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‘Many Hosts of Mycobacteria: Tuberculosis, Leprosy, and other Mycobacterial Diseases of Man and Animals’

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Comparative Mycobacteriology of the *Mycobacterium tuberculosis* complex

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Introduction

“I feel quite confident that the comparative study of tubercle bacilli will lead to some definite understanding on certain important questions, and eventually to more light on the whole subject of tuberculosis from the preventive as well as the therapeutic side.”

Theobald Smith (1898)

The *Mycobacterium tuberculosis* complex (MTBC) is a group of highly genetically related pathogens that cause tuberculosis (TB) in mammalian species. However, the very name of the complex underlines the fact that our knowledge of these pathogens is dominated by studies on the human pathogen, *M. tuberculosis*. Of course this is entirely justified; *M. tuberculosis* is a major global pathogen that exacts a horrendous burden in terms of mortality and morbidity so it is appropriate that it is the cornerstone of the complex. In the same way as *M. tuberculosis* is the best studied human tubercle bacillus, our knowledge of the animal-

adapted strains has been dominated by studies with *M. bovis*. Again, given the economic importance of bovine TB and the potential for zoonotic transmission to humans, this is entirely expected. However, taking *M. tuberculosis* and *M. bovis* as the human- and animal-adapted ‘poles’ of the complex, our focus on these pathogens to the exclusion of others members has restricted, and potentially skewed, our understanding of diversity, virulence and host adaptation within the MTBC. Referring to Theobald Smith above, have we really exploited comparative studies of the tubercle bacilli to their full potential, or have we regarded the MTBC as merely *M. tuberculosis* plus some animal pathogens of lesser import? Herein we discuss our current understanding of the make-up of the MTBC, focussing on comparisons of *M. tuberculosis* and *M. bovis* as the exemplar human- and animal-adapted strains, and look to what studies of these pathogens can teach us about the evolution of the MTBC specifically and the emergence of host adaptation in pathogens in general. We also speculate on how our current focus on *M. tuberculosis* and *M. bovis* may have hindered our appreciation of fundamental concepts such as virulence, evolution and host adaptation of the tubercle bacilli.

The *M. tuberculosis* Complex (MTBC).

The constituent members of the MTBC can be broadly split into the human and animal-adapted strains. The major human pathogens, where no obvious animal reservoir has been identified, are *M. tuberculosis* and *M. africanum* subtypes 1 and 2. The animal-adapted strains have been isolated from a range of wild and domesticated animals and are named after their host of initial/most frequent isolation, including *M. bovis* (Karlson and Lessel, 1970), *M. microti* (Wells, 1946), *M. caprae* (Aranaz et al., 1999), *M. pinnipedii* (Cousins et al., 2003), *M. orygis* (van Ingen et al., 2012), *M. mungi* (Alexander et al., 2010) and the ‘Dassie bacillus’ (Cousins et al., 1994, Smith, 1960). However, it should be noted that these species names do not define exclusivity in host range. MTBC members can infect a range of mammals to greater or lesser degrees; the central feature of host adaptation is the ability to sustain within a host population. So *M. bovis* can infect and cause disease in humans; however, the capacity of *M. bovis* to transmit between immune competent humans is severely limited compared to *M. tuberculosis* (Francis, 1950, Magnus, 1966). Conversely, the capacity of *M. tuberculosis* to sustain in human population is remarkable, with an estimated one third of the world’s population latently infected with *M. tuberculosis* (WHO, 2014). Yet, while *M. tuberculosis* has been isolated from sporadic cases of TB in cattle, elephants, dogs, cats, etc. these represent reverse zoonoses from infected humans rather than *M. tuberculosis* sustaining itself in these animal populations (Alexander et al., 2002).

The positioning of *M. canetti* (van Soolingen et al., 1997), and other smooth tubercle bacilli (STB), in the MTBC is an area of debate (Brisse et al., 2006, Smith, 2006, Becq et al., 2007). Obviously if we define the MTBC as a group of *genetically related* strains that cause TB in mammals the STB represent a problem as they are an out-group, with substantial levels of diversity compared to the other members of the complex. Comparative whole genome analysis of five STB strains isolated from patients from East Africa (Supply et al., 2013) showed that the STB have extensive genetic diversity, with multiple instances of horizontal gene transfer, genomes 10-115 kb larger than *M. tuberculosis*, and 25-fold more SNPs compared to members of the MTBC. Therefore the STB represent a distinct group of human TB pathogens; whether they also cause TB in animals is an open question. A central argument in favour of an animal reservoir is lack of evidence for human-human transmission of *M. canetti*, suggesting that the ability of *M. canetti* to sustain within the human population is limited. An alternative explanation would be an environmental reservoir (Koeck et al., 2011), such as soil or water, perhaps similar to that seen for *M. ulcerans* (Bratschi et al., 2014), but again no clear evidence is available.

There has been an increasing identification and subdivision of strains in the MTBC, with additions over the last decade including *M. orygis*, *M. mungii*, etc. This expansion of species within the MTBC is a product of the increasing sophistication of molecular methods to differentiate strains based on spoligotyping, deletion typing, and whole genome sequencing. It is beyond question that genomics has had, and will continue to have, an immense impact on our understanding of the MTBC, and we will turn to this next.

MTBC Genomics

In the current age of high throughput genomics, with the capacity to sequence multiple *M. tuberculosis* genomes in a matter of weeks, it is worthwhile taking a brief historical perspective on genomics of the MTBC. One of the first attempts to perform genomic comparison across the complex was by Mahairas *et al.* who used subtractive hybridisation techniques to identify three large scale deletions, RD1-RD3, that were absent from *M. bovis* BCG but present in the genome of virulent *M. bovis* (Mahairas et al., 1996). The publication of the *M. tuberculosis* H37Rv genome (Cole et al., 1998) heralded the start of the genomic age of the MTBC proper, with the *M. tuberculosis* genome providing for the first hybridisation-based comparative approaches using microarrays or clone-arrays (Behr et al., 1999, Gordon et al., 1999). These analyses revealed a set of deletions from *M. bovis* and BCG and were the first to throw doubt on pre-existing ideas that *M. tuberculosis* had evolved from *M. bovis* when man domesticated cattle. The completion of the genome of *M. bovis*

AF2122/97 provided further clarification of the evolution of these strains, confirming the close genetic identity between the human and bovine strains but showing that the genome of *M. bovis* had no unique genes *per se* compared to *M. tuberculosis* (Brosch et al., 2002, Garnier et al., 2003). Given the lack of substantial genetic recombination in the MTBC (Namouchi et al., 2012), the loss of genetic regions from *M. bovis* served to underline the fact that *M. bovis* could not be the progenitor of *M. tuberculosis*. The identification of the TbD1 locus as the first region found to be absent from *M. tuberculosis* H37Rv relative to *M. bovis* AF2122/97 provided a useful marker (Brosch et al., 2002), with screening for presence/absence of the TbD1 locus across *M. tuberculosis* strains allowing strains to be grouped as TbD1-intact or ‘ancient’ (a genome configuration closer to the common ancestor) or TbD1-deleted or ‘modern’ (more distant from the common ancestor such as *M. tuberculosis* H37Rv). The picture that emerged from these initial genome studies was an evolutionary scenario where all MTBC strains arose from a common progenitor, but with extant *M. tuberculosis* being closer to the common ancestor of the MTBC and hence rejecting the notion that *M. tuberculosis* in humans arose from *M. bovis* in cattle at the time of domestication (Brosch et al., 2002, Mostowy et al., 2002).

Using a limited number of complete genome sequences to identify deletions, or SNP panels, that are then screened across large numbers of isolates can generate distorted phylogenies. Gagneux and colleagues sought to address these concerns by applying multi-locus sequence typing (MLST) across the MTBC, using 89 gene fragments across 108 MTBC strains, an approach that disclosed six major lineages in the MTBC (Hershberg et al., 2008). In this phylogeny, *M. tuberculosis* showing much greater genetic diversity, and hence possibly phenotypic diversity, than previously assumed and the animal-adapted strains grouped together with *M. africanum* subtype 2. The move from MLST to whole genome sequencing provided even greater resolution to the MTBC phylogenies, with an initial analysis of 21 MTBC strains generating a phylogeny entirely congruent with previous constructs (Comas et al., 2010). Expansion of these genome analyses to encompass 259 MTBC strains and overlaying them with data on human mitochondrial haplogroups showed a striking correlation between global MTBC and human population distributions (Comas et al., 2013). These analyses suggested that the MTBC strains may have been a pathogen of humans for at least 70,000 years, with increased population sizes during the Neolithic demographic transition being a key driver in the success of the MTBC (Comas et al., 2013).

Comparative analyses of the MTBC and non-tuberculous mycobacteria (NTM), such as *Mycobacterium kansasii* and *M. marinum*, has delivered insight into the initial evolutionary steps of the tubercle bacilli (Behr, 2013, Veyrier et al., 2011). What is evident is that free

living saprophytic, or opportunistic pathogens, in the mycobacteria genus have larger genome sizes and show evidence for recombination (Doig et al., 2012, Gordon et al., 2009, Stinear et al., 2008); these features, taken in the context of the diversity of Actinobacteria as a whole, suggest that the progenitor of the MTBC was a free living saprophyte that obtained novel functions through horizontal gene transfer that provided selective advantages in the face of competition from other environmental microbiota, e.g. allowing it to resist digestion by protozoa. These traits served well for infection and maintenance within higher eukaryotes and eventually allowed adaptation to mammals as opportunistic and then obligate pathogens. Once the MTBC common ancestor was then trapped within its mammalian niche, the initial accretion of genetic material was counterbalanced by deletion events, resulting in the RD loci that act as unique markers for clades (Smith et al., 2009). Whether loss of these loci provided any selective advantage to the emerging clones, or was merely the fixation of deleterious mutations in small populations, remains to be defined.

The narrative of MTBC evolution is therefore of an environmental bacteria that host adapted to preferred mammalian host niches; *M. tuberculosis* is the pinnacle of a globally successfully pathogen that has ridden the waves of human migration out of Africa and flourished amongst burgeoning human populations. *Mycobacterium bovis* is the MTBC animal-adapted strain of choice as it infects cattle, a cornerstone of modern agriculture and a clear risk for zoonotic transmission from contaminated dairy or meat products. The exquisite ability of MTBC pathogens to infect and sustain across a diverse array of mammalian species is striking; elucidating the molecular events behind these host adaptation events will shed light not only on what makes the MTBC such a successful group of pathogens, but also the mechanism behind the emergence of new host-adapted pathogens *per se*.

Host preference

Observations of the distinct host preferences of tubercle bacilli date back to the work of Koch, Von Behring, Smith and others. In seminal work Theobald Smith examined 8 human isolates (one isolated from a pet of a TB patient), 6 isolates from cattle, and single isolates from a pig, cat, and horse for their microscopic appearance, cultural characteristics, and pathogenesis in mice, guinea pigs, rabbits, pigeons and cattle (Smith, 1898). The bovine isolates “grew less vigorously for a number of generations” in coagulated serum, while “bovine bacilli tend to remain short; human bacilli are either more slender from the start or become so during cultivation”. In terms of pathogens, bovine bacilli had ‘a much greater pathogenic activity towards rabbits, guinea- pigs and cattle’ than human isolates. This work defined the bovine tubercle bacilli as distinct from the human isolates, and furthermore

defined the distinctive virulence of the isolates in animal models (Smith, 1898). Emil von Behring took Smith’s observations an entrepreneurial step forward by generating a live attenuated *M. tuberculosis* vaccine strain for use in cattle (Bovovaccine) but which was never widely used due to concerns with residual virulence (Linton, 2005).

These classic experiments of Smith on host preference were revisited using well characterised MTBC bacilli, namely the genome sequenced strains *M. tuberculosis* H37Rv and *M. bovis* AF2122 (Whelan et al., 2010). Cattle infected with *M. tuberculosis* H37Rv and *M. bovis* AF2122 became positive to skin-test and interferon gamma release assays; however, the *M. tuberculosis* H37Rv infected cattle showed no pathological signs of disease (even though same *M. tuberculosis* H37Rv seed lot used to infect cattle caused disease in guinea pigs). Although these experimentally infected cattle did not show obvious pathological signs, cattle naturally infected with *M. tuberculosis* that show typical TB granulomatous lesions and are culture positive for *M. tuberculosis* have been identified in multiple studies in regions such as Ethiopia, Nigeria, or China (Berg et al., 2009, Cadmus et al., 2006, Chen et al., 2009). One can however argue that rather than *M. tuberculosis* sustaining in these cattle populations, they instead represent reverse zoonotic infections in countries where the burden of human TB disease is high and immune status of cattle is compromised (Ameni et al., 2011, Ocepek et al., 2005). One can argue therefore that cattle infected with *M. tuberculosis* recapitulate key presentations of TB in humans, from latent infection to active disease. The bovine-*M. tuberculosis* infection model may therefore present a unique model in defining the *M. tuberculosis*-host dynamic.

Animal-adapted strains

With the increasing resolution of genome-based phylogenies it has become apparent that the animal-adapted strains of the MTBC described to date are most closely related to MTBC lineage 5 and 6, aka *M. africanum* (Comas et al., 2010, Comas et al., 2013). Coupled with the evidence for an African origin for the MTBC, it is likely that the diversity of animal-adapted MTBC strains will only be revealed in its full glory when we isolate and characterise tubercle bacilli infecting assorted wild animals in Africa. A case in point is the isolation of a novel MTBC strain from banded mongooses in Botswana (Alexander et al., 2010). This strain, tentatively designed *M. mungi*, was unusual in that it caused high mortality in banded mongooses and appeared to be transmitted via a non-respiratory route. These characteristics may suggest that this is a newly emergent pathogen for the mongoose, or perhaps point to a particularly susceptibility in this population. A novel tubercle bacillus has also been isolated from a chimpanzee in the Cote d’Ivoire (Coscolla et al., 2013). Whole genome sequencing and comparative analyses revealed that while this strain was most similar to lineage 6, it was

part of a distinct lineage that had not been sampled before. Whether this ‘chimpanzee strain’ represents the first isolate of a distinct species remains to be seen, but both of these cases suggest the true diversity of animal-adapted MTBC awaits to be fully described.

As the identification and differentiation of animal isolates of the MTBC continues apace, we should be cautious in defining each novel animal isolate as a new species. An obvious first consideration is whether the animal represents a ‘maintenance’ host that can truly sustain the pathogen population through transmission cycles and to which the pathogen is adapted, or is a ‘spillover’ host that the pathogen has been introduced to from an exogenous source and where the pathogen cannot sustain as it is not host-adapted. The complexity of this situation can be seen with *M. bovis*, which can sustain in maintenance hosts as diverse as cattle, badgers, brush-tailed possums, wild boar, bison, white tailed deer, etc., and where a spillover host is humans. A more holistic approach is therefore to think of the host-adapted MTBC as ‘ecotypes’, a concept first explored for bacteria by Cohan (Cohan, 2002) and applied to the MTBC by Maynard Smith and colleagues (Smith et al., 2006). The bacterial ecotype concept suggested by Cohan assumes that divergence within a species is constrained by periodic selective sweeps, with a new ecotype emerging when a species adapts to a new niche such that it is then immune to selection in the ancestral population. Applying this concept to the MTBC, one can define ecotypes as evolutionarily related groups (clades) that infect and sustain within distinct host populations (each host representing a new niche); the fixed molecular differences such as RD and SNPs that are currently used to define species within the MTBC show that selective sweeps have not homogenised diversity across the clades. So the MTBC can be seen as a set of host-adapted ecotypes; e.g. the *M. tuberculosis* clade is a human-adapted ecotype. This therefore provides a framework to define the emergence of truly novel ecotypes in the MTBC and allow us to search for host-adaptive mutations. The inclination to designate each new animal isolate as a separate ‘species’ may complicate discourse around host adaptation; for example the differentiation of *M. caprae* as a distinct species from *M. bovis*, with the suggestion of ‘caprine’ host-adaptation, may be a step too far.

The application of next generation genome sequencing to clinical isolates, with its power to reveal evolutionary relatedness across strains, looks set to expand the membership of the MTBC further over the coming years. The low cost of genome sequencing compared to its resolving power, and the promise that novel MTBC strains await discovery in wild mammals, suggests that sequencing of animal isolates should become as common an occurrence as is currently being suggested for human isolates of *M. tuberculosis* (Walker et al., 2013).

Pathoadaptation and virulence factors

The key to a pathogen’s life cycle is the possession of virulence systems that enable it sustain in a host population. These virulence factors run the gamut from battering rams that act across multiple hosts, to lock-picks that open a host-specific backdoor, to set up infection. Defining virulence factors involved in host adaptation is complex, not least because ‘virulence’ depends on context; the host’s immune status and genetic background have a major affect on the outcome of infection (Casadevall and Pirofski, 2000). While great strides have been made in defining virulence factors in the MTBC, these have largely been defined on the interaction between *M. tuberculosis* mutants and murine infection models. Below we highlight some of the difficulties in this approach, and suggest that analysis of comparative virulence might provide new perspectives on MTBC virulence.

RD1

RD1 was originally identified by Mahairas and colleagues as a regions deleted from *M. bovis* BCG relative to virulent *M. bovis* or *M. tuberculosis* (Mahairas et al., 1996). Deletion of RD1 from BCG was the principal event in the attenuation of the vaccine strain due to loss of the encoded ESX-1 system (Bitter et al., 2009, Pym et al., 2002); inactivation of ESX-1 machinery or ESX-1-secreted effectors attenuate *M. tuberculosis* and *M. bovis* in animal models (Lewis et al., 2003, Wards et al., 2000). Synthesis and function of the ESX-1 type VII secretion system must demand a significant energy commitment from the bacterial cell; this presumably was a key reason for the in vitro selection of RD1-deleted variant of *M. bovis* through repeated subculture during the derivation of BCG. Hence ESX-1 can fairly be described as a locus that is essential for virulence in these strains and models. However, ESX-1 systems are clearly not required for *M. mungi*, *M. microti*, or the Dassie bacillus to sustain in their respective host populations as all of these strains have RD1-like regions deleted (Alexander et al., 2010, Brodin et al., 2002, Mostowy et al., 2004). This independent loss of a major virulence system in *M. tuberculosis* and *M. bovis* is intriguing, and could indicate a selective advantage to loss of ESX-1 in these MTBC strain-host combinations; however, what this advantage may be is unclear. Furthermore, the environmental NTM, *M. kansasii*, is highly attenuated in most experimental hosts relative to *M. tuberculosis* and *M. bovis*, yet *M. kansasii* contains the orthologous RD1 genes and can secrete ESAT-6 and CFP-10 (Arend et al., 2002). Hence, the presence of an intact ESX-1 locus is not sufficient for virulence. Furthermore, loss of ESAT-6 and CFP-10 secretion does not always attenuate *M. tuberculosis*; in work by Chen et al, site directed mutagenesis of EspA, a protein that is co-secreted with ESAT-6 and CFP-10 by ESX-1, produced a recombinant bacillus where ESAT-6::CFP-10 secretion was blocked but virulence was unaffected (Chen et al., 2013). This result

suggests that even in the well-studied mouse model there are significant gaps in our understanding of the role of ESX-1 in virulence.

MPT70 and MPT83

Two antigens that show differential expression across the MTBC are MPT70 and MPT83 (aka MPB70 and MPB83 in *M. bovis*). MPT83 is a lipoprotein that is post-translationally glycosylated, while MPB70 is secreted with no post-translational modifications (Wiker et al., 1996). *Mycobacterium bovis* shows constitutive high level expression of these antigens, while *M. tuberculosis* has low level expression but shows induction during intracellular growth (Schnappinger et al., 2003). Expression of MPB70 and MPB83 is under the control of the SigK regulon, with high-level expression in *M. bovis* resulting from a loss of negative regulation due to a mutation in the gene encoding the anti-sigma factor RskA (Charlet et al., 2005). Intriguingly, constitutive high expression of MPB70 and MPB83 is also seen in *M. orygis* by an independent missense mutation in *rskA* to that seen in *M. bovis*. The upregulation of the SigK regulon in *M. bovis* and *M. orygis* through independent mutations would suggest a selective advantage for increased expression of MPB83, MPB70 and other constituents of the regulon; however the nature of this advantage is unclear.

Insights into the function of MPB70 and MPB83 have been accumulating through a range of studies. The solution structure of MPB70 revealed it to have a novel fold with similarity to fasciclin domain proteins that are involved in protein-protein interactions (Carr et al., 2003). Chambers and colleagues (2010) used the human monocyte THP-1 cell line to show that N-acylated MPB83 peptide was a TLR1/2 agonist driving expression of matrix metalloproteinase 9 and TNF- α . Interestingly, recombinant MPB83, devoid of any post-translational modification, also stimulated the production of MMP-9 from THP-1, with this stimulation blocked by blocking antibodies against TLR1/2 (Chambers et al., 2010). This observation was extended by Chen et al (2012), who again showed that recombinant MPT83 was a TLR2 agonist that drove expression of TNF- α , IL-6, and IL-12 p40 from the murine RAW267.4 cell line (Chen et al., 2012). Their results suggested that recombinant MPT83 was as potent a TLR-2 ligand as Pam₃CysSK₄, suggesting that high-level expression of MPB83 by *M. bovis* could be a powerful driver of innate immune responses. Hence testing the virulence of recombinant *M. bovis* mutants with a functional RskA anti-sigma factor may generate valuable data on the role of these proteins during infection. However, for *M. tuberculosis*, it has been shown that inactivation of *sigK* does not attenuate in murine models (Schneider et al., 2014), suggesting either that the SigK regulon does not play a major role in *M. tuberculosis* virulence, or that the mouse model of primary progressive disease is suboptimal for understanding the role of the SigK regulon during a prolonged infectious cycle.

Lipids

Mycobacteria, whether pathogenic or harmless saprophytes, produce an esoteric array of cell wall lipids (Brennan and Nikaido, 1995). This no doubt reflects their common origin as environmental bacteria, with cell wall lipids providing the perfect coating to protect the bacterium from the vagaries of free-living such as dehydration or predator attack and with hydrophobic interactions promoting microcolony and biofilm formation. The effectiveness of lipids to provide a protective shield *in vivo* for MTBC is beyond question, but these are not simply passive defences but biologically active structures that can drive and modulate immune responses in the host. Our knowledge of host interactions with mycobacterial lipids is largely drawn from cellular *in vitro* systems or mouse models; while these have provided immensely useful, they may not provide ideal systems to address possible host adaptive roles for mycobacterial lipids.

Taking one mycobacterial lipid as an exemplar, the phenolic glycolipids (PGL) of the MTBC have known roles in virulence and immune modulation. These glycolipids are built on a core of phthiocerol dimycoserolate (DIM), with members of the MTBC having variation in the carbohydrate structures that are linked to DIM (Brennan, 2003); thus, *M. bovis* produces mycoside B, a monosaccharide variant with 2-*O*-methylrhamnose as the terminal sugar, a structure also seen in *M. microti* and *M. pinnipedii* (Malaga et al., 2008). A minority of *M. tuberculosis* strains produce a trisaccharide variant, with mutations in an assortment of glycosyltransferases, methyltransferases and polyketide synthases responsible for the variable production across *M. tuberculosis* lineages (Malaga et al., 2008, Reed et al., 2004, Simeone et al., 2013, Simeone et al., 2010). Hence variation in PGL structures across the MTBC is apparent, but this variation occurs in the context of many other genetic differences, confounding simple linkages between lipid presence/absence to virulence. To address these problems Guilhot and colleagues have used elegant genetic approaches to reprogramme the synthesis of PGL across the MTBC allowing the impact of lipid modifications to be assessed in isogenic backgrounds; for example, to switch the mycoside B variant of *M. bovis* BCG to the PGL variant expressed by *M. leprae* (Tabouret et al., 2010). Added to this, complete chemical synthesis of the PGL-tb has been recently reported, allowing the activity of this glycolipid to be studied in isolation (Barroso et al., 2012). Full chemical synthesis of the *para*-hydroxybenzoic acid derivatives (*p*HBADs), that contain an identical glycosylated phenolic moiety to PGLs, has been reported and have been used to show that *p*HBAD variants in isolation can suppress the production of IFN- γ and IL-17 by stimulated murine splenocytes (Bourke et al., 2014). Teasing apart the roles of PGL and *p*HBADs variants on host interaction across the MTBC now appears feasible.

Sulfolipids (SL) are trehalose-containing glycolipids that are only expressed by *M. tuberculosis* in the MTBC (Brennan, 2003). The role of these lipids in virulence has been ambiguous, as while SL negative mutants of *M. tuberculosis* do not show an attenuation phenotype in murine infection models (Rousseau et al., 2003), SL drives pro-inflammatory cytokine secretion by monocytes (Pabst et al., 1988). Clarity on these seemingly divergent phenotypes has been provided by a detailed analysis of SL mutants on backgrounds where the synthesis of other mycobacterial lipids (DIM, or di and poly-acyl trehalose) have also been blocked (Passemar et al., 2014). It was shown that DIM exerts a dominant effect in terms of mycobacterial virulence, with SL mutants showing no phenotype in a DIM+ background but a slight (but non-significant) decreased fitness in DIM- strains. Work by Bertozzi and colleagues has also suggested that expression of sulfolipid negatively regulates growth of *M. tuberculosis* in human THP-1 cells, but that in murine RAW264.7 cells, or a murine infection model, lack of sulfolipid does not attenuate (Gilmore et al., 2012). The attenuation defect in human cells was attributed to the production of antimicrobial peptides that are absent from murine cells. The potential of sulfolipid to act as a species-specific virulence factor warrants further attention.

Conclusions

The simplest way to assay whether a potential virulence factor has a role in experimental disease is to disrupt the responsible gene(s) and test for bacterial counts and/or pathology in a standardized infection model. While this approach has uncovered dozens of genes required for full virulence of *M. tuberculosis*, the presence of these same virulence genes in environmental bacteria and their absence in host-adapted members of the MTBC suggests that a more nuanced perspective of virulence is required. Furthermore, bacteria that are more virulent for humans can be less virulent for cattle (e.g. *M. tuberculosis*), and *vice versa* (in the case of *M. bovis*). Virulence is not linear, and by extension, bacteria of greater or less virulence are not forcibly expected to respectively enjoy more or less success.

In the case of the MTBC, to transmit between hosts, these organisms must survive host immunity and then exploit this response to cause ‘just enough’ pathology. An excessively virulent bacterium will either disseminate in the host, resulting in non-transmissible disease (e.g. TB meningitis) or simply kill the host through progressive pulmonary pathology. Conversely, an insufficiently virulent bacteria may achieve a productive infection but will not be expelled in high enough numbers to cause transmissible disease. Both extremes select against propagation of the bacteria. *M. tuberculosis* inhabits a ‘goldilocks zone’, where median time from disease to death in the pre-antibiotic era was 2.5 years. Through millennia of co-evolution, MTBC organisms have been selected to generate the appropriate amount of pathology to generate a transmissible focus of pathology, in a host

that is otherwise intact. The variants in the MTBC noted above may be the result of fine-tuned adjustments in response to hosts with gradations of natural resistance that would perturb transmission away from equilibrium. Finally, it has long been known that crowding is a risk factor for both human TB and bovine TB. It is possible that variants of the MTBC are not only adapted to cause disease in their respective hosts, but also adapted to transmit in the habitat where their hosts reside, be it burrows (*M. mungi*) or seashores (*M. pinipedii*), groups (Dassie bacillus) or herds (*M. caprae*).

The MTBC represent the ideal group of pathogens to explore concepts in One Health, with collaboration across human, veterinary and environmental spheres offering new insights into pathogen evolution, virulence, and disease transmission. In our quest for new drugs, diagnostics and vaccines to combat human TB we have underexploited the rich data available from comparative studies across the MTBC. With advances in genome sequencing technologies, and cognisant of undiscovered animal-adapted strains lying in waiting, we are now poised to look afresh at the diversity across the MTBC and the nature of host adaptation and virulence. The concepts uncovered in such an endeavour promise to illuminate our search for new disease control tools to fight TB, a fact not lost on Theobald Smith, as noted in our Introduction, nor on Emil von Behring in his studies on bovine TB (von Behring, 1901): “*I need hardly add that the fight against cattle tuberculosis only marks a stage on the road which leads finally to the effective protection of human beings against the disease*”; it is time to pay renewed heed to these calls.

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