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Visions & Reflections

Bacterial resuscitation factors: revival of viable but non-culturable bacteria

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The ability of bacteria to survive in hostile environments is essential to their persistence and pathogenesis. Some bacteria achieve this by forming spores [1], whereas others enter a state of very low metabolic activity, sometimes referred to as the Viable But Non-Culturable (VBNC) state [2, 3]. The description of the VBNC state given in this paragraph is a simplification of a complex and sometimes contentious literature [2–4]. The VBNC state is characterised by the cells being viable in terms of their ability to reduce dyes (indicating metabolic activity) or prevent uptake of dyes (indicating an intact membrane). However, VBNC cells do not form colonies on plates or grow when added to fresh media under 'standard' conditions [2, 3]. In some cases the bacteria can be cultured by using a specific protocol, and this is known as resuscitation. A resuscitation protocol is required to prove that bacteria are in a VBNC state [3]; otherwise this is only conjecture.

The VBNC state can be induced by protocols such as low temperature and/or growth in oligotrophic media (low-nutrient media such as lake water) or exposure to seawater. In at least some species the VBNC state is distinct from the starvation response, where the bacteria are still readily culturable on return to nutrient media. Physiological differences can be identified between the two states such as by proteome analysis [5] and cell wall changes [6], and these may prove to be more reliable diagnostics of the VBNC state.

Part of the importance of understanding this state comes from its similarity to the dormant state of *Mycobacterium tuberculosis* in the lungs, where the pathogen can remain without symptoms for decades. Indeed up to a third of the world's population are thought to be infected in this manner (World Health Organisation fact sheet no. 104; http://www.who.int/mediacentre/factsheets/fs104/en/). Only on the emergence of the *M. tuberculosis* from this dormant state, often when the patient's immune system is suppressed, does chronic tuberculosis develop. A large number of foodborne pathogens, including enteropathogenic strains of *Escherichia coli* and *Campylobacter jejuni*, probably persist in the environment in a VBNC state [7]. Hence, understanding how bacteria enter into and exit from the VBNC state has potential implications for detection and therapy.

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A massive advance in our understanding of dormancy/VBNC, at least in *Actinobacteria*, which comprise the GC-rich Gram-positive bacteria, including *Mycobacteria,* comes from the work of Mukamalova et al. [8–12]. They identified a protein secreted by dividing *Micrococcus luteus,* a rapidly growing *Actinobacteria,* which increased the numbers of colonies that could be formed by dormant cultures by several orders of magnitude even at picomolar concentrations [8, 9]. They called this protein resuscitation promoting factor (Rpf). In this paper we use Rpf to designate the *M. luteus* protein and *Rpf* its gene, RpfA etc. to refer to the *M. tuberculosis* homologues and rpf to designate a general member of the protein family. Using peptide sequences from the purified protein they identified a gene coding for a 220-amino acid protein containing a typical Gram-positive secretion signal at the N-terminus, which was removed during secretion [8]. Database searches showed that rpf homologues were present in other *Actinobacteria*, including *Mycobacterium tuberculosis*. They showed that *M. luteus* Rpf is active against *Mycobacteria* [9] and later that recombinant mycobacterial Rpf proteins were active against *M. luteus* as well as *Mycobacteria* [10]. Further work demonstrated that the *M. luteus* Rpf protein consists of an N-terminal domain conserved between the rpf proteins and a C-terminal LysM domain, which is dispensable for resuscitation activity but is thought to cause binding to peptidoglycan, thus localising Rpf to the cell wall [11]. In *M. luteus* (the genome of which has not been sequenced), there appears to be only one rpf, which is absolutely required for growth, as deletion of the chromosomal *Rpf* gene could only be achieved in the presence of a plasmid copy of this gene [11]. However in *M. tuberculosis* there are five genes containing an rpf domain [10]. Mutations in any one of these genes, singly, did not prevent resuscitation or growth, although such mutations did influence colonial morphology [13, 14]. Mutating *RpfA/C/D* or *RpfB/D/E* simultaneously led to cells unable to resuscitate and with reduced virulence in a mouse infection model [15]. Therefore, there is some but not complete redundancy between the *M. tuberculosis* rpfs. Very recently it was shown that deletion of the *RpfB* (Rv1009) gene of *M. tuberculosis* caused delayed reactivation in a mouse model of infection and reactivation despite no detected *in vitro* effect of the single gene deletion [16],

It was assumed at this point, particularly as rpfs were active at very low concentrations, that this protein acted as a bacterial cytokine and bound to a cell surface receptor to stimulate growth. However, work in several laboratories failed to identify such a receptor. Close examination of the sequence of the conserved rpf domain, using a meta-server that submitted the sequence to a range of fold prediction Web sites, led to a proposal that the conserved domain resembled the peptidoglycan-cleaving enzyme lysozyme [17]. This prediction was also made independently by a number of other groups as part of the 'ten most wanted' targets in 'Critical Assessment of Techniques for Protein Structure Prediction 5′ [12]. The bioinformatic prediction was experimentally confirmed by the nuclear magnetic resonance (NMR) structure of the conserved domain of *M. tuberculosis* RpfB (Rv1009) [18], which showed clear structural homology to goose [19] [e.g. Protein Data Bank (PDB) 154L] and chicken-type lysozymes [20] (e.g. PDB 3LZT), and also to the glycosylase domains of the soluble lytic transglycosylases (SLT) SLT35 [21] (e.g. PDB 1DOK) and SLT70 [22] [e.g. PDB 1QSA from *E. coli* (Fig. 1)]. The rpf domain only contains a single conserved active site glutamate, similar to goose lysozyme and lytic tranglycosylases, but in contrast to the two acidic residues of hen egg white lysozyme. Goose lysozyme is an inverting glycosidase, in contrast to the two-step retaining mechanism of hen lysozyme [23, 24], and the prediction is that Rpf would also have an inverting mechanism. The proposed mechanism for lytic transglycosylases which produce 1,6-anhydromuropeptides is that the O6 of the sugar is activated by the protein to attack the activated C1 atom after cleavage of the glycosidic bond by the glutamate [25]. However, the region of lytic transglycosylases implicated in the activation of O6 from the SLT structures [25] has no equivalent in the RpfB structure (Fig. 1). The unproven prediction is that rpfs will cleave glycosidic bonds with inversion of configuration but not form an anhydromuro derivative.

Evidence for the functional role of peptidoglycan cleavage in the action of rpfs was reported in the paper reporting the NMR structure [18], where RpfB was shown to bind a trimer of N-acetylglucosamine (NAG), and mutation of the active site glutamate of *M. tuberculosis* RpfC (Rv1884) to alanine abolished the growth stimulation of diluted *M. luteus* cultures by the protein. More detailed confirmation comes from work on the *M. luteus* Rpf [12]. A range of point mutations was made, and the peptidoglycan cleavage activity was measured both by release of fluorescamine from derivatised *M. luteus* cell walls and by lysis of *E. coli* when Rpf was expressed as a secreted protein. These correlated with three measures of the resuscitation activity of these mutants, the number of generations in 96 h after dilution of a stationary culture into minimal media of *M. luteus* with the mutant gene on a plasmid, the number of colonies of these mutant strains recovered on minimal agar media compared with nutrient media and a 'most probable number' assay when these mutants are expressed in *M. smegmatis.* Activity in zymograms and hydrolysis of an artificial substrate, 4-methylumbelliferyl-beta-DN,N ′,N″-triacetylchitotrioside (MUF tri-NAG), was also shown. This very detailed analysis unequivocally showed that peptidoglycan hydrolysis is responsible for resuscitation activity as defined by various bacterial growth assays.

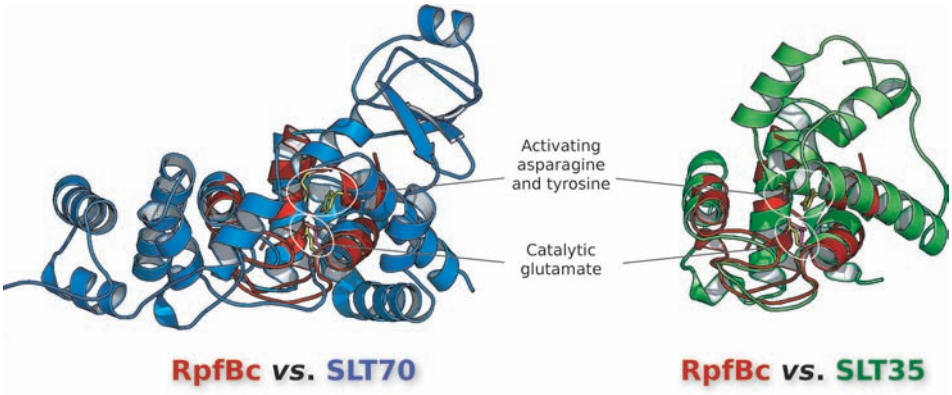

Figure 1. Structural superposition of *M. tuberculosis* RpfB core with the *E. coli* lytic transglycosylases SLT70 and SLT35. The active site glutamates coincide, but there is no equivalent in Rpfb of the asparagine and tyrosine implicated in activating O6 for formation of the anhydromuro ring.

There are some unanswered questions, however. The specific activity of the *M. luteus* Rpf was only a fiftieth of that of lysozyme against the model substrate and substantially lower in the *M. luteus* cell wall assay. Furthermore, the protein with a mutation of the active site glutamate to glutamine showed a considerable fraction of wild-type activity, and even the mutant protein with an alteration of the active site glutamate to alanine showed measurable activity. It has been shown that mutations can alter the mechanism of lysozyme from retaining to non-retaining [23], and that relative efficacy of goose- and chicken-type lysozyme depends on the exact substrate, with different efficiencies against peptidoglycan derived from different species of bacteria and against synthetic substrates [26]. Substrate functional groups have been suggested to play a compensatory role for some point mutations [26]. Significant work still remains to delineate what the substrate range and products of the rpfs are, and which are physiologically relevant.

How peptidoglycan hydrolysis causes changes in growth (resuscitation) of the bacteria is not known. Two general models have been proposed and discussed [12, 17, 18, 27]. The first is that the Rpf acts to release a 'second messenger' small molecule that signals both to the local cell and via diffusion to adjacent bacteria, possibly through a cell surface receptor. Alternatively, the role of Rpf could be to alter the cell wall in some way to overcome a mechanical block to cell division. It may be that Rpf is produced during exponential growth to routinely allow cell division, but its expression is reduced on dormancy such that its activity becomes rate limiting for division. Studies of the expression profile of rpfs support this [10, 11, 14]. Conversely, it has been shown that peptidoglycan of bacteria in the VBNC state is different from in exponential growth [6, 28, 29]. It may be that rpfs are required to cleave these modified peptidoglycans.

The VBNC state is widely found throughout bacteria, whereas clear demonstration of the resuscitation activity of Rpf is restricted to *M. luteus and Mycobacteria*, where activity both against laboratory dormant cultures and lung extracted bacteria [30] has been shown. Mutation of both Rpf genes in *Corynebacterium glutamicum* was not lethal, but the cells did show a longer lag phase [31]. More distant homologues of rpf exist in some *Firmicutes* and in other *Firmicute* species. There is clear evidence that a domain displacement event has taken place in these proteins. A different peptidoglycan-cleaving domain weakly related to *E. coli* membrane lytic transglycosylase A, designated the Sps (stationary phase survival) domain [27], has replaced the rpf domain. Clear data as to the function of Sps proteins in the growth cycle is not yet published.

The VBNC state is widespread among bacterial species, and recently evidence of a resuscitation factor in *Salmonella*, YeaZ, was published [32]. Unlike the Gram-positive Rpfs, YeaZ does not have cross-species activity [32]. Structures for YeaZ, from *E.coli* (PDB 1okj) [33], *Thematoga maritima* (PDB 2a6a) and *Salmonella typhimurium* [34] have been published from structural genomics groups. The protein YeaZ is clearly unrelated in structure to rpfs, despite some proposed sequence homology by the authors of the resuscitation factor report. YeaZ is a paralogue of Gcp, a protein found in all genomes sequenced so far, including eukaryotes [34]. Both proteins are predicted to have the actin/hsc70 fold, and this is confirmed by the structure of YeaZ. Neither have a clear secretion signal. Both are essential in many species studied, which is the reason for them being targeted in structural genomics efforts [34]. There is not much functional data for these proteins. The best-characterised Gcp is from *Mannheima haemolytica*, where it has been shown to be a secreted sialoglycoprotease cleaving glycosylated host cell surface proteins such as glycophorin A and leukocyte antigens CD34, CD43, CD44 and CD45 [35–38]. Gcp is thought to be a metallopeptidase and has conserved histidines, but these are not found in the YeaZ homologues. It is therefore not obvious what the mechanism for YeaZ

releasing *Salmonella* from the VBNC state is and whether it relates to the peptidoglycan cleavage-stimulated release from the VBNC state found in *Actinobacteria.*

Resuscitation Promoting Factors release *M. luteus* and *Mycobacteria* from the dormant state by catalysing peptidoglycan cleavage. Further work is required both to exploit the potential therapeutic application of this finding in tuberculosis, and to assess how widespread this mechanism of resuscitation is among other bacterial species.

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