Bacterial programmed cell death

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Why do we need a kill switch?

1. Possible release of genetically engineered microorganisms (GEMs) in environment; unanticipated survival and reproduction with negative ecological effects. Also uncontrolled genetic transfer processes (Ahreholtz et al. 1994).
2. Once BS has mended the crack, don’t want cells to reactivate and disrupt the repair.

Idea: we want our signal to kill the bacterium 1) at the right point in the crack-repair sequence and also 2) if the bacterium escapes from the crack.

Idea: we may need to heat concrete so that bacteria divide quickly enough.

Search words: bacteria, bacillus subtilis, autolysins, cell death, suicide, PCD, lysis, toxin

Broad possibilities for suicide strategy?


- Functions for survival in the environment are lost during laboratory cultivation?
- Killing genes/highly toxic polypeptides, plasmid R1 in *E.coli*, *hok*, *gef*, *relF* genes causing irreversible membrane damage, interfere with respiratory system. Regulated by an antisense RNA mechanism. Efficient in low cellular concentrations. Works for other species. 200 bp.
- Nucleases, e.g. from *Serratia marcescens* and *Staphylococcus aureus*. *Staph.* nuclease more stable intracellularly than *Serratia* nuclease. Like *gef*, is a universal target for all organisms.
- Alternative killing genes. Extracellular enzymes, e.g. phospholipase A to degrade bacterial membranes. Deletion of the antagonistic gene *phlB* results in conversion of *phlA* (phospholipase gene) to an effective killing gene whose induction leads to cell lysis. Source of killing genes can be from viruses.
- Chemical control. Strategies:
  1) Addition of IPTG and other chemicals (not realistic outside of lab).
  2) Grow cells in chemical not present in environment and let the suicide function become derepressed when they escape. Put *hok* gene under control of *trp* promoter. When outside tryptophan is consumed or not present, causes derepression of Hok synthesis and thus killing.
  3) Manipulate control system such that inside crack and culture, cells synthesis high levels of a regulatory protein (from a chemically inducible promoter, e.g. *lacP*) leading to repression of the suicide promoter. Induction of suicide only if levels of repressor too low (nutrients run out or cell escapes.

Also for environments containing 3-methylbenzoate (3-MB). *p_m* promoter has XylS as a regulatory protein. XylS has no activity in absence of 3-MB.
• Physical control – infection by phages. Temperature-sensitive promoters.

• Stress control: pH, extreme temperatures, toxic compounds. Fusing promoters of stress genes with a killing gene.

• Induction of killing by a stochastic switch. Recombination systems: resolvase system of plasmids and transposons. Can design molecules with relevant genetic information deleted with an appropriate frequency.

Due to recombination, transcription terminator (T) will be deleted and the promoter and suicide gene are brought into juxtaposition (by resolvase enzyme). Cell death will result.

Can shift control of the resolvase activity to the environment by replacing natural resolvase promoter with an environmentally regulated promoter.

**Chung et al. (2009) Induction of Growth Phase-Specific Autolysis in Bacillus Subtilis 168 by Growth Inhibitors, The Journal of Microbiology, 47(1), 50-59.**

How to lyse cells:

• Cell wall digesting autolysins: LytA through Lty F, ltyR
• Inhibit Peptidoglycan synthesising enzymes
• proton motive dissipators (ionophores, uncouplers, cytochrome oxidase inhibitors)
• this paper: glucose exhaustion + compound (e.g. chloramphenicol, actinomycin, ampicillin, detergents, growth inhibitors) = cell lysis

At the MAIC (minimum autolysin induction concentration) of each compound, cells starved of the primary carbon source commenced lysis. However, cells earlier or before this point did not show this behaviour - this is no good for our project.
Blackman et al. 1998 The role of autolysins during vegetative growth of Bacillus subtilis 168, Microbiology, 144, 73-82.

- N-acetylmuramoyl-L alanine amidase (amidase) – encoded by lytC (part of lytRABC divergon). Repressed by lytR. But also involved in separating cells (= no filaments)
- endo-beta-N-acetylglucosaminidase (glucosaminidase) – encoded by lytD gene


Autolysins are bacteriolytic enzymes that digest the cell wall peptidoglycan. Produced by BS for numerous cellular processes – a plethora of enzymes.

Fig. 2. Life cycle of B. subtilis. Processes that require or appear to require autolysin activity are labelled in bold capitals, and autolysins involved, where known, are indicated in parentheses after each. (a) Vegetative growth. The stages of the vegetative growth cycle apart from cell elongation are linked by broken arrows, as each step is not absolutely dependent on previous steps. Cell separation, motility and genetic competence occur primarily in stationary phase. (b) Sporulation. Events during sporulation are linked by solid arrows because they occur in a precise, genetically controlled order. Roman numerals refer to the stages of sporulation (Dif, 1989).

Therefore probably better to engineer a separate mechanism rather than over-express or induce an essential mechanism to the cell. Besides, the control of autolysin activity is quoted in this paper ‘remains a mystery’.
mazEF toxin-antitoxin module \((E. coli)\)

Stress-induced ‘suicide module’. Unstable antitoxin \(mazE\) (which is essential for cell survival) is degraded faster than the more stable toxin \(mazF\). MazE and MazF interact.

Activated by several stressful conditions that prevent the expression of MazEF, and thereby MazE synthesis → MazF is unimpeded to exert its toxic effect → cell death:

- Extreme amino acid starvation. Leads to production of the starvation signalling molecule ppGpp
- Inhibition of transcription/translation by antibiotics (rifampisin, chloramphenicol, spectinomycin)
- Damage caused by thymine starvation (provides DNA damage), mitomycin C, nalidixic acid, UV irradiation, oxidative stress.

MazF inhibits protein synthesis through its endoribonucleolytic effect on mRNA.

Bacillus subtilis has similar module called \(ydcDE\), of which the toxic product is YdcE, called Endo A which is similar in sequence, structure and function to MazF.

**skf and sdp operons (Bacillus subtilis)**

Death of a subpopulation of sporulating cultures

Nutrient limitation triggers spore formation in BS governed by Spo0A but only in part of the population (the subpopulation of Spo0A-ON cells).
Response by SPo0A-ON cells – to survive

*skf* operon is induced so cells produce killing factor and the pump that exports it. Also produce SdpC (toxic signal protein) but other genes on *sdpRI* operon make it self-resistant to toxic effects of this.

Response by SPo0A-OFF cells – to die

Produce neither killing factor nor pump, so are killed by the extracellular killing factor (i.e. from SPo0A-ON cells). Not resistant to SdpC (toxic signal protein). Cell lysis protein is said to be unidentified.

Both mazEF and skp/sdp are important in the response of bacteria to severe nutritional stress because death of part of the subpopulation may provide nutrients for the surviving cells.
Cell death without lysis


The I-CeuI homing endonuclease from the green alga Chlamydomonas moewusii creates a staggered dsDNA break in a naturally occurring sequence within the highly conserved 23s rRNA in rrn operons on the chromosomes of many bacterial species (E.coli has 7 such sites). Will not damage plasmid DNA.

Cell death occurs gradually over a 24-hour period, reducing number of contaminating viable bacteria to levels comparable with those observed in previously described suicide systems ($10^{-3}$ to $10^{-6}$).

- Can be placed under another inducible promoter system
- Causes cells E.coli VAX0813 cells to become filamentous
- Active in a wide range of pH, temperature and salt concentrations
- Possible to select by complementation of a proline auxotroph, eliminating the need for a selectable antibiotic resistance marker. No expensive inducing agents such as IPTG required.

Biological containment systems – relF


Two approaches to construction biological containment systems:

- debilitate bacterium so it can’t survive outside of certain mechanisms
- Suicide system activated under certain conditions: in this paper is based on the relF gene from E.coli controlled by inducible lac promoters.

A single copy of plasmid pSK330 at 30 °C is sufficient to bring down the number of survivors of the suicide condition to an extent that only cells containing mutant plasmids survive. Size of surviving subpopulation depends on time point and conditions of mutation. Increase efficiency of suicide system by having two independent suicide systems.

Cell lysis plasmids from bacteriophages

Kloos et al. (1994) Inducible cell lysis system for the study of natural transformation and environmental fate of DNA released by cell death.

Developed two inducible cell lysis systems:

- plasmid pDKL01 and the lysis gene E from bacteriophage φX174. This gene activates cellular autolysins that cause formation of a single pore at the cell pole or at the midpoint through which cytoplasmic contents are released. DNA released is highly instable (half-life less than 1 hour).
- plasmid pDKL02 which contains the lysis genes, S, R and Rz of bacteriophage λ. DNA released stable and can be transform nearby (non-lysed) bacteria.

Suicide system: Intracellular degradation of DNA in E.coli

Ahrenholtz et al. (1994) A conditional suicide system in Escherichia coli based on the intracellular degradation of DNA.
Release of recombinant DNA into the surrounding milieu can persist (in non-sterile soils) for weeks or months. So suicide system must combine killing of cells with the destruction of genetic material (before its release from cells)) to limit survival of GEMs and transfer of DNA to other organisms.

Cell death controlled by derepression of a nuclease gene. Chose nuc gene of Serratia marcescens which endonucleaseolytically cleaves RNA and DNA to acid-soluble material and introduces s- and ds- cuts into duplex DNA. This DNase and RNase was cloned downstream of the lambda pL promoter and controlled by the thermosensitive lambda cI857 repressor.

Efficiency of killing system is $2 \times 10^{-5}$. When two copies of killing gene = $10^8$.

Obviously can be placed under other regulators more relevant to the environment, such as those responding to starvation for specific substances.

Since cell lysis does not accompany killing, the cell entity (including production of proteins) could be preserved which the mass of nucleic acids was degraded.

**Infection with phages**


“Sophisticated bacteriophage-encoded mechanism that controls the timing of lysis during the lytic cycle such that the bacteriophage particles accumulating within the cytoplasm can be released into the surrounding environment”. Involves a ‘holin’ and an ‘endolysin’. Timing of cell lysis is dictated by the holin which controls the activity of the endolysin.

Seems like a very complicated system to work with that isn’t fully understood. Fine tuning holin to control lysis by forming a lethal membrane lesion at a specific time will be difficult.
Not all bacteriophages utilise a holin-endolysin system:

- protein antibiotics, e.g. MraY inhibits formation of first lipid-linked intermediate in cell wall biosynthesis.

Working with phages appears complicated. May show unpredictable activity even if insert inducible killing gene on phage genome to be inserted into bacterial chromosome. However, cloning their individual genes is useful (as already mentionned).

**The problem of mutations - Molin et al. 1993.**

“Laboratory model systems of induced suicide always contain a surviving subpopulation that can continue to grow even in the presence of inducer”. Main cause is mutation: of the killing gene or the expression system that inactivates the suicide function, or mutations that make cell resistant to killing function. Surviving fraction of $10^{-6}$ to $10^{-3}$ is always observed.

Possible solutions:

- Duplicate killing system: of the same or two independent systems.

**Alternative strategies - Molin et al. 1993.**

Attenuation. Disable organism so that (it is not pathogenic) or cannot survive if released to the environment.
For example, recA mutations that have reduced capacity to repair radiation damage (sun light). But there will be minimal radiation inside concrete crack. Advantages: recA is easily manipulated and recA mutants are incapable of efficient competition in the environment.

Mutations in genes whose products are essential under stress conditions. E.g. inability to utilise existing carbon sources.

Other ideas to research:

**Previous iGEM projects**

Stochastic switch (Hin recombinase). Irreversible sporulation: Give signal for sporulation to sleB and cwlJ germination-defective mutants.

Bacterial timebomb: cells commit suicide after a predetermined number of divisions.

Minicells. Genome-degradation. Insertion of plasmid –lacks exonuclease recognition sites. Gene (in plasmid) expressed using remaining cytosolic resources (cell dies after several hours).

RJC: to kill whole population, heat concrete or apply a chemical that will be soaked up through pores and kills every living organism on earth! (but doesn’t disrupt the repair). Or get bacterium (or some other organism) to release extracellular toxin that kills everything (even those cells that have mutated and therefore don’t produce the same toxin). Ethical considerations!