Kupyaphores are zinc homeostatic metallophores required for colonization of *Mycobacterium tuberculosis*

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*Mycobacterium tuberculosis* (*Mtb*) endures a combination of metal scarcity and toxicity throughout the human infection cycle, contributing to complex clinical manifestations. Pathogens counteract this paradoxical dysmetallostasis by producing specialized metal trafficking systems. Capture of extracellular metal by siderophores is a widely accepted mode of iron acquisition, and *Mtb* iron-chelating siderophores, mycobactin, have been known since 1965. Currently, it is not known whether *Mtb* produces zinc scavenging molecules. Here, we characterize low-molecular-weight zinc-binding compounds secreted and imported by *Mtb* for zinc acquisition. These molecules, termed kupyaphores, are produced by a 10.8 kbp biosynthetic cluster and consists of a dipeptide core of ornithine and phenylalaninol, where amino groups are acylated with isonitrile-containing fatty acyl chains. Kupyaphores are stringently regulated and support *Mtb* survival under both nutritional deprivation and intoxication conditions. A kupyaphore-deficient *Mtb* strain is unable to mobilize sufficient zinc and shows reduced fitness upon infection. We observed early induction of kupyaphyses in *Mtb*-infected mice lungs after infection, and these metabolites disappeared after 2 wk. Furthermore, we identify an *Mtb*-encoded isonitrile hydratase, which can possibly mediate intracellular zinc release through covalent modification of the isonitrile group of kupyaphores. *Mtb* clinical strains also produce kupyaphyses during early passages. Our study thus uncovers a previously unknown zinc acquisition strategy of *Mtb* that could modulate host–pathogen interactions and disease outcome.

Significance

*Mycobacterium tuberculosis* (*Mtb*) is the etiological agent of human tuberculosis (TB). *Mtb* can persist inside host macrophages by successfully adapting to intracellular conditions. Acquisition of balanced amounts of essential micronutrients is one such important process. Our studies have identified a metallophore produced on demand to restore *Mtb* zinc metabolic imbalance. These diacetyl-isonitrilopeptide–ligands, named kupyaphores, are specifically induced during infection and move in and out of cells to protect bacteria from host-mediated nutritional deprivation and intoxication. Furthermore, we identify an *Mtb* isonitrile hydratase homolog, expressed in low-zinc conditions, which probably facilitates zinc release from kupyaphores. Identification of this zinc acquisition strategy could provide opportunities in future to understand systemic zinc dysbiosis and associated manifestations in TB patients.

Infection of humans by *Mycobacterium tuberculosis* (*Mtb*) has been the leading cause of mortality and morbidity for more than a century (1). This debilitating chronic pathogenesis is attributed to the ability of *Mtb* to manipulate and survive within the phagocytic cells of the immune system (2). *Mtb* colonization of macrophages initiates a competitive conflict for utilization of host nutrients, including elements such as iron, zinc, copper, magnesium, and selenium (3, 4). By limiting transition metal availability to invading pathogens, the host is known to restrict pathogen proliferation, a physiological concept called “nutritional immunity” (5). The pathogen endures through these early phases of adaptation by employing mechanisms that facilitate continual access to host micronutrient supply. Paradoxically, excess free metal ions can exert toxic effects on microbial survival. In fact, studies suggest that human macrophages intoxicate intracellular Metals and micronutrients are required for colonization of *Mycobacterium tuberculosis*...
*Mtb* with a burst of free zinc and copper (6–8). It is fascinating that these two strikingly opposing outcomes are resisted by intracellular pathogens like *Mtb* for the maintenance of zinc metallostatosis.

The adaptive response strategy of bacterial pathogens to host-imposed zinc scarcity and poisoning is primarily governed by metal-sensing metalloregulatory proteins. In many bacteria, *Mtb* contains two key zinc sensor proteins that coordinate function as uptake or efflux repressors. *Mtb* zinc uptake repressor (*zur*, *Rv2359*) regulates expression of several genes (9), including those involved in zinc uptake, while the efflux regulator (*zntR*, *Rv3334*) regulates transcription of genes encoding three types of exporters (10). *Mtb* has been shown to utilize P1-type ATPases to neutralize the toxic effects of zinc in macrophages (7). *Mtb* also employs zinc sparing to overcome zinc starvation, for example, remodeling of the ribosome 70S subunit (11). Similar mechanisms of metallostatosis also govern the intracellular levels of iron during the host–pathogen interaction (12). In contrast, the general bacterial strategy of iron acquisition involving siderophore-mediated scavenging is yet to be recognized for other transition metals in *Mtb*. *Mtb* requirement of iron is accomplished by producing the siderophores—mycobactin and carboxymycobactin (13, 14). These siderophores move in and out of cells scavenging iron, releasing metal ions in the intracellular milieu, and then recycling the apo-siderophores. Ferric ion is released via reduction to ferrous ion or by modification of the siderophore scaffold using specific hydrolases (15). It is thus crucial to understand whether *Mtb* produces specialized iron siderophore-like small molecules on demand for zinc acquisition.

*Staphylococcus aureus* and *Pseudomonas aeruginosa* have been shown to produce staphylocline (16) and pseudopaline (17), respectively, for sequestering and importing metal ions, including zinc, from the host milieu to enhance the virulence and fitness of the intracellular bacteria (18). Although these molecules are not specific for zinc, opine-like metabolites have been referred to as zincophore and their biosynthesis is proposed to be widely distributed in bacteria (19). Recently, another class of low-molecular-weight metal chelating disonitrile containing bacterial metabolites SF2768 and isonitrile lipopeptides have been identified from *Streptomyces thioluteus* and *Mycobacterium* *tuberculosis* (20, 21). However, these metabolites could not be isolated from their natural hosts, but by heterologous overexpression of cognate biosynthetic gene clusters in host systems *Streptomyces lividans* and *Escherichia coli*, respectively. The biosynthetic clusters of these disonitrile-containing metabolites includes a nonribosomal peptide synthase (NRPS), fatty acyl-AMP ligase (*FAAL*), type II thioesterase, oxidoreductase and acyl carrier protein (*ACP*). Interestingly, a homologous five-gene biosynthetic cluster (*Rv0097–Rv0101*) can also be identified in the genome of *Mtb*, with the NRPS gene (*Rv0101*) being double the size (7,539 bp), as compared to other organisms. The mutant (*Δnrps*) *Mtb* strain has been shown to grow at a lower rate in immunocompetent and immunocompromised mice, causing less lung pathology, and was associated with significantly increased mice survival rates (22). Despite the role of this biosynthetic cluster in *Mtb* virulence, the metabolite produced is not known. Moreover, the metabolic function as well as significance of this large 10.8 kbp genomic locus in tuberculosis (TB) pathophysiology remains to be elucidated.

In this study, we have isolated and characterized diacyl-disonitrile lipopeptides, named kupyaphores, from human pathogen *Mtb*. Kupyaphores are stringently regulated and can move in and out of cells scavenging zinc to maintain bacterial fitness. These metabolophores are biosynthesized on demand during early phases of *Mtb* infection and here we delineate pathophysiological relevance of kupyaphores in zinc mobilization and redistribution. Further, we identify an isonitrile hydrolase homolog in *Mtb* that is expressed in low-zinc conditions, and probably facilitates zinc release from these kupyaphores.

### Results

#### Transcriptional Responses Maintain *Mtb* Zinc Homeostasis.

Analysis of genomic loci of the cryptic biosynthetic cluster *Rv0097–Rv0101* suggested classic zur-binding sites in the putative promoter regions, 61 bp upstream of *Rv0096* and 354 bp upstream of *Rv0099*. Previous studies with zur-deleted *Mtb* strain did not show significant differential regulation for any of the genes from the *Rv0097–Rv0101* cluster (9). Since these studies were carried out in the metal-rich Middlebrook 7H9 medium, we decided to examine cultures at low and transcriptional responses at varying zinc conditions in the defined minimal Sauton’s medium. The Middlebrook 7H9 medium used for culturing *Mtb* contains 6 μM concentration of ZnSO4 and we performed wild-type *Mtb* (WT *Mtb*) growth kinetics at log-order lower and higher zinc concentrations. WT *Mtb* growth curves at 0.1 μM (low), 6 μM (optimal), and 50 μM (high) of zinc showed no significant differences (Fig. 1A). WT *Mtb* intracellular total zinc levels, as measured by inductively coupled plasma mass spectrometry (ICP-MS), were also similar for all three conditions (Fig. 1B).

*Rv0099* is a member of a previously identified family of FAALs, and in the genomic context are found adjacent to polyketide synthases (PKS) or NRPS. All of these genomic clusters in *Mtb* produce virulent lipidic metabolites and show modest regulation at the transcriptional level (23, 24). With an anticipation to obtain a robust transcriptional response, we carried out five passages of WT *Mtb* in the minimal Sauton medium treated with chelax X-100 resin (Fig. 1C). Adapted *Mtb* cells were then inoculated into low-, optimal-, and high-zinc conditions. RNA was isolated and log-phase cultures and transcriptional analysis performed using an Illumina MiSeq sequencing platform for three biological replicates. After examining consistency of the raw reads (*SI Appendix*, Fig. S1A), we probed relatedness between these samples by using principal component analysis (*SI Appendix*, Fig. S1B). We observed tight clustering for all the three replicates of low- and high-zinc conditions, whereas one of the biological replicates showed greater variation from the optimal-zinc dataset.

To account for biological variation, we analyzed by including and excluding this dataset. This analysis yielded similar results for most of the significantly differentially expressed genes, many of which have been previously shown to be zinc (or metal) responsive genes. We compared differential expression analysis between 50 μM and 0.1 μM with cut off log2 fold-change > 0.5 and false-discovery rate < 0.2 and visualized the data using an MA plot (Fig. 1D). This analysis identified a small set of 19 genes to be differentially regulated. Up-regulated genes included zinc transcription repressors *smtb* and *zur* (9) and efflux zinc pump *cpc* (7), *Rv2052c* (25), and *cad II* (26). Genes belonging to the *esx-1* and *esx-3* export system showed highest down-regulation at both high- and low-zinc, when compared to optimal level of zinc. Previous study had suggested a role of these clusters in metal-dependent regulation (27). Of the FAAL-PKS/NRPS gene clusters, some of the genes belonging to mycobactin, phthiocerol dimycocerosate and the biosynthetic cluster *Rv0097–Rv0101*, showed modest up-regulation at both low- and high-zinc conditions (*SI Appendix*, Fig. S1C). Expression changes of the genes from *Rv0097* to *Rv0101* were corroborated by carrying out qRT-PCR analysis. We observed statistically significant up-regulation for four of five genes at both low- and high-zinc conditions, when compared with optimal conditions (Fig. 1E). We therefore hypothesized that this cluster may have involvement in *Mtb* zinc metallostatosis.
codes for a bimodular NRPS. We generated a NRPS knockout reading frame (Rv0101) of the Rv0097–Rv0101 gene cluster ing the biochemical function (22, 28, 29). The largest open metal homeostasis (20) has been implicated in cluster Rv0097–Rv0101 spans 10.8 kbp, which over the years of biological replicates), Mycobacterium tuberculosis of Mehdiratta et al. (Mtb) showed no growth profile differences under planktonic conditions in the 7H9 medium (21) compared to optimal zinc concentration. Data represent mean ± SEM with varying zinc concentrations: 0.1 μM, 6 μM, and 50 μM. (E) ICP-MS analysis of total intracellular zinc content of Mtb grown in these three conditions. (C) Experimental design for generation of low-metal adapted WT Mtb strain. (D) MA plot of differential gene expression analysis in a 50 μM zinc supplemented condition as compared to 0.1 μM zinc supplementation with log2 fold-change > 0.5 and false-discovery rate < 0.2 as cutoff. Genes marked in red are significantly up-regulated, while genes marked in blue are significantly down-regulated. (E) Gene-expression analysis of Rv0097–Rv0101 through qRT-PCR under low- and high-zinc conditions as compared to optimal zinc concentration. Data represent mean ± SEM (n = 3 biological replicates), P value indicated for each data point.

Fig. 1. Mtb transcriptional responses maintains zinc homeostasis. (A) Growth kinetics of WT Mtb grown in chelated Sauton's medium supplemented with varying zinc concentrations: 0.1 μM, 6 μM, and 50 μM. (B) ICP-MS analysis of total intracellular zinc content of Mtb grown in these three conditions. (C) Experimental design for generation of low-metal adapted WT Mtb strain. (D) MA plot of differential gene expression analysis in a 50 μM zinc supplemented condition as compared to 0.1 μM zinc supplementation with log2 fold-change > 0.5 and false-discovery rate < 0.2 as cutoff. Genes marked in red are significantly up-regulated, while genes marked in blue are significantly down-regulated. (E) Gene-expression analysis of Rv0097–Rv0101 through qRT-PCR under low- and high-zinc conditions as compared to optimal zinc concentration. Data represent mean ± SEM (n = 3 biological replicates), P value indicated for each data point.

Significance of Mtb nrps in Mycobacterial Physiology. Mtb gene cluster Rv0097–Rv0101 spans 10.8 kbp, which over the years has been implicated in Mtb virulence, without exactly delineating the biochemical function (22, 28, 29). The largest open reading frame (Rv0101) of the Rv0097–Rv0101 gene cluster codes for a bimodular NRPS. We generated a NRPS knockout strain in Mtb (Δnrps) to assess the role of this gene cluster in metal homeostasis (SI Appendix, Fig. S2A). The mutant strain showed no growth profile differences under planktonic conditions in the 7H9 medium (SI Appendix, Fig. S2B). However, the Δnrps Mtb mutant exhibited differences under biofilm growth conditions as reported earlier, and formed a fragile film lacking the significant reticulation of WT Mtb biofilms (29). This mutant phenotype could be reversed on complementation with an integrative shuttle cosmid vector containing an Mtb H37Rv fragment spanning Rv0096 to Rv0110 (SI Appendix, Fig. S2 C and D). We then examined the growth profiles of WT Mtb and Δnrps strains in the metal-limited chelated Sauton’s medium. The Mtb Δnrps strain displayed defective growth in the Sauton’s medium, which contains only glycerol and asparagine as sole carbon and nitrogen sources. We then examined the rescue of this growth defect by adding various divalent metals one at a time at 6 μM concentration. Δnrps growth could be restored only upon zinc supplementation to Sauton’s medium and no reversal in growth phenotype could be obtained for other metals, such as copper, magnesium, manganese, and iron (Fig. 2A).

We then evaluated growth of WT, the Δnrps mutant, and the complemented strain (Δnrps:nrps) at conditions of both low- (Fig. 2B) and high-zinc (Fig. 2C) levels. While the Δnrps Mtb mutant showed significantly reduced growth under both of these conditions, the complemented strain showed growth kinetics similar to WT Mtb. To understand whether the mutant strain had disrupted metal homeostasis, we measured metal ion concentrations within Mtb cells by using ICP-MS. At low-zinc conditions, the Δnrps mutant strain showed a significant decrease in zinc levels (Fig. 2D), whereas zinc accumulation could be observed at high-zinc conditions (Fig. 2E). The complemented Mtb strain (Δnrps:nrps) profiles were similar to WT cells. There was slight difference for copper levels between the mutant and WT (SI Appendix, Fig. S2E), and other metals—such as magnesium, cobalt, nickel, and manganese—showed no significant change across all three Mtb strains.

To understand the significance of the Rx0097–Rx0101 biosynthetic cluster in the context of Mtb pathogenesis, ex vivo infection experiments were performed with an immortalized murine bone marrow–derived RAW 264.7 macrophage cell line with WT and Δnrps Mtb strains. Within 2 h of infection, remarkable up-regulation of the Rx0097–Rx0101 gene cluster could be noted in WT Mtb cells (SI Appendix, Fig. S3A). This up-regulation response dampens in 24 h in the macrophage infection assays, suggesting an early role of the Rx0097–Rx0101 cluster during Mtb infection. Furthermore, the intracellular free-zinc levels for WT, Δnrps and complemented Mtb were measured using FluoZin-3-AM dye, a zinc-selective indicator, 2 h postinfection. To observe Mtb cells in a fluorescence microscope, all the three strains were transformed with plasmid expressing mCherry. Macrophages infected with WT Mtb showed increased punctate signals for free-zinc, as compared with infected cells (Fig. 3A). Surprisingly, weak free-zinc signals could be observed in the mutant-infected macrophages, as measured by FZ3-AM. The complemented strain (Δnrps:nrps) showed similar free-zinc levels to the WT Mtb strain as quantitated by confocal microscopy (Fig. 3B). Interestingly, almost all WT Mtb cells colocalized with free-zinc, as reported earlier (7).

To further understand these changes in zinc dynamics upon infection, we compared the zinc pools of macrophages and bacteria by performing differential lysis of WT and Δnrps infected cells. The total zinc levels were then measured by ICP-MS analysis. The total zinc levels of either of the infected macrophages (WT or Δnrps Mtb) were found to be comparable to uninfected macrophages (SI Appendix, Fig. S3B). Analysis of the total zinc pools of the internalized Mtb, however, interestingly revealed the zinc levels to be significantly low in Δnrps as compared to WT Mtb (SI Appendix, Fig. S3C). No significant difference could be observed between the two strains for any other metal ion (SI Appendix, Fig. S3 D and E). Together, the induction of Rx0097–Rx0101 biosynthetic pathway and altered intracellular zinc concentration in Δnrps Mtb strain provide evidence for the involvement of the unknown metabolite produced by this cryptic gene cluster in maintenance of bacterial zinc homeostasis.

Computational and Biochemical Analysis of Mtb nrps Biosynthetic Gene Cluster. To predict the metabolite produced by the biosynthetic operon Rx0097–Rx0101, we carried out detailed in silico analysis and also confirmed biochemical functions for three enzymes by performing in vitro assays. Retro-biosynthetic analysis based on previous studies suggested a putative pathway wherein FAAL10 (Rx0099) activates α/β unsaturated long-chain fatty acids to corresponding acyl-adenylates, which are then transferred onto the thiol group of phosphopantetheine group of ACP (Rv0100) protein (30, 31). This ACP-bound lipid chain can be modified by Rx0097 and Rx0098, as shown previously (21, 32). Mtb NRPS (Rx0101) contains two modules (bimodular), each consisting of three enzymatic domains:
The second module of this NRPS protein contains a reductase (R) domain, which releases thioester-bound lipopeptides as corresponding alcohols (35) (Fig. 4A). Protein sequence analysis of the two modules of NRPS revealed the two C domains (C1 and C2) shows weak sequence homology (similarity/identity, 35/36%).

Recently, genome mining studies have revealed similar five-gene clusters from various Actinomycetes (20). We performed dendrogram-based analysis of C domains from NRPS proteins (unimodular or bimodular) of these five-gene clusters along with other atypical C domains. This analysis divided the C domains into two discrete clusters (SI Appendix, Fig. S4A), indicating that they may have different catalytic functions. Interestingly, C2 domains from the bimodular NRPS clusters clustered along with the typical C domains, whereas C1 domains formed a separate branch. Though both the domains are of similar length and share the conserved catalytic HHXXXDG motif, structural modeling of C1 domains show substantial homology to CoA-dependent acyltransferases. All C1 domains show unique conservation of amino acid residues around the active site pocket, suggestive of functional divergence from the cognate C domains (SI Appendix, Fig. S4 B and C) and we therefore refer to these as C1*. Based on reported studies of metabolites isolated from the heterologous overexpression systems for M. marinum and S. thiolatus (20, 21), we reasoned that this unusual C1* domain may be involved in the transfer of acyl chains on both the α-amino and ε-amino groups of a lysine.

To corroborate our computational prediction, we performed biochemical reconstitution of Rv0097 and Rv0098. Our previous studies showed FAAL10 (Rv0099) to activate long-chain fatty acids as acyl-adenylates and transfer them onto the thiol group of phosphopantetheine ACP (Rv0100) protein (35). We confirmed similar activity for αβ unsaturated long-chain fatty acids. We cloned and expressed Rv0097 and Rv0098 in E. coli and purified the proteins using affinity chromatography. Enzymatic assays were then performed by using phosphopantetheine mimics, N-acetyl cysteamine (NAC) thioesters of αβ unsaturated fatty acids of different carbon lengths: C4, C8, and C12 (Fig. 4B and SI Appendix, Fig. S5). Enzymatic activities were confirmed using liquid chromatography coupled to mass spectrometry (LC-MS) assay protocols (36, 37). Addition of glycine to NAC-thioester of dodecanedioic acid in the presence of enzyme Rv0098 identified a molecular ion peak at m/z of 374.2239, corresponding to Michael addition of glycine across unsaturated fatty acyl thioester. MS/MS analysis of this ion resulted in fragment ions of m/z [M-H]- = 254.176, 74.025, and 58.030, providing confidence to the structure (Fig. 4C). Mass peak with m/z [M-H]- = 265.192 probably corresponds to the hydrated form of the fragment ion of m/z = 248.0831. No activity was observed for NAC-thioester of crotonoic and minor products were obtained with octenoic acids. When the purified protein Rv0097 was included in the above assays, a new peak corresponding to m/z of 326.2085 could be observed in high-resolution LC-MS (LC-HRMS). This molecular ion, along with fragment ions, corresponded to the isonitrile modification of the of C12 fatty acyl-SNAC moiety (Fig. 4D).

Such an isonitrile modified C12 ACP-bound acyl chain could then condense with first amino acid, which is selected by the adenylation domain (C1*-A1-T1). Computational algorithms predict the adenylation domain of the first module of the NRPS protein (C1*-A1-T1).
A1 to activate lysine, while the second A2 domain shows specificity for phenylalanine (38, 39). Finally, the acyl chain would be reductively released to the corresponding alcohol, thus producing a novel isonitrile lipopeptide.

Identification and Characterization of Diacyl-Diisonitrile Lipopeptides from Mtb. In order to isolate these molecules from Mtb, we extracted metabolites from Mtb cells grown in Middlebrook 7H9 medium and from biofilm using ethyl acetate. These samples were analyzed by LC-MS/MS using a recently developed method from our laboratory (36). Analysis of Mtb biofilm extract detected a cluster of unique peaks with m/z 707.5350, 721.5506, 735.5663, 763.5967, 833.6758, 847.6915, and 861.7071, which were absent in the Mtb planktonic cultures grown in Middlebrook 7H9 medium (Fig. 5A). The parent ion masses (I–VII) differed by a multiple of 14, typical of series with varying –CH2 group and thus can be attributed to different acyl chain lengths. The previously reported molecular formula C42H74N6O5 with m/z of 717.5612 was absent in our biofilm-analyzed samples (29). MS/MS fragmentation patterns of parent ions revealed a common set of fragments with m/z values of [M+H]+ = 266.1863, 152.1080, and 133.099, and 89.1073 (Fig. 5B). The m/z of 266.1863 along with the fragments of m/z 152.1080 and 133.099 can be assigned to phenylalaninol ion and ornithine ion, respectively. A peak of 89.1073 corresponds to decarboxylation of the ornithine ion, which suggests the backbone to be composed of a common core of ornithine and phenylalaninol dipeptide. Concomitantly, these peaks were absent in the biofilm metabolic extract of Δnrps but could be detected in the Δnrps:nrps strain (SI Appendix, Fig. S6A). Additionally, negative ion mode LC-MS analysis of the biofilm extract revealed two types of metabolites: symmetric (with identical acyl chains on the two –NH2 groups of ornithine) and asymmetric (with different acyl chains on the two –NH2 groups of ornithine) (SI Appendix, Fig. S6B and C). Recently, click chemistry-based analytical detection of isonitrile using tetrazine has been reported (40). Tetrazine treatment of WT Mtb biofilm extract rapidly changed the color from pink to yellow (Fig. 5C) and the signals from MS analysis corresponding to I–VII diminished substantially (SI Appendix, Fig. S6D). Furthermore, the universal reaction product Py-aminepyrazoles with m/z [M+H]+ = 238.1014 could be detected only in the tetrazine-treated samples (Fig. 5D).

Furthermore, to confirm the isonitrile moiety and the overall chemical structure of the metabolite, we synthesized an analog of C18 lipopeptide with amine functionality at the β

Fig. 3. Mtb nrps modulates host free-zinc dynamics. (A) Free-zinc labeling using FZ3-AM dye for uninfected, mCherry-labeled WT, Δnrps, and Δnrps:nrps Mtb strains infected murine macrophages 2 h postinfection. (B) FZ3-AM signal quantification from 75 cells for each condition. Unlike WT and Δnrps:nrps macrophages, Δnrps-infected macrophages do not show elevated free-zinc levels as compared to uninfected macrophage. Data represent mean ± SEM (n = 3 biological replicates) with P values indicated for each data point.
position. Previous studies had reported facile conversion of nitriles into amines on reaction with mild acid (41). T oward this end, protected β-amino fatty acid was coupled with L-ornithine and L-phenylalaninol sequentially, followed by deprotections resulting in the desired lipopeptide (SI Appendix, Fig. S7 A–K). Addition of formic acid to the biofilm metabolite extract resulted in disappearance of m/z for [M+H]+ ion 848.6987 (VI), having a retention time of 36 min, and a new peak could be observed at m/z for [M+H]+ ion 828.73 with a retention time at 34 min. This new peak corresponded to the chemical synthetic standard compound (Fig. 5E). Additionally, both the parent peak and synthetic standard had identical MS/MS fragments (SI Appendix, Fig. S7L). Our studies thus identify and characterize a series of diacyl-diisonitrile lipopeptides from Mtb that consists of a dipeptide core of ornithine and phenylalaninol, where the amino-groups are acylated with isonitrile-containing fatty acyl chains ranging from C13 to C19. Based on their metal acquisition functionality, we propose to name these Mtb diacyl-diisonitrile lipopeptides as “kupyaphores” (Kupya in Sanskrit refers to rare metals and phores means carrier in Latin), and this gene cluster could be recognized as kupya A-E.

Fig. 4. Biochemical analysis of Mtb Rv0097–Rv0101 gene cluster. (A) Schematic representation of putative biosynthetic steps for unknown metabolite production by Mtb Rv0097–Rv0101 gene cluster. Mtb harbors bimodular NRPS with seven catalytic domains. (B) Reaction scheme for enzymatic assays of purified Rv0098 and Rv0097 proteins with chemically synthesized C12-SNAC as substrate. (C) MS/MS spectra showing fragmentation products of glycine adduct of 2-dodecanoc-SNAC, 1, detected in the enzymatic assay with Rv0098 purified protein. No product could be detected in control reactions with no protein or no glycine. (D) MS/MS spectra of additional peak of m/z 326.2028 corresponding to isonitrile adduct of 2-dodecanoc-SNAC, 2, observed upon addition of Rv0097 purified protein to the Rv0098 assay conditions. The calculated masses for metabolites 1 and 2 are within 5-ppm mass error tolerance at M51. All assays in C and D were performed in triplicates with reproducible results each time.
We next set out to understand the temporal regulation of this zinc acquisition machinery in the mouse infection model. Previous mice infection studies with this Δnrps mutant in immunocompetent and immunocompromised mice showed reduced lung pathology with significantly increased mice survival rates (22). This phenotype was attributed to impairment in the early infection events associated with the mutant Mtb strain. We therefore carefully dissected the early infection events from day 1 to day 28 (Fig. 6A). The mutant strain indeed showed more than one log-phase growth defect till week 3 postinfection, as compared to WT Mtb (Fig. 6B). This compromised growth of mutant Mtb highlights the significant role of this gene cluster in successful establishment of TB infection.
A portion of the lung tissue was also utilized for extraction of metabolites using ethyl acetate. LC-MS analysis of the tissue organic extracts of WT Mtb- and Δnrps-infected lung tissues showed clear signals at m/z of [M+H]+ = 787.6315, which corresponds to the Mtb-specific respiratory quinone, menaquinone (Fig. 6C). This confirms the presence of Mtb in the host lung tissue. Menaquinone peak was absent in the uninfected animal’s lung tissue extract. Furthermore, we performed semi-quantitative measurements for kupyaphore masses using multiple reaction monitoring (MRM) LC-MS analysis. Signals for kupyaphores VI, II, and VII species could be detected only from the WT Mtb-infected lung tissues and these were absent in Δnrps mutant and uninfected lung tissue samples. Kupyaphore was absent on day 1 in WT Mtb and metabolite peaks built up from day 6 to day 15, then declined rapidly by day 21. Kupyaphores completely disappeared by day 28. Two series of kupyaphore peaks were absent in Δnrps mutant and uninfected lung tissue samples. Kupyaphore was absent on day 1 in WT Mtb and metabolite peaks built up from day 6 to day 15, then declined rapidly by day 21. Kupyaphores completely disappeared by day 28. Two series of kupyaphore peaks were absent in Δnrps mutant and uninfected lung tissue samples. Kupyaphore was absent on day 1 in WT Mtb and metabolite peaks built up from day 6 to day 15, then declined rapidly by day 21. Kupyaphores completely disappeared by day 28. Two series of kupyaphore peaks were absent in Δnrps mutant and uninfected lung tissue samples. Kupyaphore was absent on day 1 in WT Mtb and metabolite peaks built up from day 6 to day 15, then declined rapidly by day 21. Kupyaphores completely disappeared by day 28. Two series of kupyaphore peaks were absent in Δnrps mutant and uninfected lung tissue samples. Kupyaphore was absent on day 1 in WT Mtb and metabolite peaks built up from day 6 to day 15, then declined rapidly by day 21. Kupyaphores completely disappeared by day 28. Two series of kupyaphore peaks were absent in Δnrps mutant and uninfected lung tissue samples. Kupyaphore was absent on day 1 in WT Mtb and metabolite peaks built up from day 6 to day 15, then declined rapidly by day 21. Kupyaphores completely disappeared by day 28. Two series of kupyaphore peaks were absent in Δnrps mutant and uninfected lung tissue samples. Kupyaphore was absent on day 1 in WT Mtb and metabolite peaks built up from day 6 to day 15, then declined rapidly by day 21. Kupyaphores completely disappeared by day 28. Two series of kupyaphore peaks were absent in Δnrps mutant and uninfected lung tissue samples. Kupyaphore was absent on day 1 in WT Mtb and metabolite peaks built up from day 6 to day 15, then declined rapidly by day 21. Kupyaphores completely disappeared by day 28. Two series of kupyaphore peaks were absent in Δnrps mutant and uninfected lung tissue samples.

Moreover, to assess whether secreted kupyaphores are involved in zinc uptake from the environment, we incubated WT, Δnrps, and Δnrps:Δnrps Mtb strains with 0.5 μM radioactive zinc-65 for 4 h. After 4 h, the internalized zinc-65 levels were measured in the cells. Significantly low radioactive counts were noted in Δnrps Mtb cells, when compared to WT and Δnrps:Δnrps Mtb strains, as measured by autoradiogram (Fig. 7C and D). Together, these two studies directly demonstrate that absence of kupyaphores results in decreased zinc uptake efficiency, resulting in Δnrps mutant growth defect. We also attempted to characterize dimeric zinc-bound kupyaphores using MS. Supernatant of WT Mtb grown with zinc supplementation showed doubly charged peaks that correspond to the kupyaphore–zinc complex and were absent in WT Mtb cultures grown in the absence of zinc in chelated Sauton’s media (Fig. 7E). Our study thus demonstrates role of kupyaphore in restoration of bacterial zinc metabolic imbalance.

Putative Mechanism of Zinc Release from Kupyaphore by Mtb Isonitrile Hydratase. Since zinc is a redox-inert metal, the release of zinc from metallophores cannot follow the classic reductive release mechanism known for iron-siderophore. In the case of enterobactin, siderophore hydrolase is known to modify the scaffold mediating iron release (15). We therefore analyzed the Mtb genome to investigate the presence of putative isonitrile modifying enzyme. This enzyme activity (InhA) was first reported from Pseudomonas putida and was recently characterized (SfaF) from
the SF2768 biosynthetic cluster of S. thioluteus (42, 43). Protein sequence analysis of the Mtb H37Rv genome coding sequence with InhA identified Rv0052 with an E-value score of 5e−31. Careful analysis of Rv0052 sequence with the three-dimensional structure (PDB ID code 3NON) indicated the absence of an important catalytic site (D17) from the annotated Rv0052. The gene Rv0052 is located between 57410 and 57973 bp in the genome of H37Rv. Analysis of upstream sequences revealed another start site 57 bp upstream in-frame with annotated Rv0052. A protein coding sequence of this open reading frame shows conservation of the catalytic residue D17. This protein was conserved across all MTBC strains and was absent from nonpathogenic mycobacterial species, where the kupya cluster is absent (SI Appendix, Fig. S9A).

To examine biochemical function, we cloned and expressed Rv0052 in BL21-DE3. The protein was purified and enzymatic assays were performed along with Rv0098 and Rv0097 (Fig. 8A). A clear peak corresponding to m/z of 243.176 for the N-formamide product could be observed only in the reactions supplemented with Rv0052 (SI Appendix, Fig. S9B). MS/MS fragmentation in the negative ion mode also provided further confidence to the hydration of isonitrile unit (Fig. 8B). Interestingly, Rv0052 expression was found to be significantly up-regulated by qRT-PCR only in low-zinc conditions and showed no induction under high-zinc conditions (Fig. 8C). Next, to investigate whether Rv0052 can directly modify kupya- phores, we incubated WT Mtb culture extract with purified Rv0052. Significant reduction in kupya- phore VI mass signals could be observed upon treatment with Rv0052, with concomitant appearance of new peaks corresponding to mono- and di-formamide kupya- phore analogs of m/z for [M+H]+ = 866.7021 and 884.7216, respectively (Fig. 8 D and E). Mass peaks corresponding to formamide and diformamide kupya- phore analogs could not be detected in the absence of Rv0052 (SI Appendix, Fig. S9C). These studies clearly demonstrate that Rv0052 can modify the isonitrile moiety of kupya- phore, which reduces the Lewis base character and destabilizes the coordinate complex bond with zinc. We thus propose that this could be a putative mechanism of zinc release from kupya- phores under low-zinc conditions.

Discussion

Given that there is virtually no free-zinc in the cell and that this micronutrient cannot be produced de novo, it becomes mandatory for pathogens to acquire these from the host pools. Bacteria have therefore evolved rather intricate molecular mechanisms of metal ion sensing, uptake, efflux, and allocation to maintain homeostasis (44). We herein decipher a mechanism of zinc acquisition, as well as quenching of toxic zinc levels (SI Appendix, Fig. S9D). Previous studies have indicated other isonitrile lipopeptides to chelate copper ions (20, 21, 46). Although at this stage we do not completely rule out the ability of kupya- phore to also bind to copper, the detailed binding affinities studies for various metal ions awaits total synthesis of kupya- phores. It is rather peculiar that the identification of these zinc chelating metallophores took this long, as the iron siderophores, mycobactins, of Mtb were discovered 70 y ago (47). The secretion of kupya- phores by WT Mtb siderophore-negative strains free-zinc pools within phagosomes, as detected by zinc-selective dye. In contrast, the Δrps mutant shows significantly reduced signals and the levels of free-zinc are similar to uninfected macrophages. However, the total zinc pools within the macrophages in the case of mutant infection are maintained, suggesting that the Δrps mutant is unable to mobilize host zinc in the absence of kupya- phores. Our results thus suggest that kupya- phores can efficiently modulate host free-zinc redistribution and homeostasis; mechanisms underlying this triggered free-zinc redistribution require further investigation.

From a clinical perspective, low zinc levels have been reported in sera of TB patients as compared to healthy controls (48). Surprisingly, zinc supplementation does not restore zinc levels nor improve the clinical outcome in TB patients (49). Interestingly, studies with a mouse cachexia model of cancer have shown that proinflammatory responses modulate expression of zinc transporter, leading to a drop in serum zinc levels with concordant accumulation of zinc in tissues resulting in cachexia (50). With the discovery of kupya- phores as Mtb zinc acquisition machinery, it will be interesting to explore the dynamic processes of zinc mobilization and redistribution that could influence TB pathogenesis, and investigate relatively unexplored areas of TB-associated cachexia.

Methods

Bacterial Culture. M. tuberculosis H37Rv were routinely grown in Middlebrook 7H9 culture medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC). 0.05% Tween-80 under shaking conditions or on Middlebrook 7H11 agar with 10% OADC at 37 °C. For metal studies, Mtb strains were grown in chelated Sauton’s medium supplemented with ZnSO4 were lysed by boiling in 0.1% SDS and 0.2% HNO3 for 15 min and total metal concentrations were measured by ICP-MS (ThermoXcalibur II). Details provided in the SI Appendix.

Determination of Total Metal Ions in In Vitro Bacterial Cultures. Briefly, 0.8 to 1.0 OD WT Mtb cultures grown in chelated Sauton’s medium supplemented with ZnSO4 were lysed by boiling in 0.1% SDS and 0.2% HNO3 for 15 min and total metal concentrations were measured by ICP-MS (ThermoXcalibur II). Details provided in the SI Appendix.

Determination of Free Zinc in Macrophage Infection Studies. Macrophage cultures untreated or infected with mCherry labeled Mtb strains were stained with 0.5 μM F23-AM and fixed with 4% paraformaldehyde. Details of staining...
protocol are provided in the SI Appendix. Fixed cells were then mounted with DAPI, mCherry, and FZ3 green cells were selected for each of the triplicate samples by confocal microscopy using Zeiss LSM980 and mean pixel intensity for FZ3 signals was analyzed using ImageJ.

**Extraction and Analysis of Kupyaphores from Mtb Cultures.** Middlebrook 7H9 grown planktonic and biofilm cultures of WT Mtb, Δnrps, and Δnrps:nrps were harvested and weighed. Supernatant and cells of the WT Mtb strain from planktonic cultures of chelated Sauton’s medium
supplemented with and without zinc were also collected separately. Low-molecular-weight molecules were then extracted by ethyl acetate and analyzed by an information dependent acquisition scanning on a Sciex X500R QTOF mass spectrometer fitted with an ExionLC UPHLC system using the SciexOS software using previously reported methods (36, 37). Details are provided in the SI Appendix.

Mtb Metabolite Isolation upon Murine TB Infection. For Mtb metabolite extraction from mice, 0.1 g of lung tissue was taken from left apical lobe of uninfected and infected mice at indicated time points. Low-molecular-weight metabolites were extracted by homogenizing tissues in ethyl acetate (36, 37). All the species analyzed were quantified using the MRM-HR LC-MS method on a Sciex X500R QTOF mass spectrometer, details of which are provided in the SI Appendix.

**Radioactive Zinc-65 Uptake Assay.** An equal number of WT, Δnrps, and Δnrpsnrps Mtb cells were incubated with 0.5 μM of radioactive zinc-65 for 4 h. After 4 h, cells were lysed. Intracellular radioactive count was then measured by autoradiography and quantitated using ImageJ analysis. Mean pixel intensity so obtained was then normalized to protein content estimated by BCA for each sample. Details are provided in the SI Appendix.

**Statistical Analysis.** GraphPad Prism 8 software was used for statistical analysis. Statistical significance was analyzed by Student’s t test or one-way or two-way ANOVA, with P values mentioned for each data point when applicable. Data were plotted as the mean, with error bars representing SEM of three biological replicates for all experiments, unless stated otherwise.

**Ethics Declaration.** All mouse studies described in this paper received formal approval from the National Institute of Immunology–Institutional Animal Ethics Committee (NII-IAEC 440/17) following the guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. Studies on clinical strains isolated from TB patients described in this paper have approval from Institutional Review Board (IRB) Christian Medical College, Vellore (IRB Min No.7239). The IRB reviewed and discussed the project after going through IRB application format, patient information sheet, consent form, and other documents. Informed consent was taken from all the participants involved in the study. All of these documents were submitted to Institutional Human Ethical Committee of the Council of Scientific and Industrial Research–Institute of Genomics and Integrative Biology.

**Data Availability.** Detailed codes used for the analysis have been submitted to the GitHub repository (https://github.com/viv3kanand/MTU-Manuscript). The datasets generated during or analyzed during the current study can be...
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