REVIEW

Individuality, phenotypic differentiation, dormancy and ‘persistence’ in culturable bacterial systems: commonalities shared by environmental, laboratory, and clinical microbiology [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

For bacteria, replication mainly involves growth by binary fission. However, in a very great many natural environments there are examples of phenotypically dormant, non-growing cells that do not replicate immediately and that are phenotypically ‘non-culturable’ on media that normally admit their growth. They thereby evade detection by conventional culture-based methods. Such dormant cells may also be observed in laboratory cultures and in clinical microbiology. They are usually more tolerant to stresses such as antibiotics, and in clinical microbiology they are typically referred to as ‘persisters’. Bacterial cultures necessarily share a great deal of relatedness, and inclusive fitness theory implies that there are conceptual evolutionary advantages in trading a variation in growth rate against its mean, equivalent to hedging one’s bets. There is much evidence that bacteria exploit this strategy widely. We here bring together data that show the commonality of these phenomena across environmental, laboratory and clinical microbiology. Considerable evidence, using methods similar to those common in environmental microbiology, now suggests that many supposedly non-communicable, chronic and inflammatory diseases are exacerbated (if not indeed largely caused) by the presence of dormant or persistent bacteria (the ability of whose components to cause inflammation is well known). This dormancy (and resuscitation therefrom) often reflects the extent of the availability of free iron. Together, these phenomena can provide a ready explanation for the continuing inflammation common to such chronic diseases and its correlation with iron dysregulation. This

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Any reports and responses or comments on the article can be found at the end of the article.
implies that measures designed to assess and to inhibit or remove such organisms (or their access to iron) might be of much therapeutic benefit.

**Keywords**
Dormancy, persisters, sepsis, microbiome, inflammation, culturability, iron dysregulation

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Introduction

“It is now well established that some micro-organisms can, under certain conditions, be deprived of all visible signs of life and yet these organisms are not dead, for, when their original conditions are restored, they can return to normal life and activity.”

“Bacterial populations in both batch and continuous culture are much more heterogeneous than is normally assumed, and such cultures may consist of several types of subpopulations simultaneously differing in viability, activity and integrity of the cells.”

Consider a typical axenic flask or broth culture of bacteria (Figure 1), arguably the staple of modern laboratory microbiology. We seed a suitable growth medium with an appropriate inoculum of cells known to be capable of replicating in that growth medium. After a lag phase the number of culturable cells (the ‘viable count’), as judged by plate counts of the number of colony-forming units observable on the same medium solidified by agar or a similar material) is observed to increase, typically exponentially, for a number of generations (the growth phase or exponential phase). Apart from the changes in nutrient concentration, and for non-synchronised cultures, it is generally taken that cells pass smoothly through their cell cycles en route to doubling their numbers by binary fission. The population distribution of organisms in different parts of their cell cycle during the exponential phase is thereby unchanged and thus in a steady state (from which the cell cycle parameters can even be inferred). In time this increase in cell numbers ceases, usually because of the exhaustion of a nutrient in a closed system, or sometimes in part or whole because of the build-up of toxins. Again, after a further period, the viable or colony count decreases (often to quite low levels if such starvation is carried out for extended periods). Inoculation of a new broth culture with a similar number of viable cells from this culture usually provides a simple repeat of the previous culture, and in the absence of mutation may reasonably be anticipated, for organisms proliferating asexually, to be played out indefinitely.

The development of continuous, nutrient-limited (‘chemostat”) or feedback-controlled (‘turbidostat”) cultures was and is entirely consistent with this view of steady-state microbial doubling via homogeneous cell cycles that are common, within statistical fluctuations, to each cell. The same is true for cultures undergoing serial transfer (where there is slightly more of a focus on selection for genotypic variants that grow faster – see e.g. 12–14).

There should be nothing controversial in the above passage, but in fact it hides a variety of assumptions that themselves conceal a considerable feast of very interesting physiology. The chief one here is that – given that all cells in the culture are genetically homogeneous and see the same ’environment’, and modulo where they are in their cell cycles – all such cells are indeed supposed to represent a single population (as per Figure 2). If they do not, and as we shall see they never do15–18, we are dealing with differentiated systems. It turns out that a particular subset of typical cell cultures – a phenotypically dormant or non-growing sub-population, occurring even in non-spore-bearing bacteria – is widespread to the point of ubiquity. This leads to an exceptionally important biology with significant consequences both for our understanding of microorganisms and our ability to harness and domesticate them. Although the relevant literatures rarely cite each other or overlap, it is clear that similar phenomena are common to bacterial behaviour in the natural environment, the laboratory, and in a variety of samples of clinical interest. This theory or hypothesis that we develop here comes about from the synthesis of a large amount of data, and is summarised in Figure 3 and Figure 4.

Phenotypic differentiation to dormancy – some early indications

While dormancy and resuscitation of rotifers had been observed by Leeuwenhoek himself in 1702, some of the earliest modern indications for a physiologically significant phenotypic differentiation of microbial cultures came in the 1940s. In a conceptually simple

![Figure 1. A typical laboratory bacterial culture.](image)

After the end of stationary phase the viable count decreases over time, but very rarely to precisely zero. Some authors recognise an extended "period of prolonged decrease" during which some of the survivors undergo significant dynamics, and in which mutants are selected. Our interest here is largely in cells that have not mutated.

![Figure 2. To clarify the general concept of a population as used here, a population of individuals involves those who share certain properties (between stated values).](image)

One main population is shown. A second, smaller population is also shown; these might represent dormant cells.
Figure 3. Infographic summary of the review. (1) A bacterial system contains distinct subpopulations, that we classify as culturable, dormant and ‘non-culturable’ (2). Specific attention is given to persister cells (3), and the inter-relationship (4) between the subpopulations. Subpopulations within environmental biology are discussed (5), followed by subpopulations within laboratory cultures (6). Particular emphasis is placed on phenotypic switching between the culturable and dormant subpopulation of laboratory cultures (7). Generalized detection techniques typically fail to detect dormant cells, and we review the various reasons for this failure and discuss alternatives (8). Resuscitation of and endotoxin production by such dormant cells underpins many diseases not normally seen as having a microbial component.

Figure 4. Summary of the review in the form of a ‘mind map’ of the article.
experiment (illustrated in Figure 5), Bigger\textsuperscript{29} exposed staphylococcal cultures to concentrations of penicillin that would normally be sufficient to kill them completely (and they did kill all but 1 in a million). However, these (10\textsuperscript{6}) survivors, that Bigger\textsuperscript{28} and McDermott\textsuperscript{22} (and many modern commentators have) referred to as ‘persisters’, were not genetic mutations selected for resistance to penicillin, since when they were inoculated into fresh broth they were just as susceptible as were those in the first culture. Bigger recognised (correctly) that the only explanation that made any kind of sense was that despite being exposed to nominally the same conditions, these cells were operationally dormant (even if metabolically active\textsuperscript{22,23}) and thus phenotypically resistant to the penicillin (that anyway kills only dividing cells\textsuperscript{24,25}). Similarly, Luria and Latarjet\textsuperscript{6} noted that approximately 1% of the cells in a culture of Escherichia coli displayed a phenotypic resistance to normally sterilising doses of ultraviolet irradiation. Many similar experiments since (e.g. \textsuperscript{27–29}), discussed in more detail below, have recapitulated this basic phenomenon. (We note here that high-frequency antigenic ‘phase’ variation can occur due e.g. to changes in microsatellite DNA\textsuperscript{31}; detailed discussions of such genotypic changes\textsuperscript{31}, including those that can affect the extent of dormancy in persistent bacteria\textsuperscript{32}, are outwith the scope of the present, purely phenotypic analyses.)

**Dormancy as an operational property**

For the avoidance of doubt, and in accordance with Keilin’s description with which we opened, we shall define dormancy as:

“a reversible state of {often} low metabolic activity, in which cells can persist for extended periods without division; we shall see that this often corresponds to a state in which cells are not ‘alive’ in the sense of being able to form a colony when plated on a suitable solid medium, but one in which they are not ‘dead’ in that when conditions are more favourable they can revert to a state of ‘aliveness’ as so defined”\textsuperscript{33}.

We thus stress\textsuperscript{33} the recognition that dormancy is not solely an innate property of a bacterial cell; it is a property assessed by one or more experiments, so whether a cell appears to be dormant depends on both the cell and the experiment used to assess that dormancy. (This principle shares a similar philosophical foundation to the independence from any specific experiment, or otherwise, of the perceived state of objects within the quantum theory\textsuperscript{33–35}.) As do Postgate\textsuperscript{36,37} and Barer\textsuperscript{38–41}, we take the hallmark of a viable or living bacterial cell to be its ability to replicate or its ‘culturability’. This means that we cannot tell via culturability that a cell is alive, only (after a cell division) that it was alive\textsuperscript{41,42}. Dormant cells – even if ‘not immediately culturable’ – must by definition be resuscitable to form culturable cells. Although the term ‘nonculturable’ is quite commonly used to describe not-immediately-culturable cells it is best avoided, as we cannot try every possible combination\textsuperscript{42} of incubation conditions that might serve to resuscitate a cell in a sample. ‘Non-cultured’, ‘as-yet-uncultured’ or ‘operationally nonculurable’ are better terms. Culturable, (operationally) non-culturable and (operationally) dormant bacteria in the differentiated bacterial (cellular) system can therefore be seen as distinct subpopulations of the system, and culturable and dormant bacteria as reversible states of the same population. The relationships between such subpopulations of the bacteria within a differentiated cellular system are shown in Figure 6.

**On methods for detecting microbial presence, ‘viability’ and culturability**

Given our operational definition of dormancy as including reversible culturability, we note that different kinds of assays for the presence or activity of bacteria necessarily reflect cells in different kinds of physiological states (and can thereby be used to discriminate them). Thus direct counts with stains such as acridine orange (a list of these and other methods is given in Table 1 of 33) do not determine culturability, only presence or activity. Similarly, macro-molecular sequencing methods such as those based on rDNA and its amplification (e.g. \textsuperscript{44–49}) almost certainly reflect mainly dormant cells plus any actively dividing ones (in that ‘naked’ DNA is usually degraded fairly rapidly in serum or the environment).

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**Figure 5.** Assessment of phenotypic differentiation of a dormant subpopulation via antibiotic challenge. This kind of protocol can be used to determine if the resistant subpopulation has accumulated genetic mutations that encoded resistance or whether, as focused on here, the resistance is purely phenotypic. A detailed analysis of the shape of the time-survivor curves may also be informative\textsuperscript{83}.

**Figure 6.** The relationships between culturable, dormant and operationally non-culturable bacteria within a differentiated cellular system.
The difference between culturable counts and total sequence-based counts probably provides one of the best methods for detecting and enumerating dormant cells when they cannot yet be brought back into culture. It is particularly noteworthy (and see also 50 and below) that the amount of prokaryotic DNA in whole blood exceeds by 10–100-fold that detectable in serum,[51], implying adsorption onto or sequestration within blood cells.

We shall return to clinical and laboratory microbiology later, but it is to environmental microbiology that we now turn to discuss the culturability of typical microbes. While the same general truths undoubtedly pertain in viruses (e.g. 52,53), and in yeasts, fungi, archaea, mycoplasmas and other unicellular organisms, our focus will be on prokaryotes.

**Bacterial culturability and dormancy in environmental microbiology**

It has long been known that the number of bacteria observable microscopically exceeds, typically 100-fold, those that can readily be grown axenically in standard isolation media (i.e. to proliferate in liquid culture or to form colonies on solid media). The latter has been referred to as ‘the great plate count anomaly’,[64], and has been amply confirmed by more modern, culture-independent sequencing methods. A selection of papers and reviews serve to document both the numerical anomaly and the much greater biodiversity detectable by sequencing (e.g. 55–73). It is thus useful to discriminate (1) bacteria that have been cultured, that are typically available in culture collections, and whose growth requirements are known, from (2) bacteria that may be recognised as novel via macromolecular sequencing (typically of ribosomal DNA[64,74–77]) but that have not yet been cultured and whose growth requirements may not yet even be known. Much (sequencing) evidence indicates that the bulk of the ‘missing microbes’ or ‘dark matter’[78,79] in natural ecosystems falls into this second category,[80], and that ‘single cell’ methods may be required to culture them.[81].

Not-yet-cultured bacteria may have more-or-less fastidious growth requirements

It is an elementary observation in microbiology, and the basis for selective isolation media, that not all bacteria grow on all media and in all conditions. Leaving aside truly syntrophic bacteria (that for thermodynamic or unknown nutritional reasons require another organism for growth (e.g. 82–88)), some organisms may have quite fastidious growth requirements. A number of bacteria determined as causative of disease, whose role had originally been inferred only through microscopic observation, were later cultured and could be shown to fulfill Koch’s postulates. These include *Helicobacter pylori*,[89,90] (with an unusually high requirement for urea to fuel its alkaline urease activity[89]) and *Legionella pneumophila*,[92–95] (with an unusually high requirement for cysteine). Note that even the supposedly rich LB medium,[96] (Lysogeny Broth, often erroneously called Luria-Bertani medium, see http://schaechter.asmblog.org/schaechter/2009/11/the-limitations-of-lb-medium.html) is not in fact a particularly rich medium[97–99]. An especially nice example[100,101] is provided by *Tropheryma whippeli*, the causative organism of Whipple’s disease[102,103]. It resisted attempts (over many decades) to bring it into axenic culture until systematic genome sequencing[104,105] showed its requirements for a variety of common amino acids that it was unable to synthesise itself, the provision of which permitted its growth. The MetaGrowth database[106] is now available for similar purposes. Another good example is *Coxiella burnetii*, the causative agent of Q fever, for which a genome-derived growth medium (‘acidified citrate cysteine medium’) permitting axenic culture has now been developed[107,108]. Other examples are given by Stewart[109] and by Singh and colleagues[110], and include marine bacteria of the highly common SAR11 clade[111,112,113]. Of course these kinds of phenomena are not absolute; much evidence indicates that host stress hormones may act as growth or virulence factors for a variety of Gram-negative organisms, representing a kind of ‘microbial endocrinology’ (e.g. 112–114).

Not-yet-cultured bacteria may even be killed by our isolation media

Organisms in nature are often living in low-nutrient conditions[115–119]. It is thus reasonable (and unsurprising) that the isolation of microbes from starved, oligotrophic environments benefits from the use of

| Organism          | Comments                                             | Selected References |
|-------------------|------------------------------------------------------|---------------------|
| Bartonella spp.   | Persists inside erythrocytes                         | 330–333             |
| Brucella spp.     | Environmental and intracellular persistence and immune evasion | 334–337             |
| Listeria monocytogenes | Well-established low-GC Gram-positive intracellular saprophyte and non-sporulating persister | 338,339             |
| Mycobacterium tuberculosis | The ‘classical’ dormant bacterium, a high-GC Gram-positive; probably one third of humans carry it in a dormant state | 340–348             |
| Salmonella typhimurium | Gram-negative; non-replicating forms common in macrophages and elsewhere | 349–352             |
| Staphylococcus aureus | Low-GC Gram-positive; can escape antibiotics by hiding inside various phagocytes | 353–356             |

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Table 1. Some bacterial infections for which an intracellular, reversibly non-replicating, persistent or dormant state is well established as part of the cells’ lifestyle. Examples are given for both low- and high-GC Gram positives, as well as a number of Gram-negative organisms.
low-nutrient conditions; some manifest this ‘starvation’ through their size, as ‘ultramicrobacteria’ (see e.g. 123–129). In a similar vein, taking cells from low-nutrient natural environments directly onto, say, a highly aerobic agar plate may produce stresses that effectively kill them, so that afterwards they would not even grow on the kinds of media (as in the previous section) that would support their growth. Thus, Tanaka and colleagues showed interactions between phosphate and agar when autoclaved together that led to the production of compounds inimical to bacterial growth. Gellain may be a better solidifying agent here. However, we recognise that it may be hard to discriminate cells that we kill in the act of trying to isolate and grow them from ‘already dead’ bacteria.

**Not-yet-cultured bacteria may simply be dead and thus incapable of resuscitation**

While this possibility certainly exists, and is included for completeness, it is actually the least likely for a number of conceptual and empirical reasons. The first is that if an organism is present in a particular environment it must have been able to grow and divide in it at some point in the more or less recent past, even if the result of such growth was its utilisation of a finite amount of necessary nutrients or growth factors whose exhaustion caused replication to cease. Interestingly, in soil it seems that sequestration, rather than complete exhaustion, of nutrients is the more significant phenomenon. Secondly, it is highly unlikely that evolution could select for unidirectional growth. Thirdly, environmental organisms can be shown to metabolise even when they cannot be shown to divide (e.g. in the ‘Direct Viable Count’ method and in any number of other tests that detect metabolic activity). And finally, as we shall see in the next section, careful methods of resuscitation/cultivation do indeed allow a very significant fraction of organisms that can be isolated from a variety of environments (e.g. the gut) to be resuscitated and to grow very effectively.

**Not-yet-cultured bacteria are mainly dormant and thus resuscitable**

As indicated in the introduction, it is now well established that even laboratory cultures, that from a macroscopic point of view are growing exponentially, contain subpopulations of non-growing cells. These cells are dormant by definition, because they may later be resuscitated and grow. It is easy to ascribe an evolutionary advantage of this culture differentiation from the perspective of the benefits of having a sub-population that by not growing is more resistant to environmental stresses (e.g. 140–142). Indeed, this general kind of phenotypic differentiation strategy, in which the variance in reproductive rate is traded off at the expense of the mean, has been referred to as bet hedging and is actually adaptive. An important point here is that in many natural environments, asexually reproducing organisms such as bacteria are likely to be (spatially) close to their ancestors and descendants, such that inclusive fitness theory implies that it is entirely reasonable for them to behave altruistically, e.g. by ‘bet hedging’. This is also discussed further below.

It is also reasonable that in isolated (closed) natural environments, nutrients and thus sources of energy must be exhausted at some point, and thus for simple energetic reasons multiplication becomes impossible and a dormant state likely (if later resuscitation proves it to be so). Similarly, it is likely that in the absence of energy, nutrients and/or signalling molecules, and based on more ecological or community considerations (e.g. 157–159), it is necessary to add any or each of them to ‘prime’ bacteria to resuscitate. This has indeed been shown, including for sources of energy, iron-acquiring compounds, siderophores, cell wall muropeptides, and various signalling molecules (especially pheromones) that exist in natural environments. We note too that ‘kick starting’ dormant cells may require the synthesis of transporters necessary for the uptake of all kinds of molecules. Overall, the idea that most bacteria that may be observed in the natural environment are ‘unculturable’ is incorrect.

Finally here, and though this is obvious it is well worth rehearsing, the simple fact that we can store non-growing microbes under desiccated or frozen conditions or as agar ‘stabs’ in culture collections for extended periods means that most microbes are certainly well adapted to entering and leaving dormancy.

**Pheromonal proteins**

A related and unexpected discovery came from analyses of starved laboratory cultures of the actinobacterium *Micrococcus luteus*, in which almost all cells lost culturability. However, they were not dead but dormant, as they could be resuscitated by using a combination of weak nutrient media and a signalling molecule found in spent culture supernatants. The original studies used flow cytometry to discriminate the physiological state of individual cells (see also). By using another ‘single cell’ assay based on dilution to extinction (that avoids artefacts connected with the regrowth of ‘initially viable’ bacteria), we were able to purify the signalling molecule. It turned out to be a protein, named Rpf (for ‘resuscitation-promoting factor’). In *M. luteus* there is only one homologue, and the gene (product) is essential for both resuscitation and multiplication. Rpf contains a highly conserved 70 amino acid ‘Rpf domain’ and is widely (and probably ubiquitously) distributed throughout the actinobacteria, but with examples elsewhere. Most organisms that have a homologue have more than one. Thus *M. tuberculosis* has five homologues. Rpfs can have peptidoglycanase and muralytic activity and known crystal structures are consistent with this. These activities can certainly account for at least some of the resuscitation-promoting properties. As an extracellular protein that may be required for growth, and with a high level of immunogenicity, it is obviously an excellent candidate target for inclusion in appropriate vaccines against pathogenic actinobacteria. It is also more directly of potential utility in stimulating bacterial communication and resuscitation in a variety of cultures in both samples taken from nature and in the laboratory.

**Culturability, dormancy and persistence in laboratory cultures of non-fastidious bacteria**

Having established the frequency of occurrence of microbial dormancy in the natural environment, it is of interest to understand better the mechanisms by which microbes might effect this dormancy and potential resuscitation. Unsurprisingly, microbiologists have turned to *E. coli*, and considerable progress has been made.
The starting position is as in Figure 1 and Figure 6, to the effect that at any given moment in a typical culture a small fraction of the population is dormant. Since clearly the same fraction cannot (or is wise not to) remain in dormancy indefinitely in the presence of suitable nutrients that permit the growth of its siblings, we must invoke at least one mechanism that can cause the bacteria to ‘oscillate’ between growing and dormant states. Many simple gene expression network topologies admit this behaviour,23,24,25 including a simple feedback loop with delay,28,29, and we note that even whole cultures can exhibit oscillations and deterministic chaos.210 While flow cytometric observations (e.g. 192,271) show that even ‘homogeneous’ laboratory cultures show highly heterogeneous distributions in cellular volume (not just between X and 2X) and expression profiles (and see 272), our particular focus will be on ‘binary’ or ‘ bistable’ systems in which individual cells either are or are not operationally culturable.

Experimentally, it is also common to assess the phenotypic ability of subpopulations of cells to tolerate normally inhibitory concentrations of bactericidal drugs,273,274, this being a marker for that fraction of cells that is dormant at the stage in question. Note that the persistence phenotype is not induced by the drugs.248 Changes or transitions in the state of a particular cell in a population between the various phenotypic states is a phenomenon that may be (and is commonly) referred to as ‘phenotypic switching’.

‘Phenotypic switching’ in experimental laboratory cultures

A particularly well-developed example of this ‘bet hedging’ or phenotypic switching between physiologically dormant and growing states may be observed in laboratory cultures of organisms such as *E. coli* demonstrating ‘persistence’.249-251,252 In general, any scheme in which both a first gene product inhibits cellular proliferation and in which this first gene product may be titrated out potently by a second gene product that thereby undoes the inhibition of proliferation, can have the effect of phenotypically switching cells between growth and dormancy. This seems to be precisely what is going on, and such pairs of gene products have been referred to (somewhat misleadingly)253 as toxin-antitoxin (TA) pairs254-258. One such involves the well-known ppGppGpp system of DNA gyrase259,260, and points to the fact that in these circumstances, persisters may be quite metabolically active22,23,292,293, even if transiently incapable of reproduction. Another phenotype switching mechanism, underlying colony phenotype switching, comes from metabolic bifurcations driven by the levels of a particular metabolite.261

Any mechanisms that permit cells to communicate with each other can amplify switching effects by cell synchronisation, and by definition such ‘social’ signals act as pheromones, whose apparent ‘altruism’ can be explained on the basis of kin selection theory.255 There is considerable interest, largely outwith our scope here, in these evolutionary aspects (e.g. 297–304). Such systems are common, but far too broadly relative to the term’s origin,262 referred to as ‘quorum-sensing’. However, they do offer opportunities for limiting bacterial virulence (e.g. 306–313).

Classical clinical microbiology of culturable organisms

Until relatively recently, almost all of clinical microbiology was based on rather classical methods of plate counting263, coupled to assessment of antibiotic sensitivity. Various means of automated blood culture that assess metabolism exist (although they require typically 48–72h to show a ‘positive’).265 Positive tests, often implicitly involving culture (and not just metabolism) within the assay, would be followed by other tests seeking to identify the organisms detected, nowadays typically by nucleic acid sequence-based methods.266,267,268,269 However, these and other tests for the presence of antigens or even antibodies cannot speak to the question of culturability (and of course antigens such as lipopolysaccharide (LPS) are shed by dying cells).

The existence of bacterial DNA in even ‘healthy’ blood has long been known,272, and since naked DNA would be degraded and living cells would soon kill the host, the (seemingly) obvious conclusion that the prokaryotic DNA must reflect dormant cells seems neither to have been drawn nor acted upon.

Some well-established cases of dormancy in clinical microbiology

The idea that (typically intracellular) dormancy is a major component in some infectious diseases (including in the absence of antibiotics that may serve to light up ‘persisters’) is of course well-established, and the main purpose of this brief section is simply to remind readers of this. Such a reminder serves as a prelude to a longer discussion of the very many clinical circumstances where we consider that the role of dormant microbes is not widely appreciated, and where they are not really considered to involve a communicable or microbial component at all. Thus Table 1 shows a few organisms (and references) for which we consider that most readers would regard the idea of and evidence for dormancy as more or less uncontroversial. We do not include disease-causing infectious agents where they are better known for their ability to persist in the natural environment. Organisms such as *Legionella pneumophila* that represent significant public health issues, fall into this category, and *Legionella* and other persisters (in environments such as water system biofilms) are indeed well known (e.g. 324–328), although they too have special adaptations to an intracellular lifestyle (e.g. 329).

Generalised failure of classical techniques to detect dormant bacteria in clinical microbiology

As noted above for environmental microbiology, dormant bacteria can represent as much as 99% of the organisms that may be observed microscopically or by macromolecular sequencing, but classically (and by definition) they are not enumerated by culture-based methods that determine ‘immediate culturability’.275Such culture-based methods are also widely used in clinical microbiology. However, if we were to plate out 100 μL of a culture containing 200 bacteria/mL, of which 99% were dormant at any instant, we would expect (based on a Poisson distribution) to see fewer than 1 propagule or colony-forming unit per sample. We have noted above that it can be determined by sequencing that many of the non-cultured environmental organisms largely differ from those in standard culture collections. Certainly the examples given above in clinical microbiology, such as *Tropheryma whipplei*, were both observed microscopically and were sequenced prior to being brought into axenic culture.

The PCR method is exquisitely sensitive (down to one cell or propagule per sample), and we note that contamination artefacts
from the PCR reagents represent a real issue that must always be checked (e.g. 357–361), albeit this is no less true of blood cultures. We have rehearsed elsewhere five classes of argument that collectively make it implausible that these are all contamination artefacts; probably the most persuasive is simply the sheer number of prokaryotic DNA molecules that can be measured in blood and serum (e.g. 363–365). While some of the most recent nucleic acid sequencing methods (e.g. 366–371) do operate on single molecules, the analysis of prokaryotes usually used a broad-range PCR step to amplify small-subunit rDNA to assess their presence, whether in environmental or clinical samples. Using this, and while these methods alone cannot tell whether they were operationally dormant or dead, a very considerable number of studies have been performed in which ‘culture-negative’ clinical samples showed the presence of prokaryotes (at least as judged by sequence-based methods). This has some profound consequences.

Broad-range PCR methods indicate the widespread presence of prokaryotic DNA in culture-negative clinical samples

While PCR-based methods have long been used to assess the species involved in culture-positive samples, e.g. from blood, our interest here is in samples that are culture-negative that may yet (and indeed likely do) contain dormant cells. Among the first such indications of this was the study by Relman’s group, who showed that the blood of even healthy controls contained significant amounts of prokaryotic DNA. Table 2 lists some studies in which broad-range PCR has been used to amplify and detect prokaryotic rDNA in culture-negative samples.

In environmental microbiology, as mentioned above, there were many early indications (as observed microscopically or flow cytometrically) for the presence of bacteria that did not (or not easily) prove resuscitable or culturable. In a similar vein, many studies have shown microscopically observable organisms in culture-negative but disease-positive samples. This is true both for diseases considered to be due to microbial pathogens and, in fact, for many others normally considered non-communicable.

Microscopically observable and potentially dormant bacteria in clinical disease

Microscopic observations in tissues have been a major part of the discovery process by which certain bacteria were indeed identified as the cause of various diseases. Billings, Price, Dominguez, Mattman, Ewald and Onwuamaegbu and colleagues review

| Aims                          | Culture-negative but PCR-positive | References |
|-------------------------------|-----------------------------------|------------|
| Assessment of endocarditis    | 6 out of 29                        | 390        |
| Development of broad-range PCR| 71 out of 382                      | 386        |
| Development of broad-range PCR; limit of detection 5000 cfu/mL-1 | 10 out of 103 | 391        |
| Improved broad-range PCR method | 20 out of 24                    | 44         |
| Review                        | Many examples                      | 392        |
| Interstitial cystitis         | 14 out of 14                       | 393        |
| Endocarditis                  | 270 (36.5%) of 740                 | 394 (and see 395) |
| Endophthalmitis               | 116 out of 116 (selected)          | 396        |
| General study                 | 18 out of 394 (271 also culture-positive, PCR-positive) | 397 |
| Bacteraemia in intensive care | 48 out of 197 45 out of 94          | 398 399    |
| Sepsis/SIRS                   | 29 out of 59 38 out of 72 culture-positive 14.6% vs 10.3% (no antibiotics) 123 vs 95 | 400 401 402 403 |
| Osteoarticular samples        | 141 out of 1667                    | 404        |
| Review                        | Many examples                      | 405        |
| Various, including antibiotic-treated | 34 out of 240               | 406        |
| Meningitis                    | 26 out of 274 19 out of 21         | 407 408    |
| Orthopaedic samples           | 9% out of 125                      | 378        |
| Thoracic empyema              | 14 out of 22                       | 409        |
| Trauma                        | 28 out of 35                       | 410        |
the extensive and largely forgotten early literature. Domingue and Schlegel also mentioned that they could recover culturable bacteria, probably mainly from L forms (see 50,416,420), from lysates of normal and diseased blood. It was to be assumed that these cells were not replicating at significant rates in the blood itself. However, we can find no evidence that this was ever followed up. Our own work, summarised in 50, showed that both bacillary and coccoid cells could be found attached to and within the erythrocytes of patients with Parkinson’s disease and Alzheimer’s disease, at rather greater concentrations than in samples taken from nominally healthy controls.

In a similar way, our preliminary data show that bacteria are visible in plasma, as well as in whole blood smears in various inflammatory conditions. Here we show bacteria in platelet-rich plasma (PRP) taken from a patient with systemic lupus erythematosus and smeared onto a glass cover slip (Figure 7A and Figure 7B). We also show the same from patients with hereditary hemochromatosis (Figure 7C) and type 2 diabetes (Figure 7D). We also noted microbiota associated with erythrocytes in thromboembolic ischemic stroke (Figure 8A and Figure 8B). (Our microscopy methods are as published previously (e.g. 422–431), but fuller publications will appear elsewhere.) The ultramicroscopic evidence that these are indeed small bacteria and not say, cellular debris or microparticles (see 432) is presently mainly morphological, though we note the considerable evidence for the presence of bacterial DNA in blood (see previous sections and e.g. 51,323,433).

It is worth rehearsing the very great significance of this. With erythrocytes being present at some $5 \times 10^9 \text{mL}^{-1}$ in human blood, even if only one erythrocyte in a thousand harboured just a single dormant bacterium (that would be hard to detect microscopically, but see 433–437), the dormant bacterial load would still be $5,10^6 \text{mL}^{-1}$. This is both far from negligible, and serves to exclude the (always potentially worrisome) claim that ‘it is all contaminants’.

Figure 7. A and B) Platelet rich plasma (PRP) from a patient with systemic lupus erythematosus (SLE). A) Platelet with bacteria visible in the surrounding smear (pink arrows); B) areas in smear with bacteria (pink arrows); C) Erythrocyte with associated bacteria from patient with confirmed hereditary hemochromatosis; D) Erythrocytes with bacteria from patients with diagnosed type II diabetes. A–C Scale bar: 1 μm and D 400 nm.

Figure 8. Bacteria in whole blood from a patient with thromboembolic ischemic stroke A) Microbiota in whole blood; scale bar: 200 nm. B) Erythrocyte with bacteria; scale bar: 1 μm.
A culturable blood microbiome

A recent and highly significant paper by Damgaard and colleagues bears discussion. These workers note that while bacterial growth can normally be elicited during sterility testing in vitro from fewer than 1 in a 1000 blood units, transfusion-transmitted infections occur with a very much higher frequency (more like 10–12%).

Evidence for a microbial component in a very large variety of ‘non-communicable’ diseases

We have surveyed the literature for evidence in which a microbial component has been observed to be an accompaniment of, and probably a major contributory factor to, a variety of (typically inflammatory) diseases that are normally considered ‘non-communicable’. Rarely has the physiological state of these microbes been considered, but since it would be obvious if they were growing, it is most likely that they are indeed dormant. Table 3 summarises these highly extensive associations. While some are just associations, and we could have extended this table considerably, some studies (e.g. 450) contain very detailed aetiological arguments that leave little room for doubt. Overall, the sheer size of the Table does strongly indicate the commonality of many of the microbially based mechanisms underpinning or accompanying various autoimmune and inflammatory diseases. In conditions such as atherosclerosis, transient ischemic attacks (TIAs), and stroke, it is very easy to conceive how resuscitating bacteria might serve to block the flow of blood, for instance. At all events, our main point here is that the evidence for a microbial contribution to many diseases supposedly lacking a microbial component is both multi-factorial and very considerable. Indeed, the purpose of a synthetic review such as this is to provide such pointers for more detailed studies in individual cases. Our specific interest is with the chief mechanisms by which these supposedly dormant bacteria might resuscitate and act as triggers of disease.

Relation between iron dysregulation, sepsis and other comorbidities

Many of the diseases in Table 3 are precisely those inflammatory diseases that we have listed before as coupled to iron dysregulation. A consequence of our analysis is that iron dysregulation and sepsis (as judged either by genuine infection by culturable bacteria or their inflammatory products such as LPS) should be associated causally with these various other diseases.

This leads to a variety of predictions and postdictions that we rehearse. A purposely simple (and simplistic) indication of a plausible chain of events (for which each step is underpinned by substantial evidence) is given in Figure 9, both in general terms (for unspecified diseases) and for a couple of steps to type 2 diabetes. Figure 9 aims specifically to highlight the relationship between the ability of available iron to stimulate bacterial growth and the potential disease sequelae thereof.

Iron and sepsis

First of all, it is well established that free iron may be raised in sepsis and related conditions as may serum ferritin (that has mainly dumped its iron). We have here argued that this is likely to be a significant contributor to the relationship between overt or cryptic infection and the many iron-related inflammatory diseases discussed here and elsewhere. Note that patients suffering from iron overload diseases such as hereditary haemochromatosis are especially susceptible to infection (see e.g. 728–730 and Table 3). Certainly the idea that iron-related metabolism and siderophores are virulence factors (e.g. 731–743) is established unequivocally. In many diseases (e.g. lupus, type 1 diabetes) it is considered that patients with the disease are more prone to sepsis, but we suggest here that (as with stroke) it may more likely be the converse that is true: patients suffering from latent infections are in fact more prone to acquiring, having, or exacerbating the state of these other conditions, in a vicious cycle (see Figure 9).

Role of iron chelation in preventing sepsis

This was discussed at considerable length previously, and that discussion is not repeated here (though a few more recent and pertinent references include 736–789). However, while (shockingly, given the evidence) it does not even appear in the guidelines, there is considerable evidence that iron chelation slows, inhibits or overcomes sepsis. On this basis, iron chelation may be a suitable alternative to antibiotics in preventing multiple inflammatory diseases (such chelation may be nutritional rather than pharmacological in nature, e.g. 167). However, it is clear that we also need to learn to kill ‘dormant’ bacteria, and this usually requires that they are growing.

Utility of antibiotics in treating non-communicable diseases

It is well established that the re-use of protein motifs in natural (and directed) evolution means that most drugs, especially the more lipophilic ones, are promiscuous in the sense that they bind to multiple targets (on average six known ones for marketed drugs). This said (and while we are very far from wishing to encourage the unnecessary use of antibiotics), the prediction here is that appropriate antibiotics will prove to have clinical benefit in diseases commonly seen as non-communicable. This is certainly known to be the case for a number of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and psoriasis. Vaccination may prove equally effective.
We purposely largely confine ourselves to bacteria here, but include the occasional parasite, fungus, mycoplasma and virus. While obesity is usually seen as a cause of other diseases, rather than a disease itself, we note the influence of endotoxaemia on obesity\textsuperscript{451–456}. We note too the extensive evidence for the role of LPS in inflammation\textsuperscript{457–459}, and the experimental models (e.g. for Parkinson’s\textsuperscript{460}) where it can induce disease directly. We do not much discuss diseases such as Crohn’s disease where the extensive uncertainty over the extent of involvement of mycobacteria (e.g.\textsuperscript{461–463}) needs no extra rehearsal (albeit it serves to illustrate the difficulties of identifying the role of hard-to-cultivate bacteria in chronic diseases). Further, while similar phenomena may be observed in a variety of cancers (e.g.\textsuperscript{464–469}), for reasons of space we have determined that this must be the subject of a separate work.

### Table 3. Evidence for infectious agents in non-communicable diseases

| Disease                           | Effect of bacterial involvement                                                                                     | Class of bacteria                                      | Nature of the evidence                                                                                     | Selected References |
|-----------------------------------|---------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|---------------------|
| **AUTOIMMUNE DISEASES**           |                                                                                                                     |                                                         |                                                                                                          |                     |
| Ankylosing spondylitis            | Blood brain barrier permeability and oligodendrocyte cell death in the absence of an adaptive immune filtrate correlate with the mechanistic action of Epsilon toxin (ETX). | *Clostridium perfringens* type B, an epsilon toxin-secreting bacillus | Immunoactivity to ETX, fecal culture and PCR analysis, lysogenic bacteriophage footprint analysis (to exclude the possibility of laboratory contamination), sequencing of the patient-derived ETX gene | 474                 |
| Multiple sclerosis                |                                                                                                                     | *Chlamydia (Chlamyphilia) pneumoniae*                   | PCR, Serology                                                                                             | 475–481             |
| Rheumatoid arthritis (RA)/osteoarthritis/ reactive arthritis | Mostly antigens against these infections                                                                            | *Porphyromonas gingivalis*                              | Anaerobic cultures (from subgingival samples), PCR, ELISA                                               | 482–486             |
|                                  |                                                                                                                     | *Proteus mirabilis, Escherichia coli*                    | ELISA and other evidence                                                                                   | 450,487–495         |
|                                  |                                                                                                                     | Epstein-Barr virus cytomegalovirus                       | PCR, ELISA, in situ/hybridization, immunohistochemistry                                                   | 496–499             |
|                                  |                                                                                                                     | *Mycoplasma (arthritidis mitogen, hominis and fermentans)* | PCR, Western Blot                                                                                        | 500–502             |
|                                  |                                                                                                                     | *Staphylococcus aureus*                                  | Microbiology reports from patient records                                                               | 503,504             |
|                                  |                                                                                                                     | *Salmonella*                                              |                                                                                                          |                     |
|                                  |                                                                                                                     | *Shigella*                                                |                                                                                                          |                     |
|                                  |                                                                                                                     | *Yersinia*                                                |                                                                                                          |                     |
|                                  |                                                                                                                     | *Campylobacter*                                           |                                                                                                          |                     |
|                                  |                                                                                                                     | *Clostridium difficile*                                   |                                                                                                          |                     |
|                                  |                                                                                                                     | *Propionibacterium acnes*                                | Culture                                                                                                  | 506                 |
|                                  |                                                                                                                     | *Chlamydia trachomatis*                                  | Tissue culture inoculation Role of antibiotics                                                           | 507 508             |
| Systemic Lupus Erythematosus      | Unusual case of inflammatory monoarthritis and subsequent diagnosis of RA                                            | *Streptococcus pneumonia, Haemophilus influenza, Mycobacterium tuberculosis, Listeria monocytogenes, Klebsiella pneumonia, Staphylococcus aureus; Cryptococcus neoformans, Aspergillus fumigatus | Blood & tissue culture, patient records                                                               | 510–514             |
|                                  |                                                                                                                     | *Propionibacterium acnes*                                | Culture                                                                                                  | 506                 |
|                                  |                                                                                                                     | *Chlamydia trachomatis*                                  | Tissue culture inoculation Role of antibiotics                                                           | 507 508             |
|                                  |                                                                                                                     | Cell wall-deficient form                                  | Microscopy                                                                                               | 509                 |
|                                  |                                                                                                                     | *Streptococcus pneumonia, Haemophilus influenza, Mycobacterium tuberculosis, Listeria monocytogenes, Klebsiella pneumonia, Staphylococcus aureus; Cryptococcus neoformans, Aspergillus fumigatus | Blood & tissue culture, patient records                                                               | 510–514             |
|                                  |                                                                                                                     | Hypocomplementaemia and infection with encapsulated bacteria: patients are very susceptible to infections |                                                                                                          |                     |
|                                  |                                                                                                                     | *Propionibacterium acnes*                                | Culture                                                                                                  | 506                 |
|                                  |                                                                                                                     | *Streptococcus pneumonia, Haemophilus influenza, Mycobacterium tuberculosis, Listeria monocytogenes, Klebsiella pneumonia, Staphylococcus aureus; Cryptococcus neoformans, Aspergillus fumigatus | Blood & tissue culture, patient records                                                               | 510–514             |
|                                  |                                                                                                                     | Cell wall-deficient form                                  | Microscopy                                                                                               | 509                 |
|                                  |                                                                                                                     | *Streptococcus pneumonia, Haemophilus influenza, Mycobacterium tuberculosis, Listeria monocytogenes, Klebsiella pneumonia, Staphylococcus aureus; Cryptococcus neoformans, Aspergillus fumigatus | Blood & tissue culture, patient records                                                               | 510–514             |
| Vascularitis                      | Various reviews                                                                                                     | Possibly mainly viral, but bacteria include *Staphylococcus aureus, Treponema pallidum, Rickettsiaceae, Borrelia burgdorferi, M. tuberculosis* |                                                                                                          | 515–521             |
| Disease                           | Effect of bacterial involvement | Class of bacteria                  | Nature of the evidence | Selected References |
|----------------------------------|---------------------------------|------------------------------------|------------------------|---------------------|
| CARDIOVASCULAR DISEASES          |                                 |                                    |                        |                     |
| Atherosclerosis                  |                                 | Aggregatibacter actinomycetemcomitans | Antibiotics, Antigens, PCR | 524                 |
|                                  |                                 | Chlamydia (Chlamydophila) pneumoniae |                        | 525–529             |
|                                  |                                 | Helicobacter cinaedi               |                        | 530                 |
|                                  |                                 | Helicobacter pylori                |                        | 527                 |
|                                  |                                 | Porphyromonas gingivalis           | PCR                    | 531–536             |
|                                  |                                 | Prevotella intermedia              | PCR                    | 532                 |
|                                  |                                 | Streptococcus pneumoniae           | Inoculated animals     | 537                 |
|                                  |                                 | Toxoplasma gondii                  |                        | 538                 |
|                                  |                                 | Treponema denticola                | PCR                    | 532                 |
| Endocarditis                     | Many cell-wall-deficient forms  |                                     | Microscopy, PCR        | 539, See Table 2    |
|                                  |                                 |                                     | Benefit of antibiotic prophylaxis | 540                 |
| Hereditary haemochromatosis      |                                 | Chryseomonas, Veillonella, Streptococcus | qPCR                   | 541                 |
|                                  |                                 | Gemella haemolysans                | Blood culture (Gram stain, catalase activity and biochemical characteristics) | 542                 |
|                                  |                                 | Listeria monocytogenes             |                        | 543,544             |
|                                  |                                 | Plesiomonas shigelloides           | Blood culture; API20E system | 545               |
|                                  |                                 | Vibrio vulnificus                  |                        | 546,547             |
|                                  |                                 | Vibrio cholerae                    | Blood culture; PASCO and API20E | 548                |
|                                  |                                 | Yersinia enterocolitica            | Microbial cultures, serotype O:3, serotype 9 | 549–552 |
|                                  |                                 | Yersinia pseudotuberculosis        | Mobility test and API  | 553,554             |
| Hypertension                     | Strong positive association between periodontal infection and prevalent hypertension | Periodontal infection with A. actinomycetemcomitans, P. gingivalis, T. forsythia, and T. denticola | DNA-DNA hybridization | 555,556             |
| Myocardial infarction            | Association between dental chronic inflammatory diseases and the occurrence of acute myocardial infarction was studied | Chronic dental infection correlated positively with MI |                        | 557–559             |
|                                  |                                 | Chlamydia pneumoniae, Helicobacter pylori | ELISA to IgG; anti-infectives | 560,561 |
|                                  |                                 | Enterobacteria & influenza-like illness | Immunohistochemistry | 562                 |
|                                  |                                 | Influenza was associated with an increase in MI-associated deaths | Poissonian regression models to study the relationship between influenza and MI | 563                 |
|                                  |                                 | Streptococcus pneumoniae           | Immunofluorescence imaging | 564                 |
| Disease                                      | Effect of bacterial involvement                                                                 | Class of bacteria                                                                 | Nature of the evidence                                                                 | Selected References |
|---------------------------------------------|------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|---------------------|
| Stroke                                      | 84 different species detected in 77 patients                                                    | Community-acquired bacteremia                                                      | Population-based cohort study                                                           | 565–574             |
| Observational cross-sectional study         | Bacterial endocarditis (Organisms found included S. pneumoniae, N. meningitides and other)     | 575, 576                                                                          | 577                                                                                     |                     |
| Borrelia burgdorferi                       | ELISA                                                                                          | 579                                                                               |                                                                                        |                     |
| TIA                                         | Brucella spp.                                                                                   | Brucella agglutination and Coombs' tests in blood                                  |                                                                                        | 580                 |
| Chlamydia pneumoniae                        | Serology                                                                                        | 581–583                                                                           |                                                                                        |                     |
| Haemophilus influenzae                      | Multivariate time series analysis to assess an association between infections and stroke using the established ‘3h-algorithm’ | 584                                                                               |                                                                                        |                     |
| Mycobacterium tuberculosis                 | Cox proportional hazard regressions                                                             | 585                                                                               |                                                                                        |                     |
| Neisseria meningitidis                      | Latex agglutination test and counterimmunoelectrophoresis                                      | 589                                                                               |                                                                                        |                     |
| Staphylococcus aureus                       | Prospective observational cohort study; retrospective review;                                  | 590, 591                                                                          |                                                                                        |                     |
| Streptococcus bovis                         | Blood culture                                                                                    | 592                                                                               |                                                                                        |                     |
| Streptococcus mutans                        | PCR                                                                                             | 593                                                                               |                                                                                        |                     |
| Streptococcus pneumonia                     | Cox proportional hazard model                                                                   | 594                                                                               |                                                                                        |                     |
| Streptococcus viridans                      | Blood culture                                                                                    | 595                                                                               |                                                                                        |                     |
| Neurosyphilis also present                  | Treponema pallidum                                                                              | Serology and Treponema pallidum haem agglutination test; rapid plasma reagin test, and fluorescent treponemal antibody-absorption test | 596, 597                                                                              |                     |
| Vascular disease (aneurysmal and lesions and atherosclerotic plaques) | Numerous ‘uncultivable’ bacterial species found in atheromas                                    | 598                                                                               |                                                                                        |                     |

**DERMATOLOGICAL DISEASES**

| Disease | Class of bacteria                                                                                                                                                                                                 | Nature of the evidence                                                                 | Selected References |
|---------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------|
| Psoriasis | Streptococcus haemolyticus group A, Staphylococcus aureus, Haemophilus influenzae, Klebsiella oxytoca, Moraxella catarrhalis, Escherichia coli          | Culture from nasal/pharyngeal swab                                                  | 599                 |
| Psoriasis | Escherichia coli                                                                                                                                                                                                  | 600                                                                               |                     |
| Psoriasis | Streptococcus pyogenes, Staphylococcus aureus                                                                                                                                                                      | 601–603                                                                            |                     |

**ENDOCRINE DISEASES**

| Disease | Class of bacteria                                                                 | Nature of the evidence                                                                 | Selected References |
|---------|----------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------|
| Diabetes | Pseudomonads, Stenotrophomonas maltophilia and Ps. aeruginosa                    | PCR and antibodies                                                                    | 604, 605            |

**Blood**

| Disease | Class of bacteria                                                                 | Nature of the evidence                                                                 | Selected References |
|---------|----------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|---------------------|
| Diabetes | Pseudomonads, Stenotrophomonas maltophilia and Ps. aeruginosa                    | PCR and antibodies                                                                    | 604, 605            |
| Disease | Effect of bacterial involvement | Class of bacteria | Nature of the evidence | Selected References |
|---------|---------------------------------|-------------------|------------------------|---------------------|
| Type 1  | Urinary tract infection         | *E. coli*, *Candida albicans*, enterovirus | Urine and blood culture | 607-609 |
|         |                                 | Various proteobacteria | PCR                    | 610 |
|         |                                 | Decreased bacteroidetes |                       | 611 |
| Type 2  | Systemic antibiotics improved diabetes control | Various proteobacteria | Measured as a reduction in glycated hemoglobin or reduction in insulin requirements | 612 |
|         |                                 | Many Gram-positives | qPCR | 613 |

**NEUROLOGICAL DISORDERS**

| Disease                  | Effect of bacterial involvement | Class of bacteria | Nature of the evidence | Selected References |
|--------------------------|---------------------------------|-------------------|------------------------|---------------------|
| Alzheimer’s Disease      |                                 | Porphyromonas gingivalis | Immunolabeling and immunoblotting of brain tissue for the presence of LPS from *P. gingivalis* | 619 |
|                         |                                 | Chlamydia pneumoniae | Immunohistochemistry, Statistical correlation of a meta-analysis | 620–633 |
|                         |                                 | Spirochetal bacteria |                        |                     |
|                         |                                 | Helicobacter pylori | Histology, direct experiment | 634–636 |
|                         |                                 | Actinomyces naeslundii | Antibodies | 637 |
| Amyotrophic Lateral Sclerosis |                               | Mycoplasma infections (M. fermentas, M. genitalium, M. penetrans, M. fermentans, M. hominis, M. pneumoniae), Chlamydia pneumoniae, Borrelia burgdorferi | PCR, serology, microscopic observation | 416,638–640 |
| Autism spectrum disorders | Mycoplasmal infections (M. fermentas, M. genitalium, M. penetrans, M. fermentans, M. hominis, M. pneumonia) |                       | PCR | 641 |
|                         | Chlamydia pneumoniae (co-infection with mycoplasma and human herpes virus-6), or wall-less bacteria |                       | PCR | 642,643 |
|                         | Maternal viral infection in first trimester and maternal bacterial infection in second trimester were found to be associated with ASD |                       | Cox proportional hazards regression | 644 |
| Chronic depression       | Numerous Gram-negatives from gut, e.g. *Hafnia alvei*, *Pseudomonas aeruginosa*, *Morganella morganii*, *Pseudomonas putida*, *Citrobacter koseri*, *Klebsiella pneumoniae* |                       |                       | 645 |
| Parkinson’s Disease      | *Helicobacter pylori* | ¹³C urea breath test, odd ratios for the association between treatment for HP and risk of PD using logistic regression | 646–649 |
|                         | Toxoplasma gondii | Serology, ELISA | 650 |
|                         | *Helicobacter suis* | DNA evidence | 651 |
| Schizophrenia            | A correlation between contact with house cats in early life and the development of schizophrenia exist | Toxoplasma gondii and Herpes simplex virus type 2 | Prospective association study | 652–656 |
|                         | Prenatal exposure to bacterial infection in the first trimester increased risk of schizophrenia in the offspring |                       |                       | 657 |
| Disease | Effect of bacterial involvement | Class of bacteria | Nature of the evidence | Selected References |
|---------|---------------------------------|-------------------|------------------------|---------------------|
| Asthma  | Review                          |                   |                        | 660                 |
|         |                                 | *Branhamella catarrhalis,* *Haemophilus influenzae,* *Streptococcus pneumonia* | | 661 |
|         | Increased mast cell numbers in airways | Atypical bacteria *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* | | 662 |
|         | A significant association exists between bacterial infections and acute wheezy episodes in young children, independent of viral infection | *Streptococcus pneumoniae,* *Haemophilus influenzae,* *Moraxella catarrhalis* | | 663,664 |
|         | Lower airway infection          | *Haemophilus influenzae,* *Streptococcus pneumoniae* | | 665 |
|         |                                  |                   |                        | 666–668             |
| Chronic Obstructive Pulmonary Disease (COPD) | Haemophilus influenzae, *Streptococcus pneumoniae,* *Moraxella catarrhalis,* *Staphylococcus aureus,* *Pseudomonas aeruginosa,* *Enterobacter* spp. | | |
| OTHER INFLAMMATORY CONDITIONS | Preeclampsia | Acute atherosis | *Tannerella forsythensis,* *Porphyromonas gingivalis,* *Actinobacillus actinomycetemcomitans,* *Prevotella intermedia,* *Fusobacterium nucleatum* *Treponema denticola* | PCR | 669 |
|         | Significantly lowered risk following antibiotic treatment | *Chlamydia pneumonia* | ELISA and qPCR of genomic DNA | 45,671–674 |
|         | Significant association with periodontal disease and UTI | *Chlamydia trachomatis* | Serology | 677 |
|         |                                   | *Helicobacter pylori* | Serology | 678,679 |
| Chronic fatigue syndrome | LPS a culprit | *Hafnia alvei,* *Pseudomonas aeruginosa,* *Morganella morganii,* *Proteus mirabilis,* *Pseudomonas putida,* *Citrobacter koseri,* *Klebsiella pneumoniae* | Serology | 680–683 |
|         | Mycoplasmal infections (*M. pneumonia,* *M. fermentans,* *M. honinis,* *M. penetrans*), *Chlamydia pneumonia,* *Human herpes virus-6* | | PCR | 684 |
|         | Various enterbacteria and others | *Cell wall deficient bacteria* | | 685 |
| Vitamin D receptor (VDR) dysregulation | Evade immune destruction by invading nucleated cells where they persist in the cytoplasm. From here they down-regulated the VDR | *Multiple organisms, including mycobacteria, Borrelia.* | | 685 |
| Disease                          | Effect of bacterial involvement | Class of bacteria                        | Nature of the evidence                  | Selected References |
|--------------------------------|---------------------------------|------------------------------------------|-----------------------------------------|---------------------|
| Antiphospholipid syndrome      |                                 | *S. aureus* cross-reacting antibodies    |                                         | 687                 |
|                                |                                 | Various viral and bacterial triggers     |                                         | 688–690             |
|                                |                                 | *Toxoplasma*                             |                                         | 691                 |
| Sudden Infant Death Syndrome   | Review                          | *S. aureus* most common                  | Seasonality, bacteriology               | 692–694             |
|                                |                                 |                                         | Inflammatory markers                    | 695,696             |
|                                |                                 |                                         | Toxaemic shock indicators in serum       | 697,698             |
| Other Inflammatory Bowel Diseases | Many examples of dysbiosis of gut microbiota |                                         |                                         | 699–707             |
| Sarcoidosis                    |                                 | *P. acnes* antibodies and antigens       |                                         | 708–710             |
| Migraine                       |                                 | *H. pylori*                              |                                         | 711,712             |

**Figure 9.** An elementary systems biology model of how iron dysregulation can stimulate dormant bacterial growth that can in turn lead to antigen production (e.g. of LPS) that can then trigger inflammation leading to cell death and to a variety of diseases. While it is recognised that this simple diagram is very far from capturing the richness of these phenomena, there is abundant evidence for each of these steps, but sample references for the numbered interactions are (1) 828–831 (especially including the release of free iron from ferritin), (2) 832–834, (3) 268, 453, 455, 835–842, (4) 456, 713, 843–846, (5) 717, 116, 847, (6) 848, 849–855, (7) 856–859, (8) 860–861.
Concluding comments: on the systems properties of dormancy and virulence

We have here brought together some of the relevant elements of environmental, laboratory, and clinical microbiology. We have argued that while their languages may differ (e.g. ‘dormancy’ vs ‘persistence’), very similar phenomena have been observed in each of these spheres (plausibly underlining a commonality of mechanism). Certainly the ability to culture microbes, and not merely to observe them (whether microscopically or via their macromolecular sequences or chemical products), remains an important goal of basic microbiology. This is likely to have significant payoffs in bioprospecting (e.g. 163,783). However, we are sure that improved methods of detecting and identifying these dormant bacteria, whether this is done via chemical imaging, through macromolecular amplification and/or sequencing, or through resuscitation and culturing, will have a major role to play in increasing the awareness of their existence and importance.

Clearly dormant, persistent bacteria are likely to be relatively avirulent when they are in such dormant states, and able to bypass the attentions of the innate immune system (albeit the production of superantigens by at least some microorganisms764,795 may be what triggers autoimmune diseases). This ‘stealth’ antigenicity is probably why they have been largely unnoticed by us too786, and their routine estimation via molecular methods37 seems highly desirable. Indeed, virulence varies widely between individual strains (e.g. 788,789). Modern molecular microbiology places much emphasis on the virulence of the pathogen, with concepts such as ‘pathogenicity islands’790–795, ‘virulence genes’796,797, and the ‘virulome’798 being commonplace. However, if dormant microbes resuscitate (or are to be resuscitated) in vivo we shall need to pay much more attention to the environmental triggers that cause this to happen than we probably have so far799 (given that the pathogen genotype is fixed800,801). In other words, virulence, like dormancy, is a phenotypic as well as a genotypic property. We remain largely ignorant of the means by which an optimal immune system has been selected for (or against) by longer-term evolution on the basis of microbial exposures in early life, and how this may have changed with more recent changes in human lifestyle802–805. Nor do we understand how such microbes might enter and exit blood cells (and see 50,330,806–810) (albeit the known endosymbiotic origins811,812 of eukaryotic organelles must have presaged such mechanisms). Similarly, we do not yet know what may cause these dormant microbes to resuscitate (and/or to exit their intracellular niches). However, the potential for iron-associated replication and (e.g.) LPS production and shedding does provide a very straightforward explanation for the continuing low- or medium-grade inflammation characteristic of the many inflammatory diseases we have considered here and elsewhere167,168,429,432,717 (Figure 9).

One approach to Science is based on varying independently something considered a cause and observing its predicted effects (e.g. 178,813,814). To assess causality in microbiology it is usual (e.g. 792,815–817) to invoke what are (variously818) referred to the Henle-Koch or Koch’s postulates. These are based on the nature and presence, but not the physiological state, of an agent that might be believed to ‘cause’ (or at least contribute to) an infectious disease. Consequently, dormancy poses something of a challenge to the full completion of the required tests. Indeed a number of authors417,792,816–821 have recognised that these tests may need revision in the light of the ability to identify disease-causing microbes by sequence alone. We suspect that a key element here will be the ability to resuscitate dormant organisms in vivo and to see the effects of that on clinical disease.

As phrased by Silvers822, “Several of our contributors showed how discoveries and insights could emerge with what seemed great promise, and yet be pushed aside, discarded, and forgotten – only to re-emerge once again, sometimes many years later, and become, in their new formulation, accepted as important”. In this sense, and as presaged in the opening quotation, it seems that ideas, as well as bacteria, can remain dormant for extended periods823,824.

Author contributions
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Competing interests
No competing interests were disclosed.

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Vanya Gant

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I review Kell et al’s review relating to individuality, phenotypic differentiation, dormancy and “persistence” as a clinical microbiologist, infectious diseases doctor, with an interest in developing and assessing the impact of rapid sequence-based molecular blood and lung diagnostics in the critically ill.

This review reminded me of Mussorsky's Pictures at an Exhibition, a collection of hastily composed pieces whose theme was to take an interested individual through an art gallery, and to tarry awhile in front of 10 Tableaux, interspersed with musical elements referring to the “Promenade” through the gallery.

And so it is with Kell et al’s review. After an introductory Promenade relating to matters of bacterial dormancy and its relationship with just about any other conceivable physical state between life and death, exhaustively referenced together with the thought provoking Postgate-ian concept of the difficulties inherent in differentiating bacterial life from death if you only have an instant in time to measure it – we are then presented with several pictures, garlanded for us in extensively referenced detail by the authors. Were mindmaps not enough to capture the reader’s curiosity as to this magnum opus of a kind, we are invited to walk through Kell et al’s gallery of mental pictures depicting scenes of the Yet to be Cultured, Those bacteria that aren’t culturable yet but are certainly not dead, the biological importance of bacterial pheromones, the evils of Iron - thence to the Clinical Microbiology Room of Pictures with a liberal helping of systems biology throughout.

I am a proponent of, and believer in, the present and future potential of Nucleic Acid Technology (NAT) for pathogen detection in Clinical microbiology and I use such techniques on a daily basis. When appropriately deployed, it allows me to find those “unculturable” pathogens as drivers for individual clinical cases of infection. Perhaps strangely, this is a relatively new paradigm for most practising clinicians, and one which likely will generate fundamental discoveries highly relevant to human disease, and for all we know as equally important as Helicobacter. That such sequences should be found in blood is hardly surprising, given that human beings have between 10 and 100
times more bacterial cells than their own, living (or persisting, or dormant) on and in them. This groups’ demonstration of bacteria adhering to red cells (also in red cells) is certainly very intriguing, and such suggested “atopobiosis” is more expansively dealt with in another publication and prompts far more questions than it answers – in a good way. Another obvious question relates to how these adherent bacteria may remain undetected and intact in the presence of numerous moieties central to both innate and acquired immunity (complement and antibody to name but two) as well as escaping phagocytosis in the liver and spleen. It would certainly be interesting to look at red cells in the grave condition of erythrophagocytosis, a condition whose mechanism is in most cases obscure – it might even be that adherent bacteria “opsonize” the red cells in these cases. This reader, however, does baulk at the very serious work to be done as regards untangling the mechanistic nature of an “association” with several diseases, and certainly at this stage it would be very unwise to suggest it’s anything more than that. Further work of this nature should be approached and undertaken with extreme caution and rigor in view of the myriad possible explanations other than causative ones; the Measles vaccine/autism saga comes to mind here.

It is likely therefore that such technologies will perforce “lift the lid” on what might lie beyond the Culturable, and its relationship to human disease. This is explored in Table 3, which represents a tour de force as concerns the sheer volume of references relating to all that appears to associate human Disease and organisms, mostly bacteria.

Unfortunately, this Table doesn't work for me. Whilst it will serve me as a unique and accessible resource of information in this space, it is anarchic. Correctly described as “Evidence for agents in non-communicable diseases”, it lists, in no particular order, and with no apparently critical eye, references 470 to 712 as relevant to the Table subject stated above. This list's breadth as concerns both organisms and clinical diseases is extraordinary; and the literature quoted in a table described as “effect of bacterial involvement” ranges from unusual cases, to mechanistic assumptions of what LPS might do, to the concept of “dysbiosis” amongst many others. I was left rather dizzy from the mental exercise needed to constantly adjust to the sheer scale and variation of why a particular organism, or something it produces, might either directly causally relate to a particular disease, or perhaps through the individuals’ immune response to it; especially now we know how outbred we are as concerns immune responsiveness.

This review finishes with an impressive and lyrical chiding for Scientists, whereby those who research this field should wake up from their intellectual slumber, as might and indeed do bacteria.

This review is additionally peppered with tantalizing if perhaps sometimes unfounded assumptions, some arguable and some bordering on plain unreasonable. Certainly my eyebrow raising went into overdrive when considering Kell's conviction as concerns a Catholic Grand Unifying Theory based around the Evils of Iron, the subject of a previous equally grand Magnum Opus.

This review has to be one of the most undisciplined I have read in a long time, on occasions associating seemingly disparate observations and conflating “scientifically” determined facts with clinical issues.

Having said this, I should finish by applauding Kell et al’s review as a thumping good read. It’s fast
paced, edgy, a real treasure trove of papers for me to read at leisure, and goes way outside the usual, expected and conventional boundaries of style of prose and rigor we “normally expect” of such scientific publications. And (warts and all, and there are many) it left this reader thinking that there indeed is Life beyond dormancy within the review’s style itself, beyond the doubtless very important but less imaginative run-of-the-mill, tightly written yet dreary “Scientific Publication”. It is almost as if this review in all its unconventionality were particularly well aligned to the current state of the Art for the Uncultured in Clinical Medicine (bacteria, not Doctors) and its potential to release significant Paradigm shifts. No doubt this reviews’ readers are made up of those who have the capacity to appreciate Kells’ latest brand of emergent, imaginative systems biology style of thinking underneath what some might consider a publication of inadequate scientific rigor.

**Competing Interests:** No competing interests were disclosed.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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**Author Response 18 Aug 2015**

**Douglas Kell**, The University of Manchester, Manchester, UK

- "I review Kell et al’s review relating to individuality, phenotypic differentiation, dormancy and “persistence” as a clinical microbiologist, infectious diseases doctor, with an interest in developing and assessing the impact of rapid sequence-based molecular blood and lung diagnostics in the critically ill.

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This is a lovely analogy, which we shall let readers enjoy in the open referee’s report; we are probably not capable of recasting the review in Mussorgskian style anyway! In this regard, readers might also enjoy a little known and whimsical piece on bioinformatics that takes just such an approach: Goble C, Wroe C: The Montagues and the Capulets. Comp Func Genomics 2004; 5:623-632.
"I am a proponent of, and believer in, the present and future potential of Nucleic Acid Technology (NAT) for pathogen detection in Clinical microbiology and I use such techniques on a daily basis. When appropriately deployed, it allows me to find those “unculturable” pathogens as drivers for individual clinical cases of infection. Perhaps strangely, this is a relatively new paradigm for most practising clinicians, and one which likely will generate fundamental discoveries highly relevant to human disease, and for all we know as equally important as Helicobacter. That such sequences should be found in blood is hardly surprising, given that human beings have between 10 and 100 times more bacterial cells than their own, living (or persisting, or dormant) on and in them. This groups’ demonstration of bacteria adhering to red cells (also in red cells) is certainly very intriguing, and such suggested “atopobiosis” is more expansively dealt with in another publication and prompts far more questions than it answers – in a good way. Another obvious question relates to how these adherent bacteria may remain undetected and intact in the presence of numerous moiety central to both innate and acquired immunity (complement and antibody to name but two) as well as escaping phagocytosis in the liver and spleen. It would certainly be interesting to look at red cells in the grave condition of erythrophagocytosis, a condition whose mechanism is in most cases obscure –it might even be that adherent bacteria “opsonize” the red cells in these cases. This reader, however, does baulk at the very serious work to be done as regards untangling the mechanistic nature of an “association” with several diseases, and certainly at this stage it would be very unwise to suggest it’s anything more than that. Further work of this nature should be approached and undertaken with extreme caution and rigor in view of the myriad possible explanations other than causative ones; the Measles vaccine/autism saga comes to mind here."

These are excellent points, and we have covered some of them in the forward-looking concluding section. While they might be seen as ‘premature’ (in the sense that it requires acceptance of the basic ‘dormancy’ hypothesis in the first place) they do point to important areas where we would seek a mechanistic understanding of what is going on.

"It is likely therefore that such technologies will perforce “lift the lid” on what might lie beyond the Culturable, and its relationship to human disease. This is explored in Table 3, which represents a tour de force as concerns the sheer volume of references relating to all that appears to associate human Disease and organisms, mostly bacteria.

Unfortunately, this Table doesn't work for me. Whilst it will serve me as a unique and accessible resource of information in this space, it is anarchic. Correctly described as “Evidence for agents in non-communicable diseases”, it lists, in no particular order, and with no apparently critical eye, references 470 to 712 as relevant to the Table subject stated above. This list's breadth as concerns both organisms and clinical diseases is extraordinary; and the literature quoted in a table described as “effect of bacterial involvement” ranges from unusual cases, to mechanistic assumptions of what LPS might do, to the concept of “dysbiosis” amongst many others. I was left rather dizzy from the mental exercise needed to constantly adjust to the sheer scale and variation of why a particular organism, or something it produces, might either directly causally relate to a particular disease, or perhaps through the individuals' immune response to it; especially
now we know how outbred we are as concerns immune responsiveness."

We very much accept the point that the table could be improved with regard to ordering, and we have done so accordingly. However, we think that readers will recognise it for what it is (as does the referee), viz. as a useful resource and/or pointer to a large literature in which specialists in disease X may wish to read at least those papers we suggest as relevant to ‘their’ disease, while others will simply see it as a recognition of the widespread evidence for our more general claims.

- "This review finishes with an impressive and lyrical chiding for Scientists, whereby those who research this field should wake up from their intellectual slumber, as might and indeed do bacteria.

This review is additionally peppered with tantalizing if perhaps sometimes unfounded assumptions, some arguable and some bordering on plain unreasonable. Certainly my eyebrow raising went into overdrive when considering Kell's conviction as concerns a Catholic Grand Unifying Theory based around the Evils of Iron, the subject of a previous equally grand Magnum Opus."

As mentioned in the comments on the review of referee 1, the basis for this is the desire to produce a coherent story (in the sense used by Philosophers of Science), and (as referee 1 also states) it is well known that microbial growth in vivo is normally limited by iron availability. That iron dysregulation is also a hallmark of just those chronic inflammatory diseases that we highlight here is consistent with this view, and indeed serves to provide a simple explanation for this. Of course, as the referee indicates (and referee 1 does too), further demonstrations will benefit from varying iron levels as an independent variable.

- "This review has to be one of the most undisciplined I have read in a long time, on occasions associating seemingly disparate observations and conflating “scientifically” determined facts with clinical issues.

Having said this, I should finish by applauding Kell et al's review as a thumping good read. It's fast paced, edgy, a real treasure trove of papers for me to read at leisure, and goes way outside the usual, expected and conventional boundaries of style of prose and rigor we “normally expect” of such scientific publications. And (warts and all, and there are many) it left this reader thinking that there indeed is Life beyond dormancy within the review's style itself, beyond the doubtless very important but less imaginative run-of-the-mill, tightly written yet dreary “Scientific Publication”. It is almost as if this review in all its unconventionality were particularly well aligned to the current state of the Art for the Uncultured in Clinical Medicine (bacteria, not Doctors) and its potential to release significant Paradigm shifts. No doubt this reviews' readers are made up of those who have the capacity to appreciate Kells' latest brand of emergent, imaginative systems biology style of thinking underneath what some might consider a publication of inadequate scientific rigor."

Many thanks for these last comments; we have nothing further to add here.
Kell, Potgieter and Pretorius present a stimulating and argumentative review ranging from the interrelationships between the culturability of bacteria and their viability and any links these descriptions may have to defined physiological states, through a discussion of environmental bacteria and ultimately focusing on the human-associated microbiota, particularly those found in blood (without associated symptoms of sepsis) and their proposed roles in disease. Two central themes are developed beyond those that have been discussed extensively elsewhere: 1) the proposal that failure to culture bacteria from many samples often reflects dormancy and 2) that such dormant bacteria interact with host iron regulation to contribute to or directly cause a panoply of chronic diseases largely labelled as non-communicable.

At a general level I support the provocative stance taken by the authors. With 861 cited references, at the very least they provide a valuable resource for anyone wishing to consider the potential microbial contribution to diseases traditionally considered free of this aetiological component. Of course Helicobacter infection stands as a monument to the stupidity of dismissing this possibility in the face of carefully assembled evidence. Indeed this reviewer, who many years ago, was presented with a case of duodenal ulcer in his final medical exams, would probably have experienced quite a different career had he claimed a role for infection in causing his patient’s pathology.

In considering the specific points presented I have multiple concerns, the most significant of which I will indulge in outlining below.

Semantics present a central problem in considering bacterial viability and physiology and I broadly support the approach taken here. The authors do try to define their terms but some problems remain. In particular I take issue with the very broad application of term “Persisters” which should be reserved for cells that survive (have the potential to replicate) after exposure to an antimicrobial stress to which kills most cells in an actively growing culture of the organism concerned. Conflation of this term with “Dormancy” implies on the one hand that the persisting cells must have been dormant and on the other that dormancy and persistence represent the same physiological state in bacteria. This difficulty resurfaces later when they define dormancy but
other problems emerge before then.

I was next concerned by the extensive use of the term “Differentiation”. I completely agree that what we used to think of as uniform bacterial populations are probably never so but the degree to which subpopulations may be considered differentiated rather than reflecting a range of adaptive responses or indeed, some degree of injury, is not considered here and again I think this leads to problems in considering their hypotheses under a unitary banner downstream. I consider differentiation to require phenotypic changes that are not directly reversible, as in the case of sporulation, whereas adaptation can involve expression of a single gene that can be reversed by its subsequent repression. I do agree that cell cycle contributes to the range of phenotypes in a pure bacterial culture and that this is not the only reason for their diversity (but was not enlightened by use of the term “modulo” in this regard).

The operational definition of dormancy given deliberately leaves open the possibility of metabolic activity and seems only to require that the cell so defined should not divide; this did not allow me to recognise which operational tests might be applied to enumerate or detect dormant cells. Subsequently the detection of molecular signals indicative of bacterial presence in samples from which they were not isolated in culture is taken as evidence of dormancy. In the first case do we accept any non-dividing cell as dormant and in the second I can (and will) offer multiple alternate explanations other than dormancy. Moreover, returning briefly to the conflation between dormancy and persisters, the recent work of John McKinney and colleagues shows that antibiotic exposed persisting cells are not necessarily non-dividing cells in the mycobacterial system he studied.

Alternative interpretations of the presence of bacterial 16SrDNA sequences in blood when culture fails to detect the organisms from which they derive, include the presence of dead, injured or moribund cells. If they are shown to be repeatedly present then they must either be able to persist in the face of clearance mechanisms or be supplied at a rate equal to their clearance; both seem equally plausible to the dormancy explanation to me. Moreover, why the first three explanations offered for “Not-yet-cultured” should apply to environmental bacteriology but not to clinical samples escapes me.

I am led to the conclusion that the authors have chosen to label evidence for discrepancies between culture and nucleic acid detection of bacteria in blood to give their hypotheses a simple headline. I have no problem with the proposal that human blood and tissues classically considered sterile in the absence of overt symptoms of infection are frequently exposed to bacteria and bacterial products that in many cases contribute to serious chronic disease. However, I consider the burden of available evidence currently provides many potential explanations within the field of microbiomics/metagenomics in contrast to the dormancy hypothesis offered here. Further, I feel this broad application of dormancy to bacterial phenotypes which, even in the case of Rpf dependency, have not been shown to result from a programme of gene expression that could be considered as differentiation, diminishes the value of the term. Indeed there remains no direct proof that dormancy of *Mycobacterium tuberculosis* underpins what we call latent tuberculosis infection and it is not essential to the observed clinical or pathological pattern, notwithstanding the widespread acceptance of this view by most researches, including me.

I am not fundamentally opposed to the ideas presented by Kell and colleagues but I do not think they are assisted by lack of attention to the contradictions I have identified above.
Finally I come to the iron dysregulation hypothesis and its pro-inflammatory consequences. It is beyond my expertise to comment on the plausibility of the inorganic chemistry deployed here or to review the evidence relating to more than a fraction of the conditions listed. The importance of the struggle between pathogens and host for access to iron is beyond question. When I entered the medical field of infectious disease it was fully recognised that depriving bacteria from iron was a potential therapeutic angle and indeed iron chelation was studied. Desferrioxamine, a widely used agent in iron overload, was investigated and found to effectively deliver iron to the pathogen and the approach was set aside. More recently this agent has been identified as a major risk factor in serious fungal infection and guidance specifically recommends its avoidance. Newer agents seem not to suffer from this problem and the approach deserves renewed attention. However, I would not underestimate the ability of pathogens to outwit our pharmaceutical industry in the battle to sequester iron. While there are reasons beyond the host–pathogen tug-of war for iron to consider chelation as a therapeutic option, the potential for adverse effects is significant and I think the suggestion that omission of iron chelation from recent guidance on sepsis management is “shocking” is not justified.

Focussing briefly on the specific diseases cited and their relation to bacterial exposure in one form or another, I find that evidence cited frequently rests on what can be considered “fringe” hypotheses that have little currency in their respective fields. This is not to discourage their continued pursuit but it does weaken the strength of the authors’ argument when investigation of the supporting literature frequently leads to papers that are given little credence in the specialist field. Of course “cave Helicobacter” must remain on the table. But there, an accidental technical breakthrough led to an avalanche of convincing laboratory and clinical data.

In summary Kell, Potgieter and Pretorius have produced an interesting read which bring many important ideas to our attention. I am not convinced of the breadth of conditions to which they argue their ideas are applicable and I await with interest, demonstration of how they may be practically pursued and some selected definitive proofs that iron-driven inflammatory disease is as important as they claim.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
regulation to contribute to or directly cause a panoply of chronic diseases largely labelled as non-communicable.

At a general level I support the provocative stance taken by the authors. With 861 cited references, at the very least they provide a valuable resource for anyone wishing to consider the potential microbial contribution to diseases traditionally considered free of this aetiological component. Of course Helicobacter infection stands as a monument to the stupidity of dismissing this possibility in the face of carefully assembled evidence. Indeed this reviewer, who many years ago, was presented with a case of duodenal ulcer in his final medical exams, would probably have experienced quite a different career had he claimed a role for infection in causing his patient's pathology.

In considering the specific points presented I have multiple concerns, the most significant of which I will indulge in outlining below.

Many thanks for the above; it is perfectly accurate and we have nothing to add here.

○ "Semantics present a central problem in considering bacterial viability and physiology and I broadly support the approach taken here. The authors do try to define their terms but some problems remain. In particular I take issue with the very broad application of term “Persisters” which should be reserved for cells that survive (have the potential to replicate) after exposure to an antimicrobial stress to which kills most cells in an actively growing culture of the organism concerned. Conflation of this term with “Dormancy” implies on the one hand that the persisting cells must have been dormant and on the other that dormancy and persistence represent the same physiological state in bacteria. This difficulty resurfaces later when they define dormancy but other problems emerge before then."

This is entirely fair; we see that we occasionally elided the terms ‘dormancy’ and ‘persistence’ to imply synonymy, when either there is none or at least there is no evidence for it. We think the best solution is to add a little section pointing out the semantic difficulties, repeating the operational nature of the definitions, and specifying that in very few cases do we actually know the true physiological state of individual cells – which is what matters with regard to replicatory potential. This material mainly appears in the section defining dormancy, and its title has been extended to note the semantic issues.

○ "I was next concerned by the extensive use of the term “Differentiation”. I completely agree that what we used to think of as uniform bacterial populations are probably never so but the degree to which subpopulations may be considered differentiated rather than reflecting a range of adaptive responses or indeed, some degree of injury, is not considered here and again I think this leads to problems in considering their hypotheses under a unitary banner downstream. I consider differentiation to require phenotypic changes that are not directly reversible, as in the case of sporulation, whereas adaptation can involve expression of a single gene that can be reversed by its subsequent repression. I do agree that cell cycle contributes to the range of phenotypes in a pure bacterial culture and that this is not the only reason for their diversity (but was not enlightened by use of the term “modulo” in this regard)."
We mainly agree, and suggest what we think is a useful clarification or extension. We note again that “reversibility” is established post hoc, but there are at least two meanings involved. At one level we are discussing a reversibility of states. Let us take a spore and a vegetative cell, which obviously, for sporulating bacteria, can indeed interconvert (“reversibly”). However, another level or meaning implies a mechanistic reversibility, i.e. the path from A to B is simply traversed in the opposite direction when B reverts or interconverts to A. Not only is this not what we mean but (also for thermodynamic reasons) it is certainly not what is done (sporulation and germination in *B. subtilis* are definitely quite separate processes, as indicated by the referee, and one is not at all the reverse of the other). We have added clarificatory comments accordingly. (One might also have added, but we have not in the ms as it would distract, that similar issues apply to the ‘reversibility’ of enzymes and of biochemical pathways (gluconeogenesis is not mechanistically a reversal of glycolysis, even if the “start” and “end” states are the same molecules.)

"The operational definition of dormancy given deliberately leaves open the possibility of metabolic activity and seems only to require that the cell so defined should not divide; this did not allow me to recognise which operational tests might be applied to enumerate or detect dormant cells. Subsequently the detection of molecular signals indicative of bacterial presence in samples from which they were not isolated in culture is taken as evidence of dormancy. In the first case do we accept any non-dividing cell as dormant and in the second I can (and will) offer multiple alternate explanations other than dormancy. Moreover, returning briefly to the conflation between dormancy and persisters, the recent work of John McKinney and colleagues shows that antibiotic exposed persisting cells are not necessarily non-dividing cells in the mycobacterial system he studied."

The hallmark of the dormant macrostate, stated in quotation marks in the second paragraph of the ‘dormancy’ section, is indeed that the cells in question do not immediately grow when attempts to culture them under “suitable” conditions (that normally admit their growth), are often (but not necessarily) of low metabolic activity, but are not operationally dead since they can be resuscitated. On this basis we think that this should allow the referee or anyone else to determine the operational tests. It follows that we do not accept ‘any’ non-diving cell as dormant since only resuscitable cells can – *post hoc* – be considered dormant, and certainly a non-dividing cell it may be irreversibly injured or operationally dead. However, the presence of molecular signals (e.g. 16S) in samples from which nothing (or many fewer colonies or OTUs) may be recovered by culture is certainly an indication of the possibility of resuscitation, and hence dormancy.

The referee is entirely correct that we had missed John McKinney’s recent and very relevant work, and we mention it accordingly.

"Alternative interpretations of the presence of bacterial 16SrDNA sequences in blood when culture fails to detect the organisms from which they derive, include the presence of dead, injured or moribund cells. If they are shown to be repeatedly present then they must either be able to persist in the face of clearance mechanisms or be supplied at a rate equal to their clearance; both seem equally plausible to the dormancy explanation to me."
Moreover, why the first three explanations offered for “Not-yet-cultured” should apply to environmental bacteriology but not to clinical samples escapes me."

The referee is entirely correct with regard to the last sentence, and the whole point (or at least a major theme) of our review is precisely that what is well established in environmental microbiology has had much less impact in clinical microbiology (referee 2 makes this exact point, even more explicitly). We agree that in a steady state such cells must be supplied at a rate equal to that of their clearance, and that the fact that clearance is lower than probably expected implies a significant ability to evade the innate and adaptive immune systems. We also take it that for common organisms (not very slow growers such as certain mycobacteria) the former rates must be much lower than those typically attainable in laboratory cultures, else we would have classical sepsis. We have added a few comments on these issues accordingly, in the section entitled ‘Generalised failure of classical techniques to detect dormant bacteria in clinical microbiology’.

- "I am led to the conclusion that the authors have chosen to label evidence for discrepancies between culture and nucleic acid detection of bacteria in blood to give their hypotheses a simple headline. I have no problem with the proposal that human blood and tissues classically considered sterile in the absence of overt symptoms of infection are frequently exposed to bacteria and bacterial products that in many cases contribute to serious chronic disease. However, I consider the burden of available evidence currently provides many potential explanations within the field of microbiomics/metagenomics in contrast to the dormancy hypothesis offered here. Further, I feel this broad application of dormancy to bacterial phenotypes which, even in the case of Rpf dependency, have not been shown to result from a programme of gene expression that could be considered as differentiation, diminishes the value of the term. Indeed there remains no direct proof that dormancy of Mycobacterium tuberculosis underpins what we call latent tuberculosis infection and it is not essential to the observed clinical or pathological pattern, notwithstanding the widespread acceptance of this view by most researches, including me.

  I am not fundamentally opposed to the ideas presented by Kell and colleagues but I do not think they are assisted by lack of attention to the contradictions I have identified above."

All of the above is entirely fair, and we do not disagree. We hope that the changes we have now made to the ms to weaken the ostensible claims (and misplaced synonymies) now meet the referee's approval. For instance we have stressed that while the presence of suitable molecular sequences (e.g. 16S) implies that it is worth seeking to resuscitate the organisms from which it came, an absence would imply that it is not. A success in resuscitating organisms from a sample that initially appeared sterile would from our operational definition imply that those ones were indeed dormant, and we'd like to think that this had now been clarified.

- "Finally I come to the iron dysregulation hypothesis and its pro-inflammatory consequences. It is beyond my expertise to comment on the plausibility of the inorganic chemistry deployed here or to review the evidence relating to more than a fraction of the conditions listed. The importance of the struggle between pathogens and host for access to
iron is beyond question. When I entered the medical field of infectious disease it was fully recognised that depriving bacteria from iron was a potential therapeutic angle and indeed iron chelation was studied. Desferrioxamine, a widely used agent in iron overload, was investigated and found to effectively deliver iron to the pathogen and the approach was set aside. More recently this agent has been identified as a major risk factor in serious fungal infection and guidance specifically recommends its avoidance. Newer agents seem not to suffer from this problem and the approach deserves renewed attention. However, I would not underestimate the ability of pathogens to outwit our pharmaceutical industry in the battle to sequester iron. While there are reasons beyond the host-pathogen tug-of war for iron to consider chelation as a therapeutic option, the potential for adverse effects is significant and I think the suggestion that omission of iron chelation from recent guidance on sepsis management is “shocking” is not justified.”

The point about desferrioxamine is well made (and we mention it, with citations), but the molecule is of course in fact a natural prokaryotic siderophore, from *Streptomyces pilosus*. We have replaced the term ‘shocking’ with something more suitable.

○ “Focussing briefly on the specific diseases cited and their relation to bacterial exposure in one form or another, I find that evidence cited frequently rests on what can be considered “fringe” hypotheses that have little currency in their respective fields. This is not to discourage their continued pursuit but it does weaken the strength of the authors’ argument when investigation of the supporting literature frequently leads to papers that are given little credence in the specialist field. Of course “cave Helicobacter” must remain on the table. But there, an accidental technical breakthrough led to an avalanche of convincing laboratory and clinical data.”

It is probably a philosophical distraction to rehearse how often in science something outside the mainstream is blocked for many years by ‘vested interests’. However, we may as well mention Peyton Rous, whose discovery of a viral cause of certain cancers was sidelined for decades (he received a Nobel prize when he was 87, 40 years after first being nominated [https://en.wikipedia.org/wiki/Francis_Peyton_Rous](https://en.wikipedia.org/wiki/Francis_Peyton_Rous)). Closer to (prokaryotic) home, Barry Marshall has edited a book (Marshall BJ (ed.): *Helicobacter pioneers: firsthand accounts from the scientists who discovered helicobacters*. Melbourne: Blackwell, 2002.) whose invited contributors had all long recognised a bacterial cause of ulcers and treated their patients accordingly, on the simple grounds that the antibiotics worked! Of course Marshall and Warren (and the wider world) knew nothing of this at the time of their discovery of *H. pylori*. Under these circumstances (as here) we rely on the overall weight of evidence (as much as its place of publication) to support our views. In Philosophy of Science circles this bolstering of a view via overlapping circles of self-consistent reasoning and data is referred to as ‘coherence’. Accordingly, in this sense, we have tried to make this a coherent story, and rehearse this point in the concluding section.

○ “In summary Kell, Potgieter and Pretorius have produced an interesting read which bring many important ideas to our attention. I am not convinced of the breadth of conditions to which they argue their ideas are applicable and I await with interest, demonstration of of how they may be practically pursued and some selected definitive proofs that iron-driven
inflammatory disease is as important as they claim."

We have no further comments at this stage. Many thanks again for a very thoughtful review.

**Competing Interests:** No competing interests were disclosed.