microbiology Comment

The resuscitation promotion concept extends to firmicutes

Listeria monocytogenes is a facultative pathogenic bacterium that lives in the environment as a saprophyte but can turn into a harmful pathogen affecting humans as well as animals upon ingestion. These bacteria have the remarkable capacity to actively invade eukaryotic host cells, to multiply within them, and to spread to neighbouring cells (Freitag *et al.*, 2009). The transition from an environmental saprophyte to a pathogen is primarily controlled by the transcription factor PrfA. This regulatory protein directly activates the expression of the major virulence genes required for the individual steps of the infection cycle, i.e. invasion, escape from the phagosome, intracellular motility and cell-to-cell spread (de las Heras *et al.*, 2011). Due to its ubiquitous nature, contamination of the food processing chain by *L. monocytogenes* is a frequent event and leads to repeating listeriosis outbreaks with a high number of severe and even fatal cases (Allerberger & Wagner, 2010). Listeriosis is clearly among the deadliest gastrointestinal bacterial infections as mortality rates of up to 30 % have been reported (Ramaswamy *et al.*, 2007). Consequently, *L. monocytogenes* has become one of the most comprehensively studied human pathogens (Cossart, 2007, 2011). However, we are still far from a complete understanding of virulence of the well-characterized and sequenced *L. monocytogenes* strain EGD-e. For example, the function of roughly one-third of all *L. monocytogenes* EGD-e genes is still unknown according to the KEGG genome database (http://www.genome.jp/dbget-bin/www_bget?genome:T00066).

Like the closely related non-pathogenic bacterium *Bacillus subtilis*, *L. monocytogenes* is a soil-dwelling bacterium that frequently encounters harsh environmental growth conditions, i.e. temperature fluctuations. *B. subtilis* and other bacilli such as the pathogens *Bacillus anthracis* and *Clostridium difficile* can escape from prolonged stress by the formation of extremely resistant endospores (McKenney *et al.*, 2013). These spores can germinate and re-enter into the vegetative life cycle in response to improved environmental conditions (Fig. 1a). By contrast, *L. monocytogenes* is unable to form spores. Thus, *L. monocytogenes* and other non-sporulating bacteria must have different strategies to survive environmental challenges (see Fig. 1a). It has been suggested that many Gram-positive and Gram-negative bacteria may enter a so-called 'viable but non-culturable' (VBNC) state in response to environmental stresses (Fig. 1a; Oliver, 2005). In this physiological state the bacteria are alive and metabolically active but the cells are unable to form colonies on conventional culture media (Oliver, 2010). There is evidence that under certain conditions *L. monocytogenes* cells can also become VBNC and that these cells escape routine detection methods, which are based on bacterial growth (Cappelier *et al.*, 2007; Dreux *et al.*, 2007; Lindbäck *et al.*, 2010). Thus, the VBNC state might be an important reservoir of harmful bacteria and is of major concern for public health risk and safety assessment. Indeed, harmful bacteria like *Mycobacterium tuberculosis* can enter into the VBNC state and return to the infectious state after passaging in animal hosts (Dhillon *et al.*, 2004).

The underlying molecular mechanism of the reversal of VBNC cells to culturable cells is largely unknown. Therefore, the hypothesis that the VBNC response is a programmed response has been faced with scepticism (Bogosian & Bourneuf, 2001; Nyström, 2001). However, in recent years, a variety of chemical and biological factors have been shown to promote resuscitation of VBNC cells (Oliver, 2010). In actinobacteria (high GC-content Gram-positive bacteria) resuscitation promoting factors (Rpfs) were described that have been shown to induce resuscitation from starvation-induced dormancy of *Mycobacterium tuberculosis* and *Micrococcus luteus* cells (Mukamolova *et al.*, 1998; Shleeva *et al.*, 2004). Rpfs are small extracellular proteins with extreme potency as they are active in very low amounts (Mukamolova *et al.*, 1998). The muralytic activity of Rpfs has been proven to be essential for resuscitation of dormant cells of *Micrococcus luteus* (Mukamolova *et al.*, 1998).

Initially, Rpf proteins had not been identified in firmicute (low GC-content Gram-positive) bacteria such as *L. monocytogenes*. But later, an *in silico* analysis revealed that many firmicutes contain a related protein family with partial similarity to the actinobacterial RpfB proteins (Ravagnani *et al.*, 2005). Two paradigm members of this group of proteins are the YabE and Lmo0186 proteins from *B. subtilis* and *L. monocytogenes*, respectively. The Rpf domain present at the C terminus of the actinobacterial RpfBs is replaced by the Sps or 3D (stationary phase survival/three conserved aspartates) domain in the YabE/Lmo0186 proteins, and these domains share partial similarity with the lytic transglycosylase MltA from *Escherichia coli* (Ravagnani *et al.*, 2005). The presence of a domain that is found in proteins with muralytic activity suggested that Sps proteins are involved in cell wall

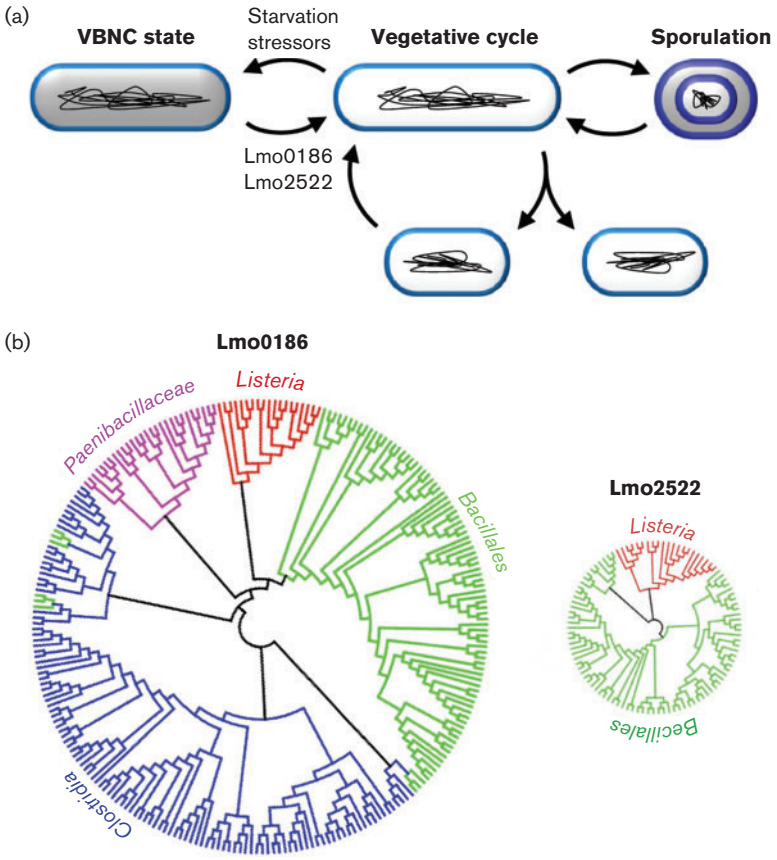

Fig. 1. (a) Schematic representation of different bacterial life styles. Bacteria like *B. subtilis* can escape from prolonged stress by forming endospores. In response to improved growth conditions the spores can germinate and re-enter into the vegetative life cycle. In contrast, non-sporulating bacteria such as *L. monocytogenes* may enter into the viable but non-culturable (VBNC) state in reponse to prolonged stress. While there is clear evidence that the resuscitation promoting factor (Rpf) protein from *Micrococcus luteus* is required for resuscitation of dormant cells it is tempting to speculate that the equivalent Sps proteins Lmo0186 and Lmo2522 of *L. monocytogenes* serve the same function. (b) Phylogenetic distribution of Rpf proteins of the Lmo0186 (SpsB/YabE) and Lmo2522 (SpsA/YocH) types. Lmo0186 and Lmo02522 were used as query sequences in a BLASTP protein–protein BLAST search (*E*-value cut-off 1e−30). This yielded 252 homologues of Lmo0186 and 106 of Lmo2522 after removal of truncated protein sequences. The protein sequences were aligned with the CLUSTAL W algorithm and a phylogenetic tree was reconstructed using the in-build tree building software of the Geneious Pro 5.5.7 software package. The trees are shown as unrooted cladograms with each end representing one protein sequence homologous to either Lmo0186 or Lmo2522.

metabolism. Indeed, recently it has been shown that the *B. subtilis* YocH protein, which also contains a Sps domain and is equivalent to the second *L. monocytogenes* Rpf protein Lmo2522, has muralytic activity and is involved in post-exponential growth phase survival (Shah & Dworkin, 2010). But as yet, YocH was the only Sps protein that had been subjected to a more detailed analysis in a firmicute bacterium even though members of both Sps domain-containing protein families are widespread among the firmicutes: YabE/Lmo0186 proteins are present in members of the class *Clostridia*, the family *Paenibacillaceae* and the order *Bacillales* as well as the family *Listeriaceae*, while occurrence of Sps proteins of the YocH/Lmo2522 type is limited to members of the order *Bacillales* and the family *Listeriaceae* only (Fig. 1b).

In this issue of *Microbiology*, Pinto *et al.* (2013) show for the first time that the Sps proteins Lmo0186 and Lmo2522 in *L. monocytogenes* are functionally equivalent to actinobacterial Rpf proteins. Although Lmo0186 and Lmo2522 are collectively dispensable for growth of *L. monocytogenes*, cells lacking both proteins had an extended lag phase when cultivated in minimal medium. The growth phenotype of the double knockout could be restored by the addition of the recombinant Sps proteins Lmo0186 and Lmo2522 to the medium. Moreover, the observation that the lack of Lmo2522 and all Sps proteins resulted in a small but significant increase in cell length during exponential growth and stationary phase, respectively, suggested a role for the two proteins in cell wall metabolism. Indeed, biochemical analysis revealed that both Sps proteins do have muralytic activity as has previously been shown for actinobacterial Rpf proteins (Mukamolova *et al.*, 1998; Shleeva *et al.*, 2004).

The work by Pinto *et al.* (2013) provides a well-grounded basis for further studies aimed at understanding the precise biological functions of Sps proteins in a human pathogen. For instance, it would be interesting to test whether lack of either Lmo0186 or Lmo2522 individually or in combination affects pathogenicity of *L. monocytogenes*. Moreover, it would be very interesting to address the question of whether the Sps proteins are needed for resuscitation of dormant *L. monocytogenes* VBNC cells (see Fig. 1a). Recently, it has been shown for *B. subtilis* that the membrane Ser/Thr kinase PrkC detects cell wall-derived muropeptides and stimulates synthesis of the Sps protein YocH, which is the homologue of Lmo2522 in *L. monocytogenes* (Shah & Dworkin, 2010). YocH is secreted into the extracellular space and the hydrolase digests peptidoglycan derived either from the same cell or from the surrounding cells. The released muropeptides may serve as signalling molecules that trigger regrowth of cells that were in a resting state (Keep *et al.*, 2006). As a PrkC homologue is also present in *L. monocytogenes* (Lmo1820), it is tempting to speculate that, similar to *B. subtilis*, Lmo2522 regulates its own expression via the PrkC-dependent pathway. Finally, an in-depth expression

analysis of the two Sps protein-encoding genes might give valuable information about which growth stage the proteins are synthesized and operate at in the important model pathogen *L. monocytogenes*.

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