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# Challenges in defining the functional, non-coding, expressed genome of members of the *Mycobacterium tuberculosis* complex

Jennifer Stiens,<sup>1</sup> Kristine B. Arnvig,<sup>2</sup> Sharon L. Kendall<sup>3</sup> and Irene Nobeli<sup>1§</sup>

<sup>1</sup>Institute of Structural and Molecular Biology, Biological Sciences, Birkbeck, University of London, Malet Street, London, WC1E 7HX, UK

<sup>2</sup>Institute of Structural and Molecular Biology, Division of Biosciences, University College London, London, WC1E 6BT, UK

<sup>3</sup>Centre for Emerging, Endemic and Exotic Diseases, Pathobiology and Population Sciences, Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, AL9 7TA, UK

§ Corresponding author: i.nobeli@bbk.ac.uk

## Keywords

Mycobacteria; *Mycobacterium tuberculosis*; non-coding RNA; RNA-seq; transcriptome

## Abbreviations

*Mtb* = *Mycobacterium tuberculosis*

MTBC = *Mycobacterium tuberculosis* complex

ncRNA = non-coding RNA

sRNA = short RNA

asRNA = antisense RNA

UTR = untranslated region

nt = nucleotide(s)

ORF = open reading frame

RBS = ribosome binding site

TSS = transcription start site

TTS = transcription termination site

## Abstract

A definitive transcriptome atlas for the non-coding expressed elements of the members of the *Mycobacterium tuberculosis* complex (MTBC) does not exist. Incomplete lists of non-coding transcripts can be obtained for some of the reference genomes (e.g. *Mycobacterium tuberculosis* H37Rv) but to what extent these transcripts have homologues in closely related species or even strains is not clear. This has implications for the analysis of transcriptomic data; non-coding parts of the transcriptome are often ignored in the absence of formal, reliable annotation. Here, we review the state of our knowledge of non-coding RNAs in pathogenic mycobacteria, emphasising the disparities in the information included in commonly used databases. We then proceed to review ways of combining computational solutions for predicting the non-coding transcriptome with experiments that can help refine and confirm these predictions.

## Introduction

A definitive atlas of expressed non-coding elements in pathogenic mycobacteria does not exist. The lists available from databases and publications overlap only partially and are only available for the reference genomes of key representatives of the *Mycobacterium tuberculosis* complex (MTBC), such as *Mycobacterium tuberculosis* (*Mtb*) H37Rv. This gap in our knowledge impacts the successful analysis of the copious amounts of genomic and transcriptomic data that have become available in the last decade. For example, in the absence of a formal annotation of the non-coding transcriptome, the easiest and most common approach to call differential expression events is to largely, or entirely, ignore information that does not relate to regions currently annotated as coding (CDS); this issue is more acute in studies focusing on non-reference *Mtb* strains or their close relatives, where non-coding annotation is scarce or non-existent. In this commentary, inspired by our own struggles to compile a definitive atlas of 'non-coding' RNA (using the term here to represent regulatory RNAs such as short RNAs, antisense RNAs and the untranslated parts of mRNA transcripts) in the members of the MTBC, we present a summary of the current information from publicly available sources, highlighting the existing gaps in the knowledge and the computational approaches used to attempt to uncover this less well understood part of the mycobacterial genome.

## Why pathogenic mycobacteria and why non-coding RNA?

Prior to the COVID-19 pandemic, mycobacterial disease was the leading cause of death by a single pathogen; causing over 1.4 million deaths, and infecting over 10 million people in 2019, worldwide (<https://www.who.int/news-room/fact-sheets/detail/tuberculosis>). The different members of the MTBC include both human-adapted (*Mtb*) and animal-adapted (*Mycobacterium bovis*, *Mycobacterium caprae*, among others) species which show distinct host preference (Brites et al., 2018). Each of these species is uniquely adapted to cause disease within their preferred hosts, and to navigate a complicated lifecycle, which requires rapid response to changing environmental conditions. Pathogenic bacteria have different programs for invasion, proliferation and survival in particular host environments. The pathogen can rapidly and transiently adapt to environmental changes brought about by host

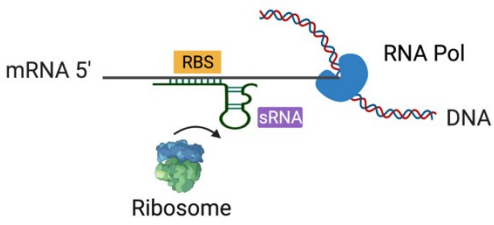

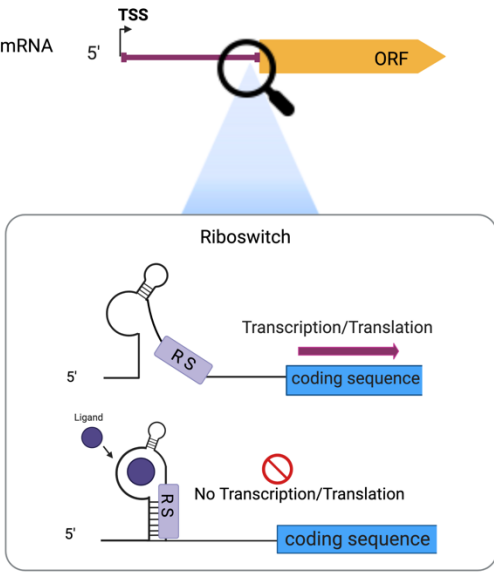
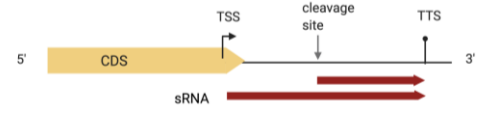
defences by regulating the effect and stability of the transcripts through the parsimonious action of post-transcriptional regulation (Chakravarty & Massé, 2019).

Though the different members of the MTBC have different tropisms, involving specific virulence profiles and metabolic changes made in response to the host environment, nearly 99% of the genomic sequence is conserved among the MTBC members (Malone & Gordon, 2017). The minor variations such as deletions and single nucleotide polymorphisms (SNPs) that vary among species members of the MTBC, and between species-specific strains, seem to have an outsized role determining these preferences (Cheng et al., 2019; Chiner-Oms et al., 2019; Dinan et al., 2014; Malone et al., 2018). These variations are not exclusively found in coding regions; indeed, (Dinan et al., 2014) have shown that SNPs in promoter regions are likely to explain many transcriptional differences between animal-adapted (*M. bovis*) and human-adapted members of the MTBC. It is thus not unreasonable to hypothesise more generally, that variations in the genomic sequence of non-coding elements could contribute to differential gene expression through both transcriptional and post-transcriptional levels of regulation (Schwenk & Arnvig, 2018).

Advances made in recent years exploring the non-coding genome, especially in the model organisms, have shown how flexible and adaptive riboregulation can be. Non-coding RNAs are often categorised by their mode of action: 'cis-acting' RNAs target or regulate the transcripts of genes proximal to the non-coding element, and 'trans-acting' RNAs act on distant gene targets. But applying these categorisations to the diversity of non-coding RNAs known in bacteria is not straightforward. For example, UTRs are typically considered cis-acting, however, they can be a source of trans-acting sRNAs, as well as containing cis-regulatory elements (Loh et al., 2009). Antisense RNAs can regulate their complementary cognate sequence (usually considered cis-encoded, despite the interaction actually occurring between the transcribed elements), but have the potential to act in trans on similar sequences elsewhere in the genome. As a full description of ncRNAs is outside the scope of this commentary, we present instead a graphical summary to describe the main types by their genomic origins, mechanisms of action and targets in Table 1. There are several recent and comprehensive reviews that describe different aspects of the constantly evolving roster of non-coding elements in bacterial genomes, but most of them focus on what has been

discovered in the model organisms (Table 2). Mycobacteria are different, in genome, physiology and lifestyle; and it appears that non-coding regulation in MTBC does not use the same accessory proteins or have the same sequence signatures as the model systems. Indeed, efforts to find an Hfq or ProQ analog acting as an RNA chaperone in mycobacteria have so far been unsuccessful (Gerrick, 2018). These differences impact not only on our ability to transfer knowledge from model organisms to the MTBC species, but also on how applicable current experimental and computational methods are to discovering new regulators in mycobacteria.

**Table 1. Origins and targets of non-coding RNA types in bacteria.**

ncRNA Type	Description	Origin	Target/ Mechanism
<p style="text-align: center;"><b>sRNA</b></p> 	<p>Short structured RNA transcripts, 30-300 nt with short binding ('seed') region</p>	<p>Usually, intergenic regions, UTRs, or antisense strands of coding genes; transcribed from own promoter, or by cleavage of longer transcripts</p>	<p>Involved in binding interactions with distant gene targets ('trans-acting') to regulate translation, including: mRNAs of other genes (e.g. UTRs of transcription factors), other sRNAs (known as 'sponge sRNAs' or 'ceRNAs'), and RNA-binding proteins</p>
<p style="text-align: center;"><b>asRNA</b></p> 	<p>RNA transcript, 75-10,000 nt long</p>	<p>Complementary strand of UTR or coding sequence of regulated gene; transcribed from own promoter</p>	<p>Cognate RNA strand ('cis'-regulatory). Regulates by binding to mRNA transcript with perfect complementarity, forming duplex RNA: altering sensitivity to RNases, action of terminators, or access to RBS (ribosome binding site), can also act in 'trans'-regulatory manner with complementary sequences transcribed elsewhere in the genome</p>
<p style="text-align: center;"><b>5' UTR</b></p> 	<p>5-100s nt long, including transcriptional start sites (TSS), ribosome binding site (RBS), alternative transcriptional terminators (TTS) and cleavage sites</p> <p>Riboswitches are structured 5' UTRs that change secondary structure in response to ligand binding, controlling either transcription or translation of downstream gene by changing access to a 'regulatory sequence' (R S) which could be an anti-terminator sequence or RBS</p>	<p>Upstream sequence of coding sequence, between TSS and start codon (alternative TSS may exist in gene locus)</p>	<p>Binding interactions with sRNAs, proteins, metabolites and second messengers ('cis'-regulation of downstream ORF)</p> <p>Antisense binding with other UTRs. Potential source of sRNAs that can act on distant RNA targets ('trans'-regulatory)</p>
<p style="text-align: center;"><b>3' UTR</b></p> 	<p>5-100s nt long, following the coding ORF of the upstream gene. Can include RNase cleavage sites, alternative transcriptional start sites (TSS) and sRNA binding sites</p>	<p>Downstream sequence between stop codon and TTS. Alternative TSS and TTS may exist in gene locus.</p>	<p>Binding interactions with sRNAs, proteins, metabolites and second messengers to regulate upstream ORF ('cis'-regulatory)</p> <p>Antisense binding with other UTRs, source of sRNAs ('trans'-regulatory)</p>

**Table 2.** Recent reviews of non-coding RNA in bacteria and their focus.

Author	Organism	Focus
Adams and Storz, 2020	Model organisms	sRNA origins/discovery
Bossi et al., 2020	Model organisms	sRNAs and Rho-dependent termination
Breaker, 2018	Model organisms	Riboswitches and translational control
Chakravarty and Massé, 2019	Model organisms	Regulatory RNA and virulence
Denham, 2020	Model organisms	Sponge RNAs (post-transcriptional regulation of sRNAs)
Hör et al., 2018	Model organisms	All regulatory RNA, global RNA-seq methods
Jørgenson et al., 2020	Gram positive and negative bacteria, including mycobacteria	sRNA-mediated regulation
<b>Ostrik et al., 2021</b>	<i>M. tuberculosis</i>	Trans-acting regulatory sRNAs in <i>M. tuberculosis</i>
Schwenk and Arnvig, 2018.	Model organisms and mycobacteria	All regulatory RNA, especially in mycobacteria
<b>Taneja and Dutta, 2019.</b>	Mycobacteria	Focus on sRNAs, and especially those involved with pathogenicity and virulence.
Toledo-Arana and Lasa, 2020	Model organisms	Overlapping transcripts in transcriptome organisation: asRNA, excludons

How many functional non-coding RNAs are there in mycobacteria?

Only a handful of sRNAs have been functionally characterised in the mycobacteria literature (Table 3). In most cases, top-down approaches, such as differential expression studies and CHIP-seq (chromatin immunoprecipitation with sequencing), have been employed to discover *Mtb* sRNAs, such as the RNAP-associated, Ms1 (Arnvig et al., 2011; Šiková et al., 2019) and the PhoP-regulated, Mcr7 (Solans et al., 2014). Verification of the transcript size and abundance by Northern blot analysis has also established the stability of many ncRNAs in *Mtb*, but identifying targets and functional associations requires extensive research. It is curious, that even among the six well-characterised examples in Table 3, there is one (Mrs1) not listed in the current official annotation of the reference H37Rv genome, available from the

corresponding NCBI annotation (GFF) file (GCF\_000195955.2\_ASM19595v2\_genomic.gff), most likely because it was a recent discovery. This annotation file currently includes 20 features labelled as non-coding RNAs, 15 of which are listed in (Arnvig et al., 2011) and 9 in (DiChiara et al., 2010)- 4 are listed in both. It also includes 10 “sequence features” which are annotated as fragments of putative small regulatory RNAs (8 matching information from DiChiara (DiChiara et al., 2010) and 2 matching information from Pelly (Pelly et al., 2012), and two “misc RNA” including a tmRNA and the ribonuclease P RNA. Although twenty or even thirty non-coding elements is almost certainly an underestimate of the total number of ncRNAs in *Mtb*, we note here that the corresponding *E. coli* reference genome annotation (GCF\_000005845.2\_ASM584v2\_genomic.gff) contains currently 72 elements labelled ncRNAs, suggesting that either functional non-coding elements are not very common in bacteria, or that, even for a well-studied organism, our understanding of non-coding regulation is incomplete.

**Table 3.** Functionally characterised sRNA in mycobacteria. \*Annotation according to Lamichlane et al., 2013.

Name (H37Rv annotation*, other names)	Mycobacterial organism	Genomic coordinates (H37Rv)	Citation	Pathway / targets
DrrS (ncRv11733, MTS1338)	<i>M. tuberculosis</i>	1960667-1960783 (+)	Moores et al., (2017)	DosR regulon / unknown
Mcr7 (ncRv002, MTB000067)	<i>M. tuberculosis</i>	2692172-2692521 (+)	Solans et al., (2014)	PhoP regulon / tatC
MrsI (ncRv11846)	<i>M. tuberculosis</i> , <i>M. smegmatis</i>	2096758-2096863 (+)	Gerrick et al., (2018)	Iron-sparing response / brfA
Ms1 (ncRv0036a, MTS2823 in <i>M.tb</i> )	<i>M. smegmatis</i> , <i>M. tuberculosis</i>	4100669-4100968 (+)	Šiková et al., (2019)	Transcription regulation/ RNAP
6C sRNA (ncRv13660c, B11)	<i>Mtb</i> , <i>Msmeg</i> , (homologues in all GC-rich gram+ bacteria)	4099386-4099478 (-)	Mai et al., (2019)	Growth, virulence (ESX-1) / panD, dnaB
Mcr11 (ncRv11264Ac)	<i>M. tuberculosis</i>	1413227 - 1413107/8 (-)	Girardin and McDonough, (2020)	Growth, metabolism / unknown
F6 (ncRv10243)	<i>M. tuberculosis</i> , <i>M.smegmatis</i>	293604 - 293705 (+)	Houghton et al., (2020)	SigF regulon / unknown

Whereas functional characterisation is ultimately needed to create a reliable list of non-coding RNAs, homology to known families of RNAs from other organisms remains the most



popular approach for predicting non-coding RNAs in the absence of experimental evidence. The RNA families described in the RFAM database (Kalvari et al., 2021) derive from the application of covariance models (and where structure information is not available, Hidden Markov Models) representing meticulously curated multiple sequence and secondary structure alignments of homologous RNAs. RFAM thus represents some of the most reliable predictions for non-coding elements in genomes and its predictions for *Mtb* H37Rv are summarised in Table 4. As conservation of structure is at the heart of RFAM families, non-coding RNAs with few or no known relatives in other species, and those that do not fold into strongly conserved structures, are unlikely to be found in RFAM. Hence, this database too is likely to miss elements that are specific to a small number of pathogenic mycobacteria or that are too short to fold into a stable structure. In general, homology-based approaches to discovering *novel* non-coding elements will be limited in pathogenic mycobacteria as there are few closely-related genomes outside the phyla. One notable exception, 6C sRNA, is well-conserved among gram-positive bacteria with over-expression leading to altered growth phenotypes in *M. tuberculosis*, *M. smegmatis* and another GC-rich bacterium, *Corynebacterium glutamicum*. Perhaps as a result, it is one of the few sRNAs for which target molecules have been identified and experimentally validated (Mai et al., 2019).

**Table 4.** Conserved non-coding RNA families and sequence listings from the RFAM database (<https://rfam.xfam.org>). Ribozymes (Group II catalytic introns and Bacterial RNase P class A), tRNAs and rRNAs have not been included in this table.

RNA Type	Family Name	Rfam ID	Number Sequences in RFAM	Length (nt)
Riboswitch	Cobalamin (B <sub>12</sub> )	RF00174	2	173-218
Riboswitch	ykok leader/Mbox (Mg <sup>+</sup> )	RF00380	2	169-174
Riboswitch	TPP/Thi-box (thiamine)	RF00059	2	110
Riboswitch	ydaO/yuA leader (Cyclic di-AMP)	RF00379	1	222
Riboswitch	Glycine	RF00504	2	90-97
Riboswitch	S-adenosyl methionine (SAM-IV)	RF00634	1	119
sRNA	Mcr7	RF02671	1	348
sRNA	npcTB_6715	RF02886	2	211

sRNA	Ms1	RF02566	1	301
sRNA	ncRv12659	RF02659	1	171
sRNA	ncrMT1302	RF02341	1	108
sRNA	b55	RF01783	1	60
sRNA/asRNA	ASdes	RF0781	2	67
sRNA	F6	RF01791	1	57
sRNA	Ms_AS-5	RF02465	1	44
sRNA/5'UTR	5_ureB_sRNA	RF02514	1	294
asRNA	ASpks	RF01782	5	68-77

Expanding our exploration to resources beyond the official NCBI annotation, further complicates the question of what is known about functional, non-coding RNAs in mycobacteria. Mycobrowser (Kapopoulou et al., 2011), arguably the most popular internet resource for the exploration of representative mycobacterial genomes, currently lists 92 non-coding RNAs, labelled as 'ncRNA' (including sRNAs and asRNAs under this moniker) for H37Rv: 40 overlap the official NCBI GFF annotation and originate from the four key publications listing experimentally-verified non-coding RNAs (Arnvig et al., 2011; Arnvig & Young, 2009; DiChiara et al., 2010; Pelly et al., 2012) and the remaining 52 overlap the list compiled by DeJesus et al. (DeJesus et al., 2017) using their in-house computational tool, *BS\_finder*, applied to RNA-seq data derived with a small-RNA sequencing protocol. Despite including annotations from nine other species and strains, including *M. bovis*, *M. smegmatis* and *M. tuberculosis 18b*, non-coding RNAs annotated with the tag "ncRNA" appear in the GFF files of only three additional species/strains in Mycobrowser, and only *M. tuberculosis 18b* has more than two ncRNAs listed. Strikingly, *M. bovis*, sharing more than >99.95% sequence identity to *M. tuberculosis*, has no other entries for RNAs apart from rRNAs, tRNAs and the same two RNAs tagged "misc\_RNA" in the *Mtb* annotation; it is highly unlikely that many of the ncRNAs present in *M. tuberculosis* do not have a counterpart in *M. bovis*; and thus the list must be assumed to be incomplete. In fact, at least 41 of experimentally-verified sRNAs found in various mycobacterial species, including in the above studies, can be mapped to the *M. bovis* genome (Dinan et al., 2014) and a sequence comparison (Supplemental Info, Table 1) finds

that only three of the listed *M. tuberculosis* ncRNAs have less than 99.0 % sequence identity in *M. bovis* (and all have greater than 92% similarity). Perhaps more worryingly, the RNase P RNA component is alternatively tagged as *ncRNA* in *M. haemophilium* and *M. orygis*, *RNase\_P\_RNA* in *M. tuberculosis* 18b and *misc\_RNA* in *M. bovis*. The lack of standardised tags and incomplete listings of non-coding elements (even within the same resource), together with the absence of a clear justification for which elements are included and why, likely adds to the confusion about non-coding regulation in mycobacteria. A more systematic approach to the annotation tags of these elements, similar to approaches suggested for consistent naming of non-coding RNA (Lamichhane et al., 2013), could go some way towards eliminating this confusion.

#### Completing the non-coding transcript atlas: computational predictions from genomic and transcriptomic data

The most extensive lists of putative non-coding RNAs in mycobacteria are the result of computational predictions based on genomic or transcriptomic data (or sometimes both). Computational prediction algorithms have been used with moderate success in other bacteria, including *Salmonella enterica* (Sridhar et al., 2010) and *Staphylococcus aureus* (Liu et al., 2018) and new tools continue to be developed with increasing sophistication. However, the utility of these tools is even more limited in their application to mycobacteria. Genomics-based methods rely on the conservation of non-coding elements across several species and, like Rfam, are likely to miss elements specific to a small subset of the genus or unique to a species. Such comparative genomics methods are typically enhanced by the search for characteristic sequence features and other signals of regulatory RNAs such as promoters, terminator structures and transcription-factor binding sites. For example, SIPHT begins with conserved intergenic sequences (defined as the sequence between two annotated genes or open reading frames (ORFs), on one strand) and looks for characteristic features of sRNAs in these regions, such as conserved promoters and rho-independent terminator motifs (Livny et al., 2008). Other genomics-based programs rely entirely on sequence features and genomic context (ignoring conservation). sRNAScanner determines intergenic sequences using genome annotation files and differentiates coding from non-coding sequences using position scoring matrices for sequence signals such as RBS and start codons (Sridhar et al., 2010). A recently published tool, the Pred-GsRNA feature of the PresRAT server, extracts intergenic

sequences, also based on genome annotation, and excludes candidates that have an 8 nt sequence found to be depleted in known sRNAs. It scores each predicted sequence with weighted Minimum Free Energy scores for predicted paired and loop regions and scores for the predicted U-rich consensus sequences typical of intrinsic terminators (Kumar et al., 2020). The server offers 405 possible 'non-genic sRNA' predictions for the *M. tuberculosis* H37Rv genome (<http://www.hpppi.icb.res.in/presrat/>). We compared the predicted sRNA coordinates with the coordinates of the 92 'stable' RNAs in the H37Rv genome on Mycobrowser (<https://mycobrowser.epfl.ch>). There were no PresRAT predicted sRNAs overlapping the boundaries of the Mycobrowser listed RNAs, except for low-ranking predictions that were over 4000 bp long, indicating that this method has limited power to recognise intergenic sRNA elements in mycobacterial genomes.

Relying on the current annotation to define the intergenic search space is problematic given that many of the start codons in the current *Mtb* annotation appear to be misannotated, with ribosome occupancy studies suggesting that there are a significant number of unannotated proteins encoded at the 5' ends of annotated genes (Shell et al., 2015; Smith et al., 2019). Furthermore, a considerable proportion of transcripts in the mycobacterial genome are either 'leaderless', meaning the transcription start site and the start codon are overlapping and the transcripts therefore lack the canonical Shine-Dalgarno sequence used to identify ORF boundaries (Cortes et al., 2013; Martini et al., 2019; Sawyer et al., 2021; Shell et al., 2015). Programs that search the intergenic regions for conserved sequence features based on sRNAs discovered in the model organisms are also less effective in mycobacteria as mycobacteria make use of a large number of alternative sigma factors which recognise diverse promoter sequences, and many lack a conserved -35 sequence (Newton-Foot & Gey van Pittius, 2013). Mycobacterial transcripts, including sRNAs, also often lack the recognisable intrinsic terminator motifs at their 3' ends typical of Hfq-binding sRNAs (Arnvig et al., 2014; DiChiara et al., 2010; Moores et al., 2017). Furthermore, identifying regions of high GC-content in order to detect RNA secondary structure in the intergenic space is even more challenging in the context of the GC-rich genome of mycobacteria. In any compact bacterial genome, tools that narrow the search space to strictly intergenic regions that lack no annotated genes on either strand, effectively ignore sRNAs and asRNAs generated from coding regions, antisense

regions or 5'/3' UTRs; this may bias our understanding of non-coding regulation in mycobacteria.

Transcriptomics-based methods are usually versions of sliding window approaches looking for abrupt increases and drops in the expression signal and using such changes to delineate the limits of putative non-coding elements. High-throughput RNA sequencing (RNA-seq) has uncovered a multitude of short transcripts from intergenic sequences, 5' and 3' UTRs and antisense to coding regions. Identifying functional transcripts in the conditions examined is the main challenge when using these data in non-coding RNA discovery. For example, sensitive methods are able to pick up expressed elements in regions of low read coverage; this signal may represent true low-abundance transcripts but it can also be the result of either technical noise or stochastic gene expression. The more sensitive computational methods will therefore inevitably over-predict putative non-coding elements. Ironically, high-depth sequencing has magnified this problem (Mao et al., 2015; Tarazona et al., 2011). Non-fragmented, size-selected libraries, where small transcripts remain intact, are superior for discerning between signal and noise for small RNA transcripts (Leonard et al., 2019; Wang et al., 2016). For all the reasons discussed above, detecting the existence of sRNAs expressed in low levels against very strongly expressed coding genes remains a computational challenge. Here, we also suggest caution when using publicly available transcriptomic data, some of which dates back to the early use of RNA-seq technologies. In particular, using strand-specific cDNA libraries sequencing, where the information about which strand the transcript originates from is preserved, is invaluable to the discovery of new ncRNAs. Preservation of the strand information avoids mis-mapping asRNAs or other overlapping sRNAs that might otherwise be mapped to a coding gene on the opposite strand.

Many labs have developed their own computational pipelines and scripts to map RNA-seq data, normalise signals and identify ncRNA transcripts across the genome (Ami et al., 2020; Dejesus et al., 2017; Gómez-Lozano et al., 2014; Miotto et al., 2012; Wang et al., 2016), whereas others have carried out this process semi-manually (Arnvig et al., 2011). Progress in the field, and an easy comparison between approaches, has been hindered by the fact that few of the labs publishing computational predictions have made their code readily available. In response to this challenge, several groups have created publicly-available prediction

programs or workflows such as *Rockhopper* (McClure et al., 2013), *DETR'PROK* (Toffano-Nioche et al., 2013), *ANNOgesic* (Yu et al., 2018), *APER0* (Leonard et al., 2019) and *baerhunter* (Ozuna et al., 2019). Users of all of these transcriptomics-based methods are required to set thresholds for separating background noise (whatever its origin) from signal in the data. Indeed, most programs need adjustment to their default parameters in order to respond to sequencing depth and signal abundance (Figure 1) but tuning these parameters can be a matter of art rather than science.

The more sophisticated among the transcriptomics-based approaches use a combination of sources, such as TSS data or conservation across species, to reduce false positives. *DETR'PROK* is a Galaxy-based workflow, coordinating over 40 publicly-available Galaxy sequence comparison tools into a pipeline which streamlines the number of user-defined parameters. However, there are still 14 different user inputs, most of which concern filtering to account for read depth and transcriptional noise (Toffano-Nioche et al., 2013). The recently published *ANNOgesic* suite of tools utilises multiple third-party software packages, as well as its own scripts to analyse RNA-seq data and filter predictions. Although, the suite includes an *sRNA-finder* module, using this module in isolation on user-generated alignment files requires specific file formats for the alignment (.wig) and several reference annotation files. Multiple levels of filtering are possible to identify *bona fide* ncRNAs, but such filtering requires downloading of tools and databases such as *RNAfold* (Denman, 1993), *BSRD* (Li et al., 2013) and the NCBI nr protein database (NCBI Resource Coordinators, 2014). In the context of validating mycobacteria ncRNA predictions, such databases may possibly be less relevant, given the lack of homology or shared sequence features between mycobacterial and other bacterial ncRNAs. Additionally, fine-tuning cut-off parameters to distinguish signal from noise is ultimately still up to the user. Somewhat surprisingly, the added complexity of such methods does not always translate into more accurate results: in limited comparisons between methods that use additional information and our own simpler, signal-only-based method, we found that our naïve approach performs comparatively well, most likely because more sophisticated methods often require more tuning of their parameters to take advantage of their added complexity (Ozuna et al., 2019). As the responsibility of parameter tuning is

left up to the user, it is obvious that methods with fewer parameters, such as Rockhopper, *baerhunter* or APERO, may be less error-prone and, ultimately, more appealing, especially to non-computational users looking for quick and easy to implement solutions. Rockhopper is an independent, Java-based tool designed for bacterial RNAseq data (McClure et al., 2013). To eliminate guesswork by the user to adjust for noise vs. signal, the program normalises for read counts using the upper quartile of non-zero gene expression values and generates a transcriptional map of the predicted non-coding elements. *Baerhunter* (Ozuna et al., 2019) and APERO (Leonard et al., 2019) are lighter tools to install, both written in R and requiring only the most commonly used BAM format alignment files and relevant reference annotations. Like Rockhopper, the output of *baerhunter* is a transcriptional map (in .gff format), and can consolidate annotations from multiple samples. APERO exploits improvements in sequencing technology by requiring paired-end reads (where each fragment is sequenced from both ends, creating two barcoded reads for each fragment) and optimising parameters for non-fragmented libraries. The output consists of a set of flat files of the predicted transcript 5' and 3' ends for each sample that can then be filtered for read counts and assembled into a genomic context.

Steps can be taken to lend support to computational predictions of sRNAs and 5' UTRs in mycobacteria. In a recent study to identify differentially expressed, verifiable sRNAs in *M. tuberculosis*, software predictions based on RNA-seq produced over 200 candidate sRNAs (Dejesus et al., 2017), 82 of which were differentially expressed by 6-fold in at least one experimental condition (Gerrick et al., 2018). Applying additional filters to the 92 'stable ncRNAs' listed in Mycobrowser, we compared their 5' boundaries with a compendium of published predicted TSSs (Cortes et al., 2013; Shell et al., 2015), and found 40 with predicted TSS within 10 nucleotides of the annotated 5' boundary. 62 of the Mycobrowser ncRNAs are putative sRNAs originating from the DeJesus et al. study (Dejesus et al., 2017), 25 of which have TSSs within 10 nucleotides of the 5' boundary. We also compared these putative sRNAs with the transcripts found to be differentially expressed in Gerrick et al (Gerrick et al., 2018), and found 17 putative ncRNAs with both TSSs and differential expression (Appendix 1). Mapping RNase cleavage sites in *M.tuberculosis*, as they were for *M.smegmatis*, could also lend support to the existence of other sRNA candidates cleaved from longer transcripts or otherwise processed (Martini et al., 2019). Sequence conservation of non-coding elements in

mycobacterial genomes outside the MTBC can help to identify *bona fide* predictions made by RNA-seq methods. A comprehensive analysis of the genomic context, structural conservation and expression profiles of non-coding RNA homologues both within the MTBC, and in the wider phyla, would be a valuable resource for the mycobacterial research community (but outside the scope of this short review). In the absence of such a resource, we have performed a sequence similarity search with each of the non-coding RNAs annotated in *M. tuberculosis* in three related genomes: one member of the MTBC (*M. bovis*), the non-pathogenic strain widely used surrogate for *Mtb*, *Mycobacterium smegmatis* and a pathogenic species outside the MTBC, *Mycobacterium abscessus*, using the web-based application, *fastA* (Madeira et al., 2019) (Supplemental Info, Table 1). 43 of the 92 Mycobrowser ncRNAs have significant (E-value < 0.01) sequence matches in both *M. smegmatis* and *M. abscessus* with sequence identities ranging from 52-87%. 18 of these have been experimentally verified by Northern blot, but 25 of them were predicted by RNA-seq methods alone. All these approaches may lend support to computational findings, but true validation of candidate ncRNAs requires experimental confirmation such as using RACE (Rapid Amplification of cDNA Ends) (Frohman et al., 1988) to identify transcript boundaries; and Northern blot to confirm the existence and size(s) of actual RNA transcripts, and to confirm expression of orthologous transcripts in related genomes. As computational tools become more specialised for the mycobacterial genome, laboratory resources can be more confidently directed to their predictions.

A further complication in defining the non-coding transcriptome is that putative non-coding elements predicted by computational algorithms may actually be (or contain) as yet unannotated ORFs; there is no way of asserting from the RNA-seq signal alone whether a transcript is coding or non-coding. Early ribosome profiling studies pointed to the presence of hundreds of small peptides encoded in the 5' UTR of mycobacterial transcripts (Shell et al., 2015), and more recent efforts have shown pervasive translation in *Mtb*, uncovering over 1000 novel ORFs (Smith et al., 2019). The majority of these were short ORFs with non-canonical features that would thus be missed by regular gene prediction algorithms. Comparing this list with the annotated ncRNAs listed in Mycobrowser, we found that two of the ncRNAs overlap with predicted ORFs (Appendix 1). Although translation of these transcripts does not necessarily render them functional, they may constitute a pool of peptides that are available to use under the right conditions. The observation that leaderless



transcripts are translated more efficiently under stress conditions (Sawyer et al., 2021) also points to the fact that mycobacterial non-canonical ORFs may play increasingly important roles in conditions of nutrient starvation or other stresses.

Can we improve the identification of non-coding elements in mycobacteria?

There is limited scope for improving the computational methods used to predict non-coding RNA from the currently available mycobacterial genomic and transcriptomic data. In our experience, both lack of specificity and sensitivity of current methods can be accounted for by the signal (or absence of it) in the raw data. One problem is that in a compact mycobacterial genome, overlapping signal from UTRs and ORFs may confuse algorithms and stop them from correctly predicting the limits of transcripts. In such cases, some level of manual curation is often needed, guided by visualisation on a genome viewer such as Artemis (Carver et al., 2012) or IGV (Robinson et al., 2011). Another source of problems is the use of short reads in the currently most popular sequencing protocol. Typical Illumina RNA-seq fragments are 75-150 base pairs long and are mapped in overlapping segments, preferentially using paired-ends, to infer a longer transcriptional unit. Many genes in mycobacteria, including sRNA and asRNA, are transcribed as polycistronic transcripts, where multiple sequential genes are transcribed into a single mRNA transcript. The individual overlapping transcripts of varying lengths are often difficult to detect with standard RNA-seq (Figure 1). The development of specialised RNA-seq methods, such as dRNA-seq (Sharma et al., 2010) to enrich for the 5' end of primary transcripts and map TSSs, and Term-seq (D. Dar et al., 2016) to find 3' termini, offers information that can be used to address the issue of overlapping signal from distinct transcripts. Moreover, ribosome profiling (Ingolia et al., 2009) will continue to be instrumental in resolving ambiguities in annotation of ORFs versus non-coding elements in untranslated regions (Shell et al., 2015; Smith et al., 2019). Although such information can already be integrated in a subset of computational pipelines (Yu et al., 2018), the corresponding data is only available for a limited number of reference mycobacterial strains.

Perhaps one of the most promising new technologies for studying whole transcriptomes are based on long-read sequencing. Pac-Bio SMRT or Oxford Nanopore Technologies sequencing can achieve reads several thousand nucleotides long, resolving issues associated with errors

in the assembly of short reads. The selection of primary RNA transcripts that have not been fragmented in cDNA library preparation make it possible to reconstruct an entire transcriptome with a high level of confidence. In addition, the ability to sequence full polycistronic transcripts allows the surveying of dynamic changes to the structure of bacterial operons in response to a change in the conditions of growth (Yan et al., 2018). Nanopore sequencing goes one step further in making it possible for native RNA molecules to be sequenced, allowing post-transcriptional modifications of individual nucleotides to be detected, that would otherwise be lost during reverse transcription to cDNA (Grünberger et al., 2020). The technologies are still evolving, but with bioinformatics improvements to resolve technical issues of noise from the nanopore and saturation (Soneson et al., 2019), long-read sequencing will become a valuable tool for studying transcriptomes. The longer reads will certainly improve our mapping of 5' and 3' UTRs, as well as our understanding of the dynamic nature of bacterial transcriptional units, but issues with discriminating coding from non-coding elements, sRNAs from UTRs and identifying original versus processed or degraded transcripts will remain a problem.

One, perhaps less obvious, way in which new sequencing technologies may prove instrumental in improving the prediction of non-coding RNAs is their role in improving the assembly of genomic sequences against which RNA-seq reads are mapped. Currently, *Mtb* transcript mapping relies on the cultured genome, H37Rv, which shows considerable differences compared to clinical and field strains, or isolates, that have adapted to different environmental pressures (O'Neill et al., 2015; Shockey et al., 2019). As SNPs in promoter regions and small insertions/deletions may play a major role in regulating the expression of non-coding elements (Dinan et al., 2014), it is clear that using the correct genomic sequence is important when analysing transcriptomes of non-reference strains. Sequencing and assembly of potentially thousands more strains are being facilitated by technologies offering portable sequencing platforms and we can expect the number of available mycobacterial genomes to increase manifold in public databases in the next few years, as a result of the increase in popularity of such methods.

Having the correct genomic sequence available is important but correct annotation is arguably just as important, given how many algorithms rely on the annotation of coding

elements to make predictions of non-coding ones. Homologous predicted sRNAs are sometimes annotated as protein-coding or non-coding in different genomes, and could, in fact, be dual-function sRNAs (Vanderpool et al., 2011). This is especially obvious when trying to compare non-coding elements or small ORFs in different lineages of *Mtb* (Arnvig & Young, 2012). To improve annotation efforts, the idea of assembling MTBC pangenomes that differentiate core genes (including non-coding ones) present in all lineages, from accessory genes present in a subset and unique genes present in only one strain or lineage, is an appealing one (Vernikos et al., 2015). Although members of the MTBC are assumed to share very high sequence identity, this assumption is rooted primarily on comparisons of reference sequences and less so on circulating strains. For example, the sequencing of *M. bovis* strains that cannot be classified in current clonal complexes, suggests that diversity within this species may be higher than previously thought (Zimpel et al., 2020). Pangenomic projects to date have primarily focussed on identifying differences in antibiotic liability/resistance among clinical strains of *Mtb* (H. A. Dar et al., 2020; Rufai et al., 2020), but whole genome sequencing projects of clinical and field strains to assemble lineage-specific pangenomes for both human and animal-adapted MTBC members would allow comparisons and provide a more accurate picture of the extent of riboregulation and its effect on host-specificity and other phenotypic differences (Zimpel et al., 2017).

Finally, it is worth pointing out that the quest for an atlas of the mycobacterial non-coding transcriptome may need to be reconsidered in view of the fact that the number of non-coding RNAs we discover, just like the number of peptides we discover, is closely linked to the growth conditions and the sensitivity of the methods we use. There is likely expression detectable at every nucleotide of the genome, if we use a sensitive enough method to detect it. However, what transcripts are functional or able to acquire function given a set of conditions is the important question here. To answer this, more targeted experiments are needed, but computational methods will be crucial in focusing the efforts towards the most promising subjects for investigation.

## Conclusions

A definitive answer to the question, “How many non-coding RNA elements exist in mycobacterial genomes?”, is not yet possible. Although several computational methods have been developed to support this area of research, our knowledge is currently limited by the availability and quality of raw data. We believe the key to constructing an atlas of the mycobacterial non-coding universe is recognising both the diversity of the individual genomes, and the dynamic nature of the corresponding transcriptomes. Integration of existing and new sequencing technologies and close collaboration between experimental and computational groups should allow us to progress faster towards this goal.

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## Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure legend

**Figure 1. Challenges of predicting non-coding expressed elements from transcriptomic signal alone.** Coverage views from two real and one hypothetical Mtb transcriptomic dataset: Illumina high-throughput sequencing datasets from Bioproject accession numbers PRJNA278760, sample SRR1917694 **(A)** and PRJNA390669, sample SRR5689230 **(B)**; diagrammatic illustration of long-read sequencing coverage of the same region **(C)**. The two RNA-seq samples differ in their sequencing depths: average, non-zero, sequencing depth for the region displayed is 55.6 for (A) and 312.8 for (B). The blue rectangle below the x-axis indicates the genomic region covered by DrrS (MTS1338), an annotated M.tb sRNA of 109 nts. This stable (and by far most abundant) form is cleaved from longer transcripts found in Northern Blots of 160-400+ nts (Moore et al., 2017). Both RNA-seq datasets (A & B) display a gradual drop in coverage at the 3' end. In such cases, automatic computational prediction of the correct transcript length is challenging for any algorithm but here the prediction is further complicated by the fact that multiple overlapping transcripts of different length most likely co-exist in the data. Even in the deeply sequenced sample, where the presence of overlapping transcripts could be conjectured, most algorithms would call a single transcript, without additional knowledge of transcription start and termination sites for the refinement of computational predictions. In the absence of such additional data, long-read sequencing might be helpful: as illustrated in diagram (C), long reads whose starts and ends can be unambiguously defined should be helpful in identifying the presence of multiple overlapping transcripts expressed from a single locus. Image created using the Integrated Genome Viewer (Robinson et al., 2011) and BioRender.com.



2 **Appendix 1.** List of *M. tuberculosis* 'stable regulatory RNAs created from Mycobrowser (<https://mycobrowser.epfl.ch>) H37Rv annotation file, release 4  
3 (*Mycobacterium\_tuberculosis\_H37Rv\_gff\_v4.gff*). Presence of a predicted TSSs (Cortes et al., 2013; Shell et al., 2015) within 10 nts of start position and overlap with predicted  
4 sORFs (Smith et al., 2019) are indicated. \*nc Locus ID refers to annotation as Lamichhane et al., (Lamichhane et al., 2013), and missing locus names were assigned based on  
5 mapping coordinates to H37Rv reference sequence (AL12345.3).

Name	nc Locus ID	Tuberculist	Start	End	Width	Str	Citation	Verif	RNA-seq	Diff Expr	Rfam	TSS	sORF
ncRv10071	ncRv10071	MTB000100	80240	80440	201	+	DeJesus et al, 2017		*	*		TSS	
ncRv10071c	ncRv10071c	MTB000101	80254	80344	91	-	DeJesus et al, 2017		*	*		TSS	
ncRv10128	ncRv10128	MTB000102	156452	156567	116	+	DeJesus et al, 2017		*	*			
ncRv10128c	ncRv10128c	MTB000103	156521	156568	48	-	DeJesus et al, 2017		*	*			
ncRv10150c	ncRv10150c	MTB000104	177236	177285	50	-	DeJesus et al, 2017		*	*			
ncRv0179	ncRv0179	MTB000105	209683	209841	159	+	DeJesus et al, 2017		*	*			
ncRv0186c	ncRv0186c	MTB000106	218320	218379	60	-	DeJesus et al, 2017		*	*			
ncRv10243	ncRv10243B	MTB000107	293603	293663	61	+	DeJesus et al, 2017		*	*		TSS	
F6	ncRv10243A	MTB000051	293604	293705	102	+	Arnvig and Young, 2009; DiChiara et al, 2010	Northern RLM-RACE		*	RF01791	TSS	
ncRv0441c	ncRv0441c	MTB000108	530246	530353	108	-	DeJesus et al, 2017		*				
ncRv10467	ncRv10467	MTB000109	558815	558884	70	+	DeJesus et al, 2017		*			TSS	
mcr19	ncRv0485	MTB000060	575033	575069	37	+	DiChiara et al, 2010	Northern					
ncRv0490	ncRv0490	MTB000110	579290	579408	119	+	DeJesus et al, 2017		*	*		TSS	
ncRv10609	ncRv10609B	MTB000111	704185	704246	62	+	DeJesus et al, 2017		*		RF01783	TSS	
B55	ncRv10609A	MTB000052	704187	704247	61	+	Arnvig and Young, 2009	Northern /RLM-RACE			RF01783	TSS	
ncRv10637	ncRv10637	MTB000112	733361	733459	99	+	DeJesus et al, 2017		*	*		TSS	
ncRv0638	ncRv0638	MTB000113	734118	734244	127	+	DeJesus et al, 2017		*	*			
ncRv0641	ncRv0641	MTB000114	736166	736284	119	+	DeJesus et al, 2017		*	*			
ncRv10666	ncRv10666	MTB000115	759479	759610	132	+	DeJesus et al, 2017		*	*		TSS	
ncRv10685	ncRv10685	MTB000116	786021	786074	54	+	DeJesus et al, 2017		*	*			
ncRv10699	ncRv10699	MTB000117	800242	800359	118	+	DeJesus et al, 2017		*	*			

ncRv0724	ncRv0724	MTB000118	815417	815685	269	+	DeJesus et al, 2017		*			
ncRv0810c	ncRv0810c	MTB000119	905075	905164	90	-	DeJesus et al, 2017		*			TSS
ASdes	ncRv0824	MTB000053	918264	918458	195	+	Arnvig and Young, 2009	Northern, RLM-RACE			RF01781	TSS
ncRv10860	ncRv10860	MTB000120	958459	958509	51	+	DeJesus et al, 2017		*	*		
ncRv0897	ncRv0897	MTB000121	1000719	1000826	108	+	DeJesus et al, 2017		*			
ncRv0952	ncRv0952	MTB000122	1063969	1064101	133	+	DeJesus et al, 2017		*	*		
ncRv10996	ncRv10996	MTB000123	1113606	1113664	59	+	DeJesus et al, 2017		*	*		
ncRv11042c	ncRv11042c	MTB000124	1165548	1165613	66	-	DeJesus et al, 2017		*	*		
mpr5	ncRv11051	MTB000061	1175225	1175315	91	+	DiChiara et al, 2010	Northern				TSS
ncRv1072	ncRv1072	MTB000125	1197082	1197179	98	+	DeJesus et al, 2017		*			TSS
MTS0858	ncRv1092c	MTB000074	1220388	1220487	100	-	Arnvig et al, 2011	Northern	*			TSS
ncRv11144c	ncRv11144c	MTB000126	1271918	1271961	44	-	DeJesus et al, 2017		*			
ncRv11147c	ncRv11147c	MTB000127	1275610	1275674	65	-	DeJesus et al, 2017		*	*		
mcr10	ncRv1157	MTB000072	1283693	1283815	123	+	DiChiara et al, 2010	Northern, RLM-RACE				
ncRv11179c	ncRv11179c	MTB000128	1313343	1313452	110	-	DeJesus et al, 2017		*	*		TSS
ncrMT1234	ncRv11196	MTB000075	1340578	1340625	48	+	Pelly et al, 2012	Cloned fragment	*			
ncRv11199	ncRv11199	MTB000129	1342888	1342941	54	+	DeJesus et al, 2017		*	*		
mpr6	ncRv1222	MTB000062	1365274	1365365	92	+	DiChiara et al, 2010	Northern				
mcr11, MTS0997	ncRv11264c	MTB000063	1413094	1413224	131	-	Arnvig et al, 2011; DiChiara et al, 2010	Northern, RLM-RACE	*	*	RF02341	TSS
ncRv11264c	ncRv11147c	MTB000130	1413105	1413227	123	-	DeJesus et al, 2017		*	*		TSS
ncRv1298	ncRv1298	MTB000131	1455386	1455461	76	+	DeJesus et al, 2017		*	*		
ncRv11298	ncRv11298	MTB000132	1455406	1455461	56	+	DeJesus et al, 2017		*	*		
mcr3	ncRv11315A	MTB000064	1471619	1471742	124	+	DiChiara et al, 2010	Northern, RLM-RACE				*
ncRv11315	ncRv11315B	MTB000133	1473385	1473503	119	+	DeJesus et al, 2017		*	*		TSS
ncRv1329	ncRv1329	MTB000134	1497132	1497220	89	+	DeJesus et al, 2017		*	*		



mcr15	ncRv1364c	MTB000065	1535417	1535716	300	-	DiChiara et al, 2010	Northern , RLM-RACE					
MTS1082	ncRv11373	MTB000076	1547129	1547268	140	+	Arnvig et al, 2011	Northern	*			TSS	
ncRv1389	ncRv1389	MTB000135	1564297	1564499	203	+	DeJesus et al, 2017		*	*			
ncRv1501	ncRv1501	MTB000136	1692646	1692731	86	+	DeJesus et al, 2017		*			TSS	
ncRv1617	ncRv1617	MTB000137	1816131	1816235	105	+	DeJesus et al, 2017		*	*			
ncRv1621c	ncRv1621c	MTB000138	1821646	1821753	108	-	DeJesus et al, 2017		*				
G2	ncRv11689c	MTB000054	1914962	1915190	229	-	Arnvig and Young, 2009	Northern , RLM-RACE			RF01798	TSS	1915107-1915187
AS1726	ncRv1726c	MTB000055	1952291	1952503	213	-	Arnvig and Young, 2009	Northern , RLM-RACE					
MTS1338, DrrS	ncRv11733A	MTB000077	1960667	1960783	117	+	Arnvig et al, 2011	Northern , RLM-RACE	*				TSS
ncRv11733	ncRv11733B	MTB000139	1960667	1960774	108	+	DeJesus et al, 2017		*				TSS
ncRv11793	ncRv11793	MTB000140	2030986	2031038	53	+	DeJesus et al, 2017		*	*			
ncRv1821	ncRv1821	MTB000141	2068863	2068962	100	+	DeJesus et al, 2017		*	*			
ncRv11846, MrsI	ncRv11846	MTB000142	2096766	2096867	102	+	DeJesus et al, 2017		*	*			TSS
AS1890	ncRv1890	MTB000056	2139419	2139656	238	+	Arnvig and Young, 2009	Northern , RLM-RACE					
ncRv12023	ncRv12023	MTB000143	2268164	2268231	68	+	DeJesus et al, 2017		*	*			TSS
ASpks	ncRv2048	MTB000057	2299745	2299886	142	+	Arnvig and Young, 2009	Northern , RLM-RACE					
mcr5	ncRv2175c	MTB000066	2437823	2437866	44	-	DiChiara et al, 2010	Northern					
ncRv12220	ncRv12220	MTB000144	2489205	2489252	48	+	DeJesus et al, 2017		*				
mcr16	ncRv2243c	MTB000073	2517032	2517134	103	-	DiChiara et al, 2010	Northern Northern					
mcr7	ncRv0024	MTB000067	2692172	2692521	350	+	DiChiara et al, 2010	Northern , RLM-RACE			RF02571	TSS	
ncRv12459	ncRv12459	MTB000145	2762409	2762484	76	+	DeJesus et al, 2017		*				
ncRv12557	ncRv12557	MTB000146	2877751	2877808	58	+	DeJesus et al, 2017		*	*			
mpr11	ncRv12560	MTB000068	2881252	2881320	69	+	DiChiara et al, 2010	Northern					
mpr12	ncRv12562	MTB000069	2882185	2882276	92	+	DiChiara et al, 2010	Northern					

ncRv12641	ncRv12641	MTB000147	2966410	2966450	41	+	DeJesus et al, 2017		*	*		
ncRv12783c	ncRv12783c	MTB000148	3092761	3092886	126	-	DeJesus et al, 2017		*	*		TSS
ncRv2986c	ncRv2986c	MTB000149	3343113	3343216	104	-	DeJesus et al, 2017		*			TSS
ncRv2993c	ncRv2993c	MTB000150	3350950	3351074	125	-	DeJesus et al, 2017		*	*		TSS
ncRv13003c	ncRv13003c	MTB000151	3363029	3363152	124	-	DeJesus et al, 2017		*	*		TSS
ncRv3220	ncRv3220	MTB000152	3595951	3596059	109	+	DeJesus et al, 2017		*			
ncRv13303	ncRv13303	MTB000153	3690941	3691059	119	+	DeJesus et al, 2017		*	*		
ncRv13418cA	ncRv13418Ac	MTB000154	3837297	3837458	162	-	DeJesus et al, 2017		*	*		TSS
ncRv13418cB	ncRv13418Bc	MTB000155	3837346	3837458	113	-	DeJesus et al, 2017		*	*		TSS
ncRv3461c	ncRv3461c	MTB000156	3880231	3880294	64	-	DeJesus et al, 2017		*			
ncRv3520	ncRv3520	MTB000157	3956291	3956550	260	+	DeJesus et al, 2017		*			
mpr17	ncRv13596	MTB000070	4040879	4040938	60	+	DiChiara et al, 2010	Northern				TSS
ncRv3648c	ncRv3648c	MTB000158	4088267	4088350	84	-	DeJesus et al, 2017		*	*		
mpr18	ncRv13651	MTB000071	4093468	4093522	55	+	DiChiara et al, 2010	Northern				
ncRv13660c	ncRv13660c	MTB000159	4099384	4099477	94	-	DeJesus et al, 2017		*			
B11, 6CsRNA	ncRv13660c	MTB000058	4099386	4099478	93	-	Arnvig and Young, 2009; DiChiara et al, 2010	Northern, RLM-RACE			RF01066	TSS
MTS2823, Ms1	ncRv13661	MTB000078	4100669	4100968	300	+	Arnvig et al, 2011	Northern /RLM-RACE	*		RF02566	TSS
C8, 4.5S RNA	ncRv13722Ac	MTB000059	4168154	4168281	128	-	Arnvig and Young, 2009; DiChiara et al, 2010	Northern /RLM-RACE				TSS
ncRv13722c	ncRv13722Bc	MTB000160	4168192	4168281	90	-	DeJesus et al, 2017		*			TSS
ncRv3804c	ncRv3804c	MTB000161	4265583	4265765	183	-	DeJesus et al, 2017		*	*		4265642-4265773
ncrMT3949	ncRv3842	MTB000079	4314798	4314891	94	+	Pelly et al, 2012	Cloned fragment	*	*		
MTS2975	ncRv13943	MTB000080	4317073	4317165	93	+	Arnvig et al, 2011	Northern	*			TSS

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