Predictive microbiology theory and application: Is it all about rates?

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**ABSTRACT**

We review early work on the microbial growth curve and the concept of balanced growth followed by commentary on the stringent response and persister cells. There is a voluminous literature on the effect of antibiotics on resistance and persistence and we call for a greater focus in food microbiology on the effect of biocides in the same context. We also raise potential issues in development of resistance arising from “source–sink” dynamics and from horizontal gene transfer. Redox potential is identified as crucial in determining microbial survival or death, and the recently postulated role for reactive oxygen species in signalling also considered.

“Traditional” predictive microbiology is revisited with emphasis on temperature dependence. We interpret the temperature vs growth rate curve as comprising 11 regions, some well-recognised but others leading to new insights into physiological responses. In particular we are intrigued by a major disruption in the monotonic rate of inactivation at a temperature, slightly below the actual maximum temperature for growth. This non-intuitive behaviour was earlier reported by other research groups and here we propose that it results from a rapid metabolic switch from the relaxed growth state to the stringent survival state.

Finally, we envision the future of predictive microbiology in which models morph from empirical to mechanistic underpinned by microbial physiology and bioinformatics to grow into Systems Biology.

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1. Introduction

This paper is based on a presentation of the same title given at 7ICPMF in September 2011 (McMeekin, Olley, Ratkowsky, & Ross, 2011). In the presentation we discussed rates and time scales in microbiology, early studies on microbial growth rate curves and the concept of balanced growth. This was followed by consideration of the stringent response and persister cells, topics which are under-represented in the food microbiology literature. Attention was then focused on modelling studies with emphasis on temperature dependence models. Finally, we asked if the quantitative information embedded in predictive models could be effectively integrated with microbial physiology and “omics” technologies.

In answer to the question posed in the presentation title, “…is it all about rates?” we were confident to respond positively when the question addressed the use of predictive models to estimate the safety and shelf-life of foods. However, our response was equivocal when the question was framed in the context of integrating predictive models with microbial physiology studies and the data deluge emanating from “omics” studies. Here we extend the scope of the 7ICPMF presentation by incorporating recent literature (much of it published since September 2011) in an attempt to provide a definitive response to the objective of successful integration of traditional and futuristic predictive modelling.

2. Rates: all pervasive and all persuasive?

Time scales in microbiology range from milliseconds for enzyme catalysed reactions (Stockbridge, Lewis, Yuan, & Wolfenden, 2010) to doubling times of 7 min for Clostridium perfringens to days, weeks or months for psychrophiles growing under optimal conditions to more than 3.5 billion years for life to reach the current level of adaptive evolution. The last time frame has been achieved only as a result of the sub-second rates of enzyme catalysed reactions. For example the enzyme catalysed decarboxylation of orotidine-5′-phosphate, the final step in the synthesis of pyrimidines and thus nucleic acids, has a half-life of 0.017 s. Without the enzyme the half-life of the same reaction at 20 °C is 78 million years (Stockbridge et al., 2010).

In microbiology we consider rates, with time as the universal denominator to describe the development and decline of microbial populations. Much of the early work on microbial growth was carried out by the Paris School (Monod, 1942, 1949) and the Copenhagen School of Maaløe, Kjeldgaard, Neidhardt and Schaechter. Schaechter...
rates have important physiological and practical implications. For populations of cells, it is important not to lose sight that zero net growth rates are regions of zero or very slow growth they are crucial to the continuation of a lineage as will be evident when the physiology of the stringent response and of persister cells are considered. The physiology of the lag phase identifies it as a distinct growth phase that prepares cells for exponential growth (Rolfe et al., 2012).

3. The stringent response – paradigm lost in food microbiology

Even major changes in the physiology of the cell can occur in seconds. A good example is the transition from the relaxed response state to that of the stringent response and the converse switch which occur in 20–30 s (Cashel, 1975). This is equivalent to the half lives of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (ppGppp), the alarmones that give an unequivocal clue to cells to switch from one state to the other (Lund & Kjeldgaard, 1972). Thus, in a few seconds the purpose of cellular metabolism is totally reversed from a focus on growth (the relaxed state) to a focus on survival (the stringent [response] state; SR).

The SR has been shown to play a significant role in processes as different as biofilm formation, quorum sensing, antibiotic resistance, and virulence regulation (Navarro Llorens, Tormo, & Martinez-Garcia, 2010). Perhaps, it also provides an explanation for the Jameson Effect? Here we posit that the component of a mixed culture to approach MPD first produces sufficient ppGpp not only to signal its entry into the SR state but also that of its competitors. Relief from the SR state can be achieved by inoculation into a fresh batch culture or prevented by growth in continuous cultures in which the alarmone is continually diluted and nutrients are continually added. However, if a continuous culture is set up in a retentostat (a chemostat with 100% feedback of biomass) very slow or zero growth rates ensue (Chesbro, Evans, & Eifert, 1979; Goffin et al., 2010). This phenomenon can again be attributed to the programmed objective of the stringent response physiological state: survival at any cost. During transition from the exponential to the stationary phase the cytoplasmic concentration of the general stress factor (RpoS) increases in Gram negative cells (Gentry et al., 2003; Lange & Hengge Aronis, 1991, 1994) and cell density and quorum sensing compounds have been proposed to have a role in the mechanism of transition (Hengge-Aronis, 2002). However, Ihssen and Egli (2004) argued that, as other factors (growth rate, carbon source availability and metabolite concentration) also change during the transition, quorum sensing alone is insufficient to explain the transition. They concluded, on the basis of both batch and continuous culture experiments, that RpoS expression is not controlled by quorum sensing, that specific growth rate plays a prominent role and that ppGpp is a possible intracellular signal linking RpoS and specific growth rate. From an ecological standpoint this “egoistic” strategy of self-determination by individual cells was deemed to be eminently sensible compared with the more risky option of depending on signals from other cells. The line of reasoning developed by Ihssen and Egli (2004) was supported by Putrykus, Murphy, Philippe, and Cashel (2011) who concluded that ppGpp is the major source of growth rate control in Escherichia coli.

4. Persister cells – “stealth bombers” in the microbial survival armoury?

Cells with slow or zero growth rates confer a very distinct competitive advantage on microbial populations that, as a result of minimal metabolism, are extremely difficult to inactivate. The term, “bacterial persistence,” was introduced by Bigger (1944) who reported the inability of ampicillin to “sterilize” cultures of...
"super-survivors" were described by Kolter (1999). Research on persistence continues apace with organisms including E. coli (Shah et al., 2006), Pseudomonas aeruginosa (Mulcahy, Burns, Lory, & Lewis, 2010; Silver, 2011), and Mycobacterium tuberculosis and Candida albicans (Fauvart, Grootet, & Michiels, 2011).

Balaban, Merrin, Chait, Kowalik, and Leibler (2005) made a significant advance using a microfluidic device and microscopy to distinguish between normal, rapidly growing cells and slow growing persister cells. Persistence was shown to arise from pre-existing heterogeneity in bacterial populations with phenotypic switching occurring between fast growing cells and slow growing cells. Importantly, this demonstrated that mutation was not the mechanism of persistence development because cells sub-cultured from persisters remained sensitive to ampicillin. For a perspective on non-inherited antibiotic resistance see Levin and Rozen (2006). A microfluidic device also proved useful to track accelerated antibiotic resistance in connected environments (Zhang et al., 2011). The device was designed to mimic naturally occurring bacterial niches in the mammalian body. A spatially complex microenvironment was suggested to lead to accelerated evolution as a result of a stress gradient in which a mutant, that acquires resistance to a local stress, will have increased fitness in the population. The outcome was that four single nucleotide polymorphisms were established in 10 h from an inoculum containing as few as 100 cells.

Biofilms are widely reported in the literature to provide protection to cells contained in the extracellular matrix making them more resistant to environmental insults than planktonic cells (Costerton, Stewart, & Greenberg, 1999). Apart from physical protection supposedly afforded by the biofilm, tolerance to antibiotics is increased as a result of slow growth rates (Gilbert, Collier, & Brown, 1990). But, this is not a unique feature of biofilms and, contrary to popular opinion, slow growing planktonic cells can also display enhanced antibiotic tolerance (Spoering & Lewis, 2001). These workers also showed that, while the biocide peracetic acid was less effective in killing biofilm cells than exponential phase planktonic cells, stationary phase planktonic cells were more resistant than biofilm cells. Thus, the physiologically slow state and growth rate of stationary phase cells are key elements in persistence.

While the vast majority of persister cell studies have been concerned with antibiotics, the peracetic acid effects described by Spoering and Lewis (2001) expose the commonly held misconception that sessile cells are more resistant to sanitisers and disinfectants used in the food industry without reference to the role of their physiological state. The discussion in Spoering and Lewis (2001) is particularly pertinent: “Unlike conventional antibiotics biocides... kill cells directly”. Treatment with peracetic acid did not result in persister cells which can survive antibiotic levels 100–1000 times higher than the minimum inhibitory concentration (MIC). The mechanism of killing by antibiotics is indirect and involves activation of a programmed cell death response. By contrast, direct lethal damage caused by biocides resulted in total kills at only four times the MIC (Lewis, 2001).

A link back to the stringent response is found in Rodionov and Ishiguro (1998) and references therein, e.g. Sarubbo, Rudd, and Cashel (1988), in which steady state growth rates were shown to be affected by ppGpp production. Recent research on the stringent response and persisters includes Wang and Wood (2011) and Yamaguchi and Inouye (2011) who demonstrated a role for toxin/antitoxin systems in biofilm and persister cell formation. Kim et al. (2011) who screened a chemical library to locate a single compound that caused persisters to revert to antibiotic sensitive cells and Nguyen et al. (2011) who concluded that starved cells and biofilm cells of P. aeruginosa increase antibiotic resistance by active responses to starvation rather than the passive effects of growth arrest. The protective mechanism described was controlled by the SR and reduced levels of oxidative stress. When the protective mechanism was inactivated, the sensitivity of biofilm cells increased by several orders of magnitude and the efficacy of antibiotic treatment was markedly increased in experimental infections. The reduction in oxidative stress is consistent with the work of Belenky and Collins (2011) and Shatalin, Shatalina, Mironov, and Nudler (2011).

5. Greater focus on biocides in food microbiology?

The number of publications on the role of the SR and persister cells in surviving antibiotic challenges greatly outnumbers those concerned with detailed mechanisms of survival of cells when challenged with biocides. The possible effect of four antimicrobial substances widely used in the food industry was addressed by EFSA (2008) which wrote that “despite a long history of use, there are currently no published data to conclude that the application of chlorine dioxide, acidified sodium chlorite, trisodium phosphate and peroxy acids to remove microbial contamination from poultry carcasses will lead to the occurrence of acquired reduced susceptibility to these substances.” The same conclusion was reached in relation to the development of resistance to therapeutic antibiotics arising from use of the four biocides. However, the panel recognised that uncertainties exist and recommended that research on other biocides should continue. Essentially the same conclusions were reached earlier by an expert panel convened by IFT (Davidson & Harrison, 2002). A similar opinion was expressed by Meyer (2006), by Kastbjerg et al. (2009) who measured internal pH levels in a persistent strain of Listeria monocytogenes and by Kastbjerg et al. (2010). The response to an acid challenge on a single cell level was the same whether cells were planktonic or attached. Thus, persistence of the strain in food processing environments was deemed unlikely to result from selection of a more acid tolerant sub-population. Park and Chen (2011) examined inactivation of STEC strains producing extracellular cellulose by degrading the cellulose with cellulase and subsequent treatment with acetic and lactic acids and sanitisers. Cellulase alone had no effect on survival but strains with reduced cellulose were more susceptible to organic acids and sanitisers. This finding is consistent with that of Poole (2002) who reviewed the literature on biocide resistance. In this the author indicated that impermeability (with target alteration and efflux mechanisms) were the major survival strategies.

5.1. Connected microenvironments and source–sink dynamics (SSD)

The fascinating results of Zhang et al. (2011), revealing accelerated evolution in connected microenvironments, requires consideration of similar microcosms in agricultural and food environments. In the former, examples include: soil pores and structural features of plant roots, shoots and leaves which are associated with the vexed question of internalisation of human pathogens in plant tissues. In food environments, biofilms are an example of connected microenvironments with gradients of pH and oxygen tension created by microbial metabolism. Similarly, solid, multi-component foods such as salami also may represent temporally connected microenvironments as pH, osmotic and redox conditions change systematically and markedly during fermentation and maturation.

In addressing the question we should remember that Zhang et al. (2011) exclusively examined accelerated emergence of bacterial antibiotic resistance and, as discussed above, properly
applied sanitisers and disinfectants do not appear to lead to formation of persistor cells. Nevertheless, expert panels and individual authors who have written on the potential to induce increased resistance to commonly used cleaning agents add a caveat to their general conclusion that this is unlikely. The caveat normally recommends that further research is required because current knowledge is deficient.

Zhang et al. (2011) discussed the “source—sink” model of evolution that, like the stringent response, does not appear to be well represented in the food microbiology literature. A “sink” is a species habitat in which local mortality exceeds local reproduction. In a sink, a population can only be maintained with continuous migration from the “source” habitat where reproduction exceeds mortality (Hermens & Hwa, 2010). Characteristically, sinks occur near the limits of a species’ range. In source—sink dynamics (SSD), sources provide poorly adapted immigrants to the sink and those that can adapt through mutation are able to colonise the sink. SSD are reported to lead to accelerated bacterial drug resistance, resistance of fruit flies to insecticides and, relevant to this discussion, the evolution of virulence (Chattopadhyay et al., 2007; Denneh, Friedenberg, McBride, Holt, & Turner, 2010; Sokurenko, Gomulkiewicz, & Dykhuizen, 2006). SSD studies have also provided advances in understanding evolutionary processes with several publications on biological fitness landscapes revealing evolutionary pathways and why these pathways were selected (Lobkovsky, Wolf, & Koonin, 2010; Poelwijk, Kiviet, Weinreich, & Tans, 2007; Rowe, Wedge, Platt, Kel, & Knowles, 2010).

How are we to interpret source—sink dynamic situations in relation to the potential development of biocide resistance? Are they the result of an adaptive, phenotypic process resulting in habituated cells which will return to their original state on removal of the stress? Or, are they the result of mutations which become fixed in a newly competitive population and will be passed on to progeny of the lineage. Perhaps both strategies are in operation providing the population with an increasingly diverse array of options for survival? Laland, Sterenyi, Odling-Smee, Hoppitt, and Uller (2011) offered a philosophical treatment of cause and effect in biology, observing that “proximate” (immediate) outcomes such as physiological responses have traditionally been separated from “ultimate” (evolutionary) outcomes (Mayr, 1961).

5.2. Horizontal gene transfer and gene duplication influence the rate of prokaryotic evolution

Laland et al. (2011) concluded that it is time for the proximate—ultimate dichotomy to be replaced in biological sciences by a new “reciprocal” concept of causation. They proposed that the microbial survival imperative is serviced by adaptive, phenotypic responses which are induced rapidly and become redundant when a crisis passes as retention would place an unnecessary energy drain on the cell; plus an investment in long term (evolutionary) responses. Neither of these strategies is static, and the extent and importance of horizontal gene transfer (HGT) both in proximate and ultimate outcomes is increasingly recognised (see Koonin, Makarova, & Aravind, 2001; Smillie et al., 2011 and references therein). Treangen and Rocha (2011) compared the roles of HGT and gene duplication showing that the vast majority of expansions in protein families are due to transfer. Transferred genes persist longer while duplicated genes are expressed more, suggesting that transfer and duplication have different (but complementary) roles in evolutionary adaptation of prokaryotes; transfer enables new functions and duplication leads to increased gene dosage.

Smillie et al. (2011) concluded that a global network of gene exchange among members of the human microbiome was driven by ecology rather than geography or phylogeny. They reasoned that genes recently transferred between bacteria occupying well-defined niches are likely to reflect adaptation to that niche. Perhaps, this provides additional support for the view of Bass-Becking (1895–1963) that “everything is everywhere but the environment selects” (de Wit & Bouvier, 2006). Other examples included “massive” gene exchange between archaeal and bacterial hyperthermophiles (Aravind, Tatusov, Wolf, Walker, & Koonin, 1998), transfer of carbohydrate-active genes from marine bacteria to Japanese gut microbiota (Hehemann et al., 2010) and high levels of HGT by Shewanella baltica in a nutrient rich pelagic environment leading to its designation by Caro-Quintero et al. (2011) as the “ultimate r-strategist.” The potential of HGT to cause rapid transfer of genetic material was well illustrated by Stecher et al. (2012) who demonstrated that gut inflammation induced by S. Typhimurium triggered blooms of commensal E. coli. These were infected, with 100% efficiency in vivo, by a conjugative colicin plasmid from S. Typhimurium and out-competed the inhibitory effects of the commensal microbiota. Conversely, the ecological competition for colonisation of a niche was “won” by the indigenous soil microbiota that prevented establishment of invading E. coli O157:H7 (van Elsas et al., 2004).

6. Redox potential—an arbiter of growth or death and associated rates

Above we were concerned with microbial survival and presented several examples of widely contrasting time scales and rates from rapid physiological responses to eons of evolutionary time. One of the major events during evolution was the microbial driven switch from anaerobiosis to the highly oxygenated world that we currently inhabit. But, while aerobic metabolism was essential for development of more complex life forms there was also a downside because of the generation of reactive oxygen species (ROS) that cause cell senescence and death.

Only recently have we started to realise the enormous importance of the anaerobic “nether regions” in soils, sediments and permafrost where billions of tonnes of carbon are captured in huge microbial populations. Estimating the extent and rate of release of carbon dioxide from these carbon sinks is one of the most active research areas in biology at this time. The applicability of models, originally derived to estimate the microbial stability and safety of foods, to the often-called “big picture” science applications such as climate change, environmental degradation and bioremediation and biotechnology will be discussed later.

An inevitable consequence of anaerobic conditions is a marked reduction in energy yield from fermentative metabolic pathways compared to respiratory metabolism. However, for the microbial legions stable, anaerobic and cold conditions with minimal maintenance metabolism offer remarkable longevity that some consider to approach immortality (Price & Sowers, 2004). Hamanaka and Chandel (2011), Anastasiou et al. (2011) and Guerra et al. (2011) drew attention to reduction in reactive oxygen species (ROS) and reducing conditions as factors promoting cell survival. A similar conclusion was reached earlier by Chesney, Eaton, and Mahoney (1996) in protecting cells from the effects of chlorine. Intracellular glutathione protected E. coli against hydrogen peroxide, hypochlorous acid and chloramines and glutathione deficient mutants were approximately twice as sensitive to killing by each of the compounds.

While ROS are well known for their role in cell stress, Dickinson and Chang (2011) suggested that an alternative role as signal molecules may contribute to physiology and increased fitness. The signalling function is achieved by:
• co-localisation of the ROS sources and targets. Many ROS species are inherently unstable and reactive, e.g., the cellular half-life of [OH] is \( \sim 10^{-9} \) s and for \( \text{H}_2\text{O}_2 \) is \( \sim 1 \) ms;
• modulation of local redox buffering capacity by millimolar concentrations of glutathione provides substantial redox buffering for many ROS but reacts too slowly with \( \text{H}_2\text{O}_2 \). Buff-
ering the latter is achieved by peroxiredoxin proteins that cycle between reduced dithiol proteins and oxidised disulphide forms controlled by glutathione and \( \text{H}_2\text{O}_2 \) respectively.

Reduction of disulphide bonds also features in releasing the antimicrobial activity of human defensin-1 (hBD1) against the opportunistic pathogens and gut commensals (Schroeder et al., 2011) prompting the authors to postulate a general defence mechanism regulating the colonisation of mucosal membranes and skin surfaces.

The recent studies referred to above accord with earlier studies attributing the viable but non-culturale state to ROS (Bloomfield, Stewart, Dodd, Both, & Power, 1998; Dodd, Sharman, Bloomfield, Booth, & Stewart, 1997; Imlay, 1995; Vartoukian, Palmer, & Wade, 2010).

7. Quantifying rates in predictive microbiology

To this point the discussion has been concentrated on aspects of microbial physiology that affect growth and inactivation rates and rates of recovery from the effects of environmental insults. From this point we will move to consider predictive models more closely in sections 8—10. Finally we will attempt to integrate modelling and ecophysiology through the development of a mechanistic model, and the enormous opportunities offered by “omics” technologies, systems biology and bioinformatics.

The scheme proposed by Whiting and Buchanan (1993) which classified predictive mathematical models in food microbiology as ‘primary’, ‘secondary’ or ‘tertiary’ is logical and has stood the test of time. Commonly used primary models that describe sigmoidal bacterial growth curves include the Logistic, Gompertz and Baranyi models. Details and comparisons of these and other primary models were presented by McKellar and Lu (2004). Secondary growth models describe the effect of environmental factors growth or inactivation rate and, while most attention has been given to modelling temperature dependence, multi-factorial models have been developed and the paper by Mejljholm et al. (2010) is in the “must read” category as it evaluates the performance of such models. These include one in which the combined effects of up to nine environmental factors on the growth of \( \text{L. monocytogenes} \) were considered. Ross and Dalgard (2004) provided a comprehensive account of secondary models to that time.

Whiting and Buchanan (1993) defined tertiary models as algo-
rithms incorporated into software to integrate the effect of envi-
ronmental variables on microbial responses and to provide predictions of the outcomes. The most developed tertiary models are all now available via the internet and include

• the Pathogen Modeling Program (http://pmp.arsserc.gov/ PMPHome.aspx),
• SymPrevius (http://www.symprevius.net/),
• Seafood Spoilage and Safety Predictor (http://sssp.dtuaqua.dk/) and
• ComBase (http://www.combase.cc/index.php/en/).

The Microbial Resources Viewer (MRV: http://mrv.nfri.afric.go. jp) was developed by the National Food Research Institute, Japan (Koseki, 2009) to complement ComBase. Both the SSSP and the MRV provides growth/no growth data sets with growth rate contour plots. This allows users to identify conditions at the growth/no growth boundary visually as well as quantitatively and the user friendly visual feature suggests that Koseki (2009) should also be placed in the “must read” category.

8. Modelling the effects of temperature on growth

As noted above, temperature has often been the primary factor to measure in predictive microbiology because it is the factor likely to fluctuate to the greatest extent as product moves through the supply chain. The major “competitors” for interpreting the effects of temperature on microbial ecology in foods are Arrhenius-type and Bélehradék-type models. Whilst the goodness-of-fit of these models have been compared in many studies, differences are often minimal. The conclusion is that both logarithmic and square root transformations of data are very effective in homogenising variance. Subsequently, arguments may arise about the relative merits of a model on the basis of other criteria. For example does the model have a mechanistic basis? Arrhenius-type models have their origin in chemical reaction kinetics and this has been sufficient to endow them with a “mechanistic aura” despite the fact that they do not provide an adequate description of biological reaction kinetics. This view is encapsulated in the title of a paper by Knies and Kingsolver (2010) “Errorenous Arrhenius: modified Arrhenius model best explains the temperature dependence of ectotherm fitness.”

Differences of opinion on the goodness-of-fit of models also arise within a particular model type. Huang (2010, 2011) described a “new” temperature dependence model for bacterial growth. The motivation for the study was the discrepancy between \( T_{\text{min}} \) and the theoretical minimum temperature for growth and the actual minimum temperature at which growth is observable (“MINt”). The author posited that \( T_{\text{min}} \) should reflect biological reality (i.e., MIN) and, in an attempt to make the estimates similar or the same, changed the exponent of the square-root model from 2.0 to 1.5. Furthermore, the idea of a theoretical minimum temperature has an important and universally accepted precedent in science, the concept of absolute zero, introduced by Lord Kelvin.

To assist in preventing further misinterpretation Ross, Olley, McMeekin, and Ratkowsky (2011a, 2011b) proposed to retain the current notation, \( T_{\text{min}} \), for the theoretical minimum temperature and introduced the abbreviation MIN for the measurable minimum temperature. Despite the clear definitions of \( T_{\text{min}} \) and \( T_{\text{max}} \) by McMeekin, Olley, Ross, and Ratkowsky (1993), uncertainty about the definition of \( T_{\text{min}} \) as a theoretical temperature was also evident in the advent of cardinal temperature models. These were first proposed by Lobry, Rosso, and Flandrois (1991), Rosso, Lobry, and Flandrois (1993), and Rosso, Lobry, Bajard, and Flandrois (1995) in each of which \( T_{\text{min}} \) was defined as the minimum growth temperature and \( T_{\text{max}} \) as the maximum growth temperature. Bajard, Rosso, Fardel, and Flandrois (1996) presented the first report from the Rosso group in which \( T_{\text{min}} \) and \( T_{\text{max}} \) were correctly defined as “the minimal temperature where growth can theoretically not be observed” and \( T_{\text{max}} \) as “the maximal growth temperature above which growth is theoretically not possible.”

Bajard et al. (1996) highlighted the unexpected growth rate response of \( \text{L. monocytogenes} \) at suboptimal temperatures. Whilst \( \text{E. coli} \), \( \text{Clostridium botulinum} \) and \( \text{P. aeruginosa} \) growth rates were well described by the square root model at suboptimal tempera-
tures, the response of \( \text{L. monocytogenes} \) deviated from linearity. A change in temperature response kinetics was proposed between 10 \(^{\circ}\)C and 15 \(^{\circ}\)C and square root models were fitted to data at temperatures <10 \(^{\circ}\)C and >15 \(^{\circ}\)C. The \( T_{\text{min}} \) value using the low temperature rates was \( \sim -5 \) \(^{\circ}\)C for both strains, while for the high temperature range data the estimates were \( \sim -4 \) \(^{\circ}\)C and 1 \(^{\circ}\)C. The authors emphasised the need to use the low temperature range
estimate of $T_{\text{min}}$ as the high temperature range estimate would lead to “fail dangerous” predictions.

No explanation for the particular behaviour of *L. monocytogenes* was offered and, to the best of our knowledge, none has been advanced since. Here we propose that the response is a function of the dual lifestyle of the organism. On one hand it is an intracellular pathogen and, on the other an environmental organism with remarkable survival capability. The physiological basis for this duality may possibly be explained by thermosensitive riboswitches. Cossart (2011) reviewed transcriptional complexity and RNA regulation in *L. monocytogenes* noting that many virulence factors are regulated by PrfA under the control of a thermosensor. This results in optimal PrfA expression at high temperatures and translational repression at low temperatures with maximum expression of virulence genes at 37 °C. A thermosensitive link between virulence and nutrient availability in the transition from saprophyte to parasite was also established by Toledo-Arana et al. (2009). Conversely, flagella activity, an ecological advantage in saprophytic mode was greater at low temperatures (Kamp & Higgins, 2009; Mauder, Williams, Fritsch, Kuhn, & Beier, 2008). The majority of temperature dependence studies on virulence have been conducted at more than Kuhn, & Beier, 2008). The majority of temperature dependence used in biology and exponents other than two have been suggested.

Toledo-Arana et al. (2009) occurred at 10–12 °C whereas the change in growth rate noted by Bajard et al. (1996) occurred at 10 °C–15 °C. Data is needed to bridge the temperature gap as *L. monocytogenes* moves from an environmental to a parasitic lifestyle.

8.1. Why select an exponent of 2?

The square root model is a special case of Bělehraděk’s temperature function first introduced in 1926, in which the exponent is two (Ross, 1993). The Bělehraděk model became widely used in biology and exponents other than two have been suggested. Spencer and Baines (1964) used an exponent of one for fish spoilage but Olley and Ratkowsky (1973) showed that it was accurate only over a narrow temperature range from 0 °C to 6 °C. Further work on deterioration of fish was reported by Ohta and Hirahara (1977) who found an exponent of two was suitable to describe the temperature dependence of nucleotide degradation. In turn this led to the application of square root kinetics to microbial growth rates (Ratkowsky, Lowry, McMeekin, Stokes, & Chandler, 1983; Ratkowsky, Olley, McMeekin, & Ball, 1982).

Subsequently, the square root model was extended to include terms for water activity (McMeekin et al., 1987) and pH (Adams, Little, & Easter, 1991). Importantly, this did not change selection of 2 as the appropriate exponent or the estimate of $T_{\text{min}}$ but MINt increases with increasing levels of NaCl and decreasing water activity.

From the 1980’s the great majority of power-law models have used an exponent of 2 across several fields of biology. Examples include Jefferies and Brain (1984), for pollen tube penetration; Li (1984), for marine phytoplankton photosynthesis (for further discussion on this see McMeekin et al., 1993, App. 4.5, p. 340); Smith (1985), for coliform growth rates; Li and Dickie (1987), for marine phytoplankton growth rate; Blankenship, Craven, Lefler, and Custer (1988), for *C. perfringens* growth during cooling and Griffiths and Phillips (1988) for spoilage of milk.

8.2. Why have theoretical $T_{\text{min}}$ and $T_{\text{max}}$ values?

So, what are the advantages of the dichotomy of theoretical and measurable minimum and maximum temperatures? Below we suggest several, some practical and some that may add to the development of theory in microbial ecophysiology (McMeekin et al., 2008).

- The value of $T_{\text{min}}$ is fixed and does not depend on other environmental variables such as water activity or pH. However, MINt values vary markedly depending on other factors;
- A constant $T_{\text{min}}$ value provides a convenient way to construct a relative rate curve, the basis of technologies to predict the microbiological safety and stability of foods;
- There is a continuum of $T_{\text{min}}$ values describing the thermal adaptation of bacteria across all temperature categories, psychrophiles, psychrotropes*, mesophiles and thermophiles;
- A constant $T_{\text{min}}$ leads to a simple multiplicative model to describe the combined effect of several hurdles (the Gamma hypothesis);
- The form of the model suggests that the combined effect of several hurdles is additive and not synergistic when the cells are growing;
- Synergistic effects occur at the growth/no growth interface and beyond when cell physiology switches from growth to survival mode;
- The gap between $T_{\text{min}}$ and MINt increases with progressively harsher conditions due to energy diversion to deal with additional hurdles.

(*n.b., The term psychrotroph has gained almost universal approval in the food microbiology literature to describe a cold tolerant microorganism but the etymology of the word is incorrect as —troph, from which psychrotroph is derived is a suffix referring to nourishment. The correct suffix to indicate cold tolerant is —trophic referring to turning, as in heliotrope or geotropic).

9. The elements of a growth rate versus temperature plot for the full biokinetic temperature range

The response of growth rate to temperature plotted as the square root of growth rate versus temperature is shown in Fig. 1. This has the advantage of homogenising the variance and
providing a linear response in the sub-optimal region. It also emphasises the juxtaposition of $T_{\text{min}}$ and $T_{\text{Mint}}$ and of $T_{\text{max}}$ and $\text{MAXt}$.

9.1. Revelations from a temperature dependence plot

Several features of the growth curve require further comment. The shaded regions of the curve indicate where estimates of growth rate are variable, but for different reasons. Between points 2 and 3 variability arises from the distribution of generation times (described by a gamma function) when the variance is proportional to the mean response time squared (Ratkowsky, Ross, Macario, Dommett, & Kamperman, 1996). At point 7 variable estimates are explained by maximum demand on cell physiology to produce sufficient proteins and other macromolecules to service the requirements of new cells. The term optimal temperature may be considered correct to describe the maximum rate of growth but is a misnomer for optimal metabolic efficiency, which is in the $T_{\text{mes}}$ region (points 4–5).

The apparently anomalous response at 9–10 where inactivation occurs at temperatures less than $\text{MAXt}$ is counter-intuitive but has been reported by others (Cornet, Van Derlinden, Cappuyns, & Van Impe, 2010; Niemela & Oivanen, 1992; Van Derlinden, Lule, Bernaerts, & Van Impe, 2010). Physiological explanations were not advanced and here we propose that the perturbation is caused by rapid induction of the stringent response when the purpose of cellular metabolism is suddenly and unequivocally reversed from growth to survival.

Note also the skewed shape of the temperature dependence plot which indicates that specific growth rates rise gradually to a maximum and then decline rapidly in the superoptimal region. The same pattern is also observed when rate is plotted against temperature (Chen & Shakhnovich, 2010). The rapid decline is due to increasing irreversible denaturation of proteins as $\text{MAXt}$ is approached and identifies the superoptimal temperatures as a region where large declines in rate are achieved by small shifts in temperature.

10. Moving towards a mechanistic model

Ratkowsky, Olley, and Ross (2005) reported on the similarity of temperature dependence of bacterial growth and the stability of globular proteins using a kinetic model incorporating thermodynamic terms for protein denaturation. The underlying concept relied on a term for the positive change in heat capacity during protein unfolding.

The thermodynamic model was developed using 35 data sets for psychrophilic, psychrotrophic, mesophilic and thermophilic bacteria resulting in biologically meaningful estimates of the thermodynamic parameters. Subsequently the same 35 data sets were used by Kries and Kingsolver (2010), Chen and Shakhnovich (2010), Ghosh and Dill (2010) and Dell, Pawar, and Savage (2011). The probable mechanism of protein unfolding (denaturation) is a change in tertiary structure in response to exposure of hydrophobic amino acids to water in the cell.

The thermodynamic approach was further advanced by Corkrey, Olley, Ratkowsky, McMeekin, and Ross (2012) using a Bayesian hierarchical modelling approach to analyse 95 data sets (including those considered earlier) drawn from the Archaea, Bacteria and Eukarya, i.e., representing the three domains of life on earth. The new model, as in Ratkowsky et al. (2005), was based on the master reaction hypothesis and the results implied that temperature dependent growth rates of all unicellular organisms respond according to the assumptions of the model.

11. Bioinformatics — the highway to predictive Systems Biology?

To date predictive microbiology has been concerned with predicting the rates of biological processes, particularly microbial growth and inactivation, almost exclusively using empirical models. More recently we have seen development of mechanistic models, such as the thermodynamically based temperature dependence model proposed by Corkrey et al. (2012). We have also experienced the “stamped” into ‘omics-based technologies by which enormous amounts of data are accumulated potentially allowing detailed descriptions of the inputs and outputs and control of complex metabolic pathways in cells. Thereby, the era of systems biology has arrived but its application rests on being able to deal effectively with the “data deluge.” Thus, biologists now need to recruit the quantitative skills of bioinformaticists to provide the link between data accumulation, organisation and storage, and interpretation. This is exemplified by a trend in major journals to spin off separate sections or, in some cases, new journals within a publishing group to deal with Computational Biology.

Mechanistic models and systems biology sit naturally together so it is appropriate that they should evolve in parallel. In striving for a comprehensive description of metabolic co-ordination, how these are affected by environmental conditions and how they are controlled and fine-tuned by genetic and epigenetic events in the cell, it is appropriate that our focus shifts from empirical, predictive microbiology models to mechanistic, predictive systems biology models. The latter have the potential to reveal unifying themes in biological sciences.

The rate of publication of papers linking genomics, proteomics, metabolomics etc. to microbial ecology and physiology is increasing markedly and one of particular relevance to the content presented here is that by Vieira-Silva and Rocha (2010) which dealt with the implications of growth rate for ecological genomics and metagenomics. The research outcomes were summarised as follows: “Prokaryotes have evolved a set of genomic traits to grow fast, including biased codon usage and transient or permanent gene multiplication for dosage effects. ... these traits can be used to predict minimal generation times from the vast majority of microbes that cannot be cultivated. ... this inference can also be made with incomplete genomes and thus be applied to metagenomic data to test hypotheses about the biomass productivity of biotypes and the evolution of microbes in the human gut after birth. Our results also allow better understanding of evolution between growth rates and genomic traits and how they can be manipulated in synthetic biology. Growth rates have been a key variable in microbial physiology studies in the last century, and we show how intimately they are linked with genome organisation and prokaryotic ecology”. The authors summary makes a persuasive case that linking ecophysiology with genomics will advance knowledge rapidly.

Vieira-Silva, Touchon, Abby, and Rocha (2011) also used proteomics to study the effect of growth rate on the evolutionary rates of essential proteins showing that the relative frequency of highly expressed proteins in the proteome increased steeply with growth rate. The positive correlation between expression levels of highly expressed proteins and growth rates was confirmed in 74 proteobacteria. Analyses of the ribosome and the replisome suggested that growth-related sequence conservation is associated with synthesis of highly expressed proteins, consuming a major part of the cellular energy budget and leading to strong conservation of sequences coding for highly expressed proteins. For other papers using proteomics to predict evolutionary trends, ecological distribution and population physiology see Wang, Yafremava, Caetano-Anolles, Mittenthal, and Caetano-Anolles (2007) and Mueller...
et al. (2010). The use of proteomics in food safety research was addressed by D’Alessandro and Zolla (2012) and the contribution of metabolomics and transcriptomics to unravel the stress response of E. coli was demonstrated by Jozefczuk et al. (2010). Papp, Notebaart, and Pal (2011) described systems-biology approaches for predicting genomic evolution judging that the models: “Hold the promise of transforming evolutionary biology into a more predictive discipline” with the ability to “make specific and reliable predictions on the outcome of metabolic evolution, both in short term evolution and on macroevolutionary time scales.” The genomic basis of trophic strategy in marine bacteria was elucidated by Lauro et al. (2009) and Raes, Letunic, Yamada, Jensen, and Bork (2011) combined biochemical, geographical and metagenomic data to develop a molecular trait-based view of ecology.

Finally, considerable concern has been expressed about the effect of climate change on global patterns of biological diversity and the search is on for predictors of diversity. Tittensor et al. (2010) undertook this challenge in the case of marine biodiversity across taxa. They concluded that across 13 major species groups from zooplankton to marine mammals that sea surface temperature was the only environmental predictor highly related to diversity across taxa. They concluded that across 13 major species groups from zooplankton to marine mammals that sea surface temperature was the only environmental predictor highly related to diversity across taxa. We expect that the model of Corkrey et al. (2012), proposing universal thermodynamic constraints to temperature limits for microbial, based on protein stability, will find utility in assessing the effect of changing temperatures on microbial growth rates, microbial diversity and species survival in marine, and perhaps other, environments.

12. Conclusions

In the conclusion given at 7ICPMF in September 2011 we were unable to present unequivocally that successful integration of traditional and futuristic predictive modelling was possible. Now, after an extensive additional literature search, including many papers published subsequent to 7ICPMF we are more confident that this goal is achievable.

The first reference cited in this paper, Stockbridge et al. (2010), provided an estimate of time required for evolution of enzymes. So, having started the paper by considering enzymes, which as proteins, require to be folded correctly to function we need to consider what drives the folding process. Is it purely under thermodynamic control or is there a kinetic element involved (Govindarajan & Goldstein, 1996; Mallam & Jackson, 2012)? The answer is that, while the rate of protein folding is accelerated by molecular chaperonins it is essentially a spontaneous process that, despite being slow proceeds without misfolding (Mallam & Jackson, 2012). In turn, this confirms the view of the 1972 Nobel Prize winner Anfinsen (Anfinsen, 1973) that the most probable conformation of a folded protein in any given environment is determined solely by its amino acid composition. Rothman and Skefman (2011) highlighted the very significant advance in knowledge arising from elucidation of the role of molecular chaperonins in accelerating rates of protein folding noting that this work was complementary to that of Anfinsen. Further, it is consistent with our opening comments on the crucial part played by enzymes in determining rates of biological evolution.

Now, the loop is nearly closed in that protein folding can be described by a mechanistic, thermodynamically based, temperature dependence model (Corkrey et al., 2012) which can be applied equally well to all three domains of unicellular life. At this juncture we intend to extend our studies to determine if the effect of other constraints on microbial growth, such a water activity and pH, can be modelled thermodynamically and to extend temperature dependence studies to multicellular life forms. Given similar outcomes it seems reasonable to project widespread applications for thermodynamically based models not only in microbiology, but also in protein stability, thermal biology, ecological theory, biogeochemistry, extremophile biology and climate science. We are excited by these prospects and pleased that the transition from empirical to mechanistic predictive models has its genesis, in part, in food microbiology.

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