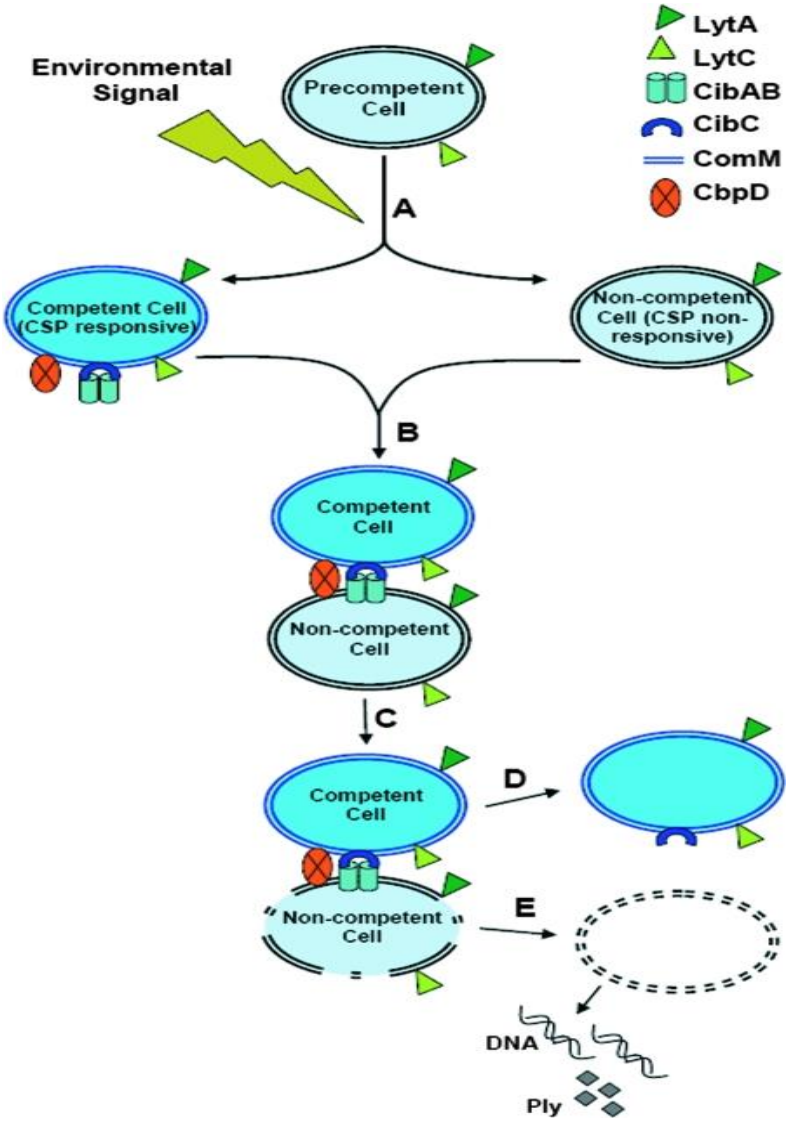
Streptococcus pneumoniae is a Gram positive pathogenic bacterium that causes pneumonia in humans. Although PCD in bacteria was initially thought to play an altruistic role wherein death of a few cells could benefit their kin, later, in certain bacterial populations undergoing cell death some cells in the population were reported to kill other sibling cells. One recently

identified example of this kind of cell death is the phenomenon of fratricide during competence development of *S. pneumoniae*. The ability of a population of *S. pneumoniae* cells to become competent is regulated by the ComDE two-component regulatory system (Fig 1.10 and 1.12) [48,49]. Induction of the competent state turns on the expression of proteinaceous toxins that lyse non-competent clones that are present in the same niche (Fig 1.10). The accumulation of a peptide pheromone called competence-stimulating peptide (CSP) (encoded by the comC gene), which is secreted by the growing *S. pneumoniae* culture, is sensed by ComD (a membrane-bound histidine kinase). When the extracellular concentration of CSP reaches a threshold level, it binds to ComD and triggers its autophosphorylation. The phosphoryl group is then transferred to the cognate response regulator ComE, which in turn upregulates the expression of the "early" com genes. One of these early com genes encodes sigma factor X (ComX), which subsequently regulates the expression of the "late" com genes, including the genes necessary for DNA binding, uptake, and recombination. Although this developmental process has been well studied for many decades, relatively little was known about how donor DNA was made available during competence development in the environment. A breakthrough in this field was made when it was shown, by measuring the release of either β-galactosidase or chromosomal DNA into the culture supernatant, that a lysing subpopulation of cells appeared during natural competence development in *S. pneumoniae* [50]. The emergence of this lysing subpopulation is dependent on the ComCDE regulatory system and results in the release of chromosomal DNA that could be used as a source of donor DNA for natural transformation [50] (Fig 1.10 and 1.12).

It was also shown by co-cultivation experiments using mutants deficient in various components of the ComCDE system that two populations of cells are present during competence development: one population of competent, non-lysing cells that lyse the second

population of noncompetent cells [51]. In other words, during competence development, donor DNA is provided by heterolysis/allolysis (lysis of one bacterial cell that is caused by another cell) as opposed to autolysis (lysis of self) [23,23,51]. The phenomenon of competence-induced cell lysis was subsequently named “pneumococcal fratricide,” defined as the intraspecies-specific killing of cells that occurs during the development of competence in *S. pneumoniae* [23].

Figure 1.10



Reference: Rice and Bayles, 2008 [23]

Fig.1.10: Fratricide during competence development in *S. pneumoniae*. (A) An environmental signal(s) leads to the emergence of two subpopulations, competent cells (CSP responsive) and noncompetent cells (CSP nonresponsive), presumably via a bistable regulatory mechanism. Competent cells express the lytic factor CbpD, putative two-peptide bacteriocin CibAB, and immunity proteins ComM and CibC, whereas the murein hydrolases LytA and LytC are expressed by both competent and noncompetent cells. **(B)** Cell-to-cell

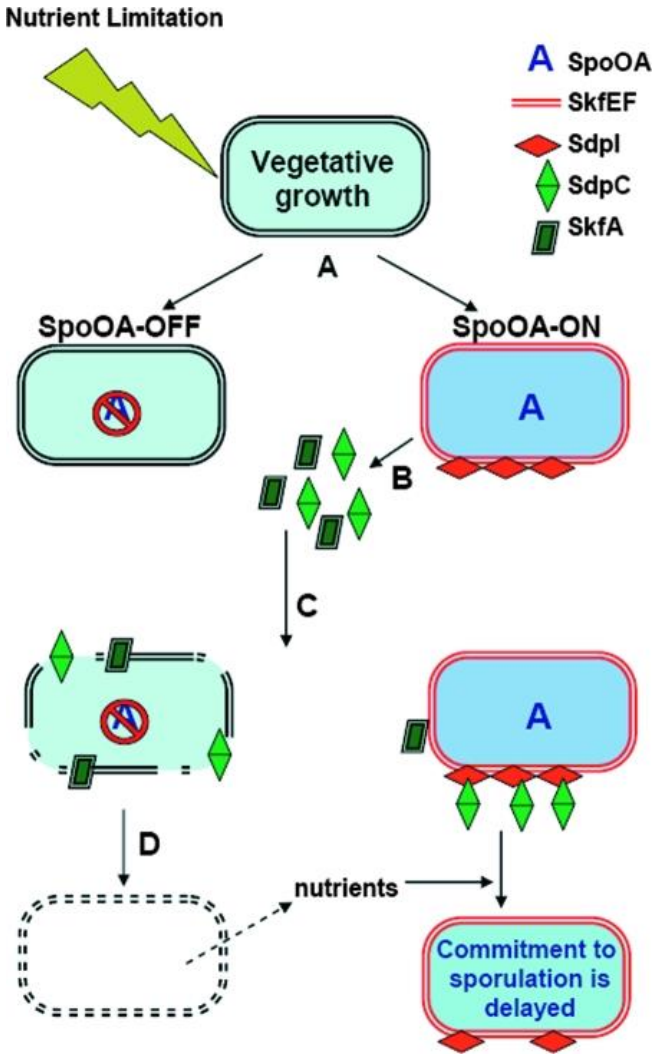
contacts between competent and noncompetent cells allow access of CibAB and CbpD to the noncompetent cells. **(C)** CibAB triggers the lytic action of CbpD, LytA, and LytC. **(D)** Competent cells are protected from the actions of these enzymes by the expression of immunity factors CibC and ComM. **(E)** Noncompetent cells lack these immunity proteins and undergo lysis, releasing DNA (used for genetic transformation of competent cells) and virulence factors (Ply) [23].

Interestingly, recent studies of the pyruvate oxidase (encoded by *spxB*) produced by *S. pneumoniae* have also revealed an important role for this enzyme in cell death during stationary phase of his organism [52]. Stationary phase cells harbouring a mutation in *spxB* gene were observed to have increased viability due to the absence of hydrogen peroxide (ROS), generated as a product of this enzyme's activity. Furthermore, the death process induced by hydrogen peroxide exhibited features similar to those of apoptosis in eukaryotic organisms, including alterations in membrane characteristics and increased degradation of DNA.

1.9 Programmed Cell Death in *Bacillus:* Cannibalism

Bacillus is a Gram positive soil bacterium. Cannibalism during *B. subtilis* sporulation is also an example of fratricidal cell killing within a population [23] (Fig. 1.11). Sporulation is a differentiation process whereby a dormant cell (the endospore) is produced which is able to survive harsh environmental conditions till the time growth conditions improve.

Figure 1.11



Reference: Rice and Bayles, 2008 [23].

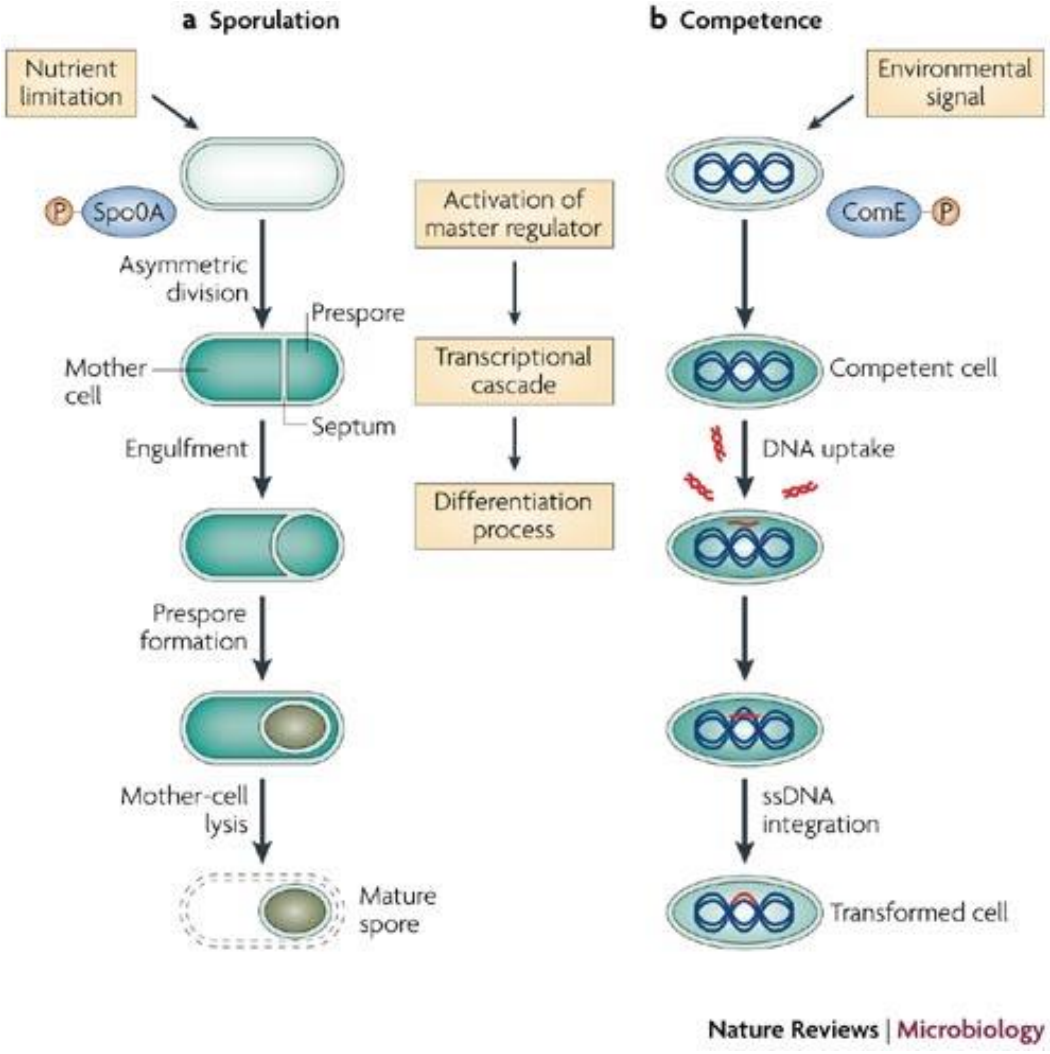
Fig.1.11: Cannibalism during *B. subtilis* sporulation. (A) Nutrient limitation leads to the emergence of two subpopulations, SpoOA-ON (cells that have entered the sporulation pathway but have not yet passed the irreversible stages of sporulation) and SpoOA-OFF (nonsporulating cells), via a bistable regulatory switch. **(B)** SpoOA-ON cells express the killing factors SkfA (sporulating killing factor) and SdpC (sporulating delay protein) as well as their cognate immunity proteins SkfEF and SdpI. **(C)** SpoOA-OFF cells lack the immunity

proteins SkfEF and SdpI and therefore are susceptible to the lethal action of SkfA and SdpC; SpoOA-ON cells are protected from killing by the immunity proteins SkfEF and SdpI. **(D)** SpoOA-ON cells are able to delay their commitment to sporulation by feeding on the nutrients released from their dead siblings (SpoOA-OFF cells) [23].

In *B. subtilis*, sporulation normally occurs as a last-resort response to nutritional stress, but entry into sporulation is also regulated by other signals such as cell density and the cellular redox state [53]. The response regulator protein SpoOA (stage 0 sporulation) acts as master regulator that governs entry into sporulation. The various signals and inputs required for entry of sporulation feed into the SpoOA protein [53]. In a given population of genetically identical cells, only a subset actually initiates sporulation and this developmental process is irreversible once the asymmetrically positioned division septum (separating the prespore and mother cell) is formed [23]. The "decision" by an individual cell to enter into sporulation is regulated by a 'bistable switch' that controls the phosphorylated state of SpoOA and explains why some cells contain active Spo0A and others do not [23,54]. Since this developmental process is very energy consuming, cells need a means to delay spore formation for as long as possible in case the nutritional stress is only short-term. For example, if favourable growth conditions were to resume, cells that have irreversibly committed to sporulation would be at a growth disadvantage relative to vegetative cells that could rapidly reinitiate cell division [23]. A mechanism by which this is accomplished in *B. subtilis*, whereby cells that have entered the sporulation pathway (but not fully committed to sporulation and have not yet crossed the irreversible step of sporulation i.e. engulfment) are able to block sibling cells from sporulating and kill these cells in order to feed on their nutrients, thereby delaying commitment to sporulation, has been identified [55,56]. This mechanism was termed cannibalism and is similar to fratricide in that two groups with distinctive fates ("killer" cells

verses “victim” cells) arise from a genetically identical population of cells [23,48,55]. The operons *skf (*sporulating killing factor) and *sdp* (sporulating delay protein) have high affinity for Spo0A and encode killer proteins SkfA (has high sequence similarity to bacteriocin-like proteins) and SdpC respectively [48,55] (Fig 1.11). Sporulating cells express SkfA and SdpC as well as their cognate immunity proteins SkfEF and SdpI. The non-sporulating cells lack these immunity proteins, and are therefore, susceptible to the lethal action of SkfA and SdpC. The sporulating cells are able to delay their commitment to sporulation by feeding on the nutrients released from their dead siblings (SpoOA-OFF cells) (Fig 1.11 and 1.12). Although the most important stimulus for sporulation in *B. subtilis* is nutritional stress, additional physiological signals, such as high cell density and DNA damage, are also integrated through Spo0A [48].

Figure 1.12



Reference: Claverys, and Havarstein, 2007 [48].

Fig.1.12: Sporulation and competence are multilevel controlled adaptive responses. In each case, initiation signals activate a master transcription regulator, Spo0A and ComE, respectively. Spo0A triggers the asymmetric sporulation division, which produces two distinct cells with different fates — the smaller prespore (also known as the forespore), which develops into the prespore through a process called engulfment, and the mother cell, which is necessary for spore formation and ultimately lyses to liberate the mature spore. ComE

triggers the expression of the com regulon, which includes genes encoding the DNA uptake and recombination machinery. Competent cells can therefore take up exogenous DNA in the form of single-stranded (ss) fragments. Homologous recombination proteins enable the formation of heteroduplex intermediates in genetic transformation if and when homologous DNA is internalized. Differentiating cells are shown in dark green [48].

1.10 Programmed cell death in *Caulobacter crescentus*

Caulobacter is a Gram negative bacterium and is commonly found in fresh water bodies [57]. Recently, Bos et al. screened for an SOS-induced factor that caused cell death and identified a previously uncharacterized protein, which they named bacterial apoptosis endonuclease (BapE) [58]. The authors reported that wild-type *Caulobacter crescentus* encodes this novel endonuclease that fragments the chromosome when the DNA is extensively damaged. Following DNA damage, bacterial cells typically induce the SOS response, which arrests the cell cycle and activates DNA repair pathways. To investigate the *C. crescentus* response to DNA damage, the authors used two different approaches to induce the SOS response: deletion of *lexA* (which encodes a repressor of the SOS response) and treatment of cells with the DNA crosslinker mitomycin C (MMC). A subpopulation of both lexA-null cells and MMC-treated cells were positive for DiBAC4 and TUNEL staining, which are markers for membrane depolarization and chromosomal fragmentation, respectively. This evidence indicated that DNA damage promotes apoptosis-like death in *C. crescentus* [58,59]. Deletion of the BapE encoding gene or a reduction in its cellular level had no phenotype in wild-type cells; however, the authors observed that DiBAC4 and TUNEL staining were significantly reduced in SOS-induced BapE-deficient cells, indicating that BapE is necessary for mediating cell death. Interestingly, purified BapE digested plasmid DNA in vitro in a sequence-

nonspecific manner. Furthermore, overexpression of BapE in vivo resulted in perturbed chromosome morphology and fragmentation [58].

To determine when and how *C. crescentus* makes the decision to switch from a DNA repair programme to a cell death programme following DNA damage, quantitative real-time PCR was used to measure the induction kinetics of SOS-responsive genes over time after MMC treatment. This analysis revealed that genes involved in cell division arrest and DNA repair were expressed early after MMC exposure and reached a plateau or declined at later time points. On the contrary, *bapE* induction was delayed, but its expression reached a high level at later time points. Additionally, the cell viability assays revealed that low levels of BapE resulted in reversible cell division arrest, whereas, high levels led to irreversible cell death. Thus, cell division arrest and repair pathways appear to be induced early; however, when DNA damage persists, the cell death programme is favoured owing to a rise in BapE levels and subsequent chromosome fragmentation [58].

1.11 Programmed cell death in *Xanthomonas*

Xanthomonas is a Gram negative, aerobic bacterium. Majority of its species are reported to be plant pathogens. A PCD dependent on a caspase-3 like protein has been demonstrated earlier in *Xanthomonas campestris* pv. *glycines* strain AM2 [1,5,60–62]. It was induced when this organism was grown in protein rich media like Luria bertani medium or nutrient broth with no other carbohydrate source added to the media. This phenomenon was not observed in cells grown in starch minimal medium. Addition of starch or glucose to LB medium was found to inhibit PCD [60,62]. A protein cross-reacting with anti-human caspase-3 antibody was also observed to be associated with PCD in this organism [62]. The *Xanthomonas* caspase 3-like protein appeared in cells at around 4 h of incubation and peaked at around 24 h

before finally diminishing at around 54 h of incubation. Interestingly, caspase enzyme activity was detected 12–13 h after incubation (in LB medium) which peaked at around 18 to 20 h. Addition of starch at the beginning or during the period of exponential growth in LB cultures of XcgAM2 terminated the synthesis of this protein indicating that starch acted as the repressor of biosynthesis of the *Xanthomonas* caspase, thereby preventing the organism from undergoing PCD. The cells undergoing PCD also displayed the other markers of eukaryotic apoptosis including PS externalization and the presence of nicked DNA in culture supernatant as evidenced by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) assay. Moreover, caspase-negative mutants of *Xanthomonas* (XcgM42) obtained by N-methyl-N-nitro-N-nitrosoguanidine (MNNG) treatment did not display PCD [62].

1.12 Significance of PCD in bacteria

PCD is a genetically regulated process of cell suicide. Bacteria live as unicellular organisms and the idea of PCD in bacteria seems counterintuitive. This compels us to think about the possible evolutionary advantage to encode for a self-destruction program in bacteria. With the immense studies on biofilms bacteria are no longer looked upon as loners, rather they behave in a multicellular manner. PCD triggered in response to nutrient deprivation in a few cells of genetically identical clones benefits the entire population. This altruistic aspect is reflected in the PCD of the mother cell during sporulation in *Bacillus subtilis* and MazEF toxin-antitoxin dependent death in *E. coli* cells [63]. Secondly, a viral infection in a few cells acts as a trigger for self-destruction to save the genetically identical population [63]. Thirdly, PCD prevents the propagation of defective genome by eliminating damaged cells, thereby, acting as the guardian of the bacterial genome [63].

1.13 Apoptosis in unicellular eukaryotes

Many unicellular eukaryotes like *Leishmania, Blastocystis, Trypanosoma, Plasmodium* and *Dictyostelium discoideum* and *Saccharomyces cerevisiae* undergo PCD displaying its typical features [64–70]. *Leishmania* was first reported to undergo PCD when treated with certain anticancer drugs like amphotericin B and camptothecin [66,67]. Camptothecin was found to hyperpolarize mitochondria leading to oxidative stress in the cells. This was accompanied by the release of cytochrome c and activation of CED3/CPP32 group of proteases. Besides this, other markers of PCD like nuclear condensation, DNA fragmentation and cell shrinkage were also observed. *Blastocystis hominis* undergoes PCD when treated with metronidazole or surface-reactive cytotoxic monoclonal antibody [64,65]. It shows the characteristic features of PCD like chromosomal condensation, externalization of PS, although DNA laddering was not observed [65]. *Plasmodium falciparum* has been reported to undergo PCD in response to chloroquine treatment [70]. *Trypanosoma brucei rhodesiense* undergoes apoptosis when treated with concanavalin A [71].

1.14 Programmed cell death in yeast

Budding yeast, *Saccharomyces cerevisiae*, has been used as a model organism in several areas of cell biology. The functional advantage of yeast being a eukaryote coupled to the easy handling like that of bacteria made yeast a preferred research tool. Apoptosis was first discovered in yeast under oxidative stress in 1997 [72,73]. Since this discovery several yeast orthologs of mammalian apoptotic proteins like YCA1 (yeast caspase), AIF1 (apoptosis-inducing-factor-1) have been identified [69,72,73]. Both exogenous and endogenous triggers have been reported to induce apoptosis in yeast. Hydrogen peroxide, hypochlorous acid, acetic acid, high salt, heat stress, UV irradiation, heavy metals, certain drugs like aspirin,

paclitaxel have been reported to induce apoptosis in yeast [69]. Moreover, the heterologous expression of the human key apoptotic inducer Bax (pro-apoptotic factor) in yeast was also found to induce apoptotic cell death with the release of cytochrome c [74]. On the contrary, heterologous expression of Bcl-2 or Bcl-xL (anti-apoptotic factor) prevented the Bax-induced lethality and improved the resistance of yeast cells to H_2O_2 and acetic acid stress [74,75]. DNA damage (due to ROS generation), replication failure (e.g. during aging), defects in cellular processes like chromatid condensation and N-glycosylation, were also reported to induce apoptosis in yeast [76,77]. ROS have been identified as one of the small signalling molecules regulating yeast apoptosis. Probable cellular sources of ROS include the electron transport chain in mitochondria, the endoplasmic reticulum and the iron coupled Fenton and Haber-Weiss reactions.

1.15 Programmed cell death in higher eukaryotes

Apoptosis in higher eukaryotes is a well-studied phenomenon. It is essential for the maintenance of homeostasis and involves the interplay of several proteins. The most important role is played by caspases (cysteine-aspartic proteases) [2]. They cleave at a specific site in the target proteins. Till date 14 caspases have been identified in humans. They are highly conserved throughout the evolution. All known caspases possess a cysteine residue in the active site, and cleave substrates at Asp-Xxx bonds (i.e. after aspartic acid residues). Since they bring about the most visible changes in cell morphology characteristic of apoptosis, they are considered to be the main executioners of this process. Activation of caspases does not result in total degradation of cellular proteins; rather it selectively cleaves a restricted set of target proteins, usually at one or few positions in the primary sequence after an aspartate residue. Caspase-3 is known to cleave ICAD (inhibitor of caspase-activated

DNase) which leads to the activation of CAD (caspase-activated DNase) resulting in the characteristic DNA ladder pattern, the hallmark of apoptosis [2]. Caspases are mainly classified either as initiator or executioner based on their function. Caspase 1, 4, 8 and 9 belong to initiator caspases and aid in initiating the apoptotic cascade, whereas, caspases 3, 6 and 7 belong to executioner caspases as they cleave the downstream target proteins like ICAD, PARP and lamins.

1.16 Activation of Caspases

Caspases are synthesized as zymogens (enzymatically inert) [2]. In humans these are reported to be composed of three domains: an N-terminal prodomain, the p-20 and p-10 domains. The mature enzyme is a heterotetramer containing two p20/p10 heterodimers. There are following two pathways for activation of caspases:

a) Intrinsic pathway (mitochondrial pathway)

The intrinsic apoptotic pathway is characterized by permeabilisation of the mitochondria and release of cytochrome c into the cytoplasm [2]. Cytochrome c then forms a multi-protein complex known as the 'apoptosome' and initiates activation of the caspase cascade through caspase 9 which cleaves and activates caspase-3 and -7.

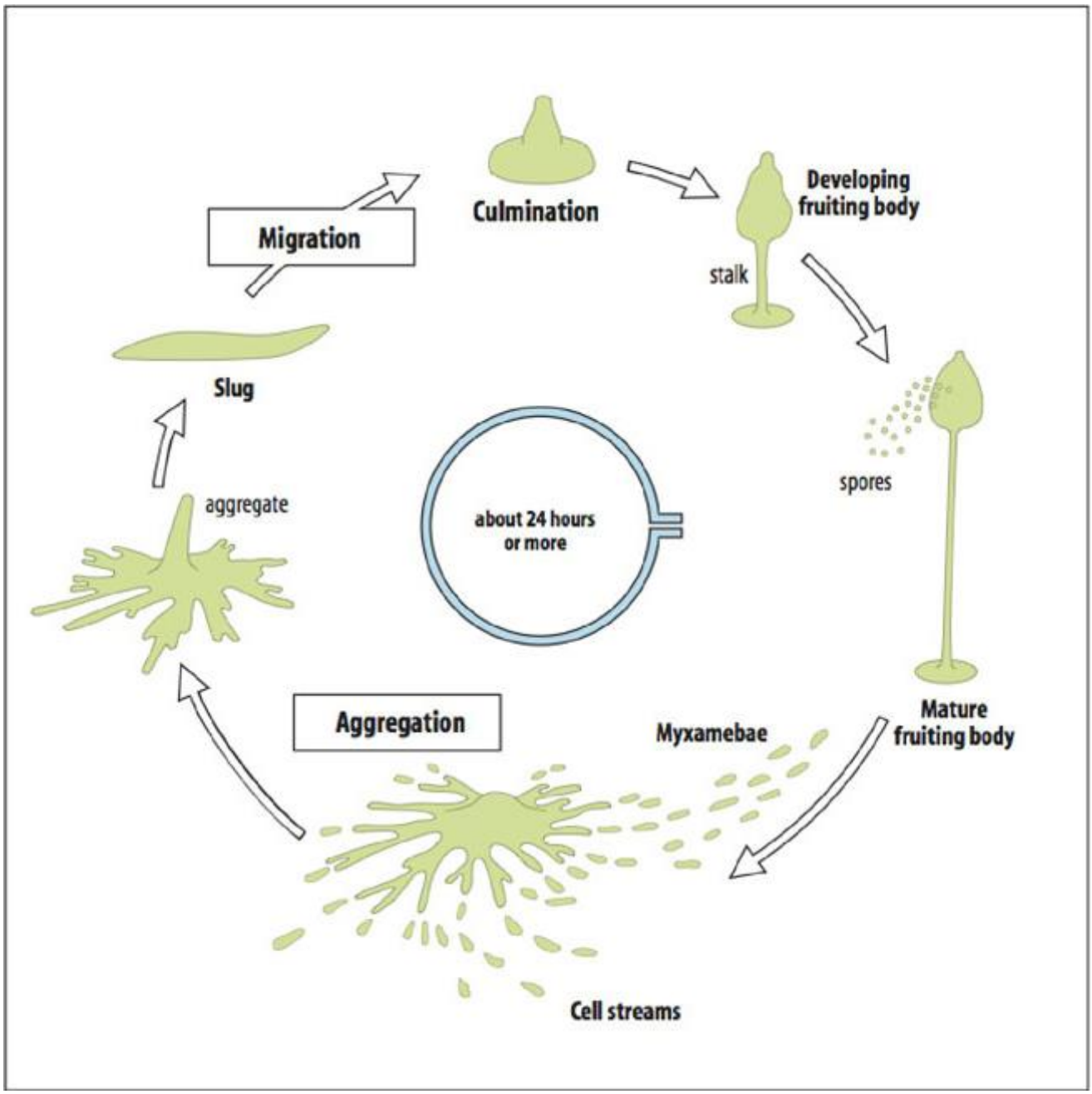
b) Extrinsic pathway (death receptor pathway)

Upon ligand binding the death receptors [(Fas Associated Death Domain (FADD) or Tumour associated Receptor death domain (TRADD)] aggregate to recruit procaspase-8 resulting in formation of a Death inducing signaling complex (DISC) [2]. This activates procaspase-8 which in turn activates other executioner caspases like caspase-3 or cleave BID (BH3-interacting domain death) eventually leading to the formation of apoptosome as described above.

1.17 Caspase-independent cell death

Dictyostelium discoideum is a protist which multiplies vegetatively as a unicellular organism in nutrient rich conditions (Fig 1.13). But under starvation conditions *Dictyostelium* cells aggregate due to periodic cAMP signals produced by a few cells which act as aggregation center (Fig 1.13) [1,78]. Individual cells chemotactically move towards the increasing cAMP level. *Dictyostelium* cells upon aggregation differentiate and morphogenize into a multicellular structure, called sorocarp, containing a mass of spores supported by a stalk [79]. The cells in the stalk undergo PCD which involves chromatin condensation but differs from apoptosis because it involves massive vacuolization, lacks DNA fragmentation and has been found to be independent of caspases.

Figure 1.13



Reference: http://www.mun.ca/biology/desmid/brian/BIOL3530/DB_05/fig5_33.jpg

Fig. 1.13: Life cycle of *Dictyolstelium*

1.18 Necroptosis or Programmed Necrosis

Necroptosis is a form of programmed necrosis. Necroptotic stimuli (e.g. anticancer drugs, ionizing radiation and calcium overload) promote the interaction of the RIP1 (receptor-interacting protein 1) kinase and RIP3 (receptor-interacting protein 3) death domain containing kinase under conditions in which caspase-8 is not active [80–82]. This RIP1/RIP3 complex, known as complex IIb, mediates necroptosis. Caspases have no positive role in necroptosis. So far, an analogous class of executioner proteins has not been identified for necroptosis. Necroptosis is characterized by mitochondrial dysfunction, cell swelling (oncosis), organelle swelling membrane permeabilization and release of cytoplasmic content in the extracellular space [83,84]. Unlike apoptosis, DNA fragmentation does not occur during this process.

1.19 Autophagy

Autophagic vesicles are commonly observed in necroptotic cells. Autophagy has been proposed as a clean-up mechanism for necroptosis. Autophagy is a self-degradative process (self-eating) that is important for balancing sources of energy at critical times in development and in response to nutrient stress [85,86]. Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles, such as mitochondria, endoplasmic reticulum and peroxisomes, as well as eliminating intracellular pathogens [85]. Currently, 32 different autophagy-related genes (Atg) have been identified by genetic screening in yeast. Significantly, many of these genes are conserved in mammals, plants, worms, flies, and slime mould, emphasizing the importance of the autophagic process in responses to starvation across phylogeny [85].

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