REVIEW ARTICLE

Screening for Biomarkers of Actinobacteria Associated with Water-Damaged Buildings – Part 1

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Acronyms

CIRS	Chronic Inflammatory Response Syndrome
GENIE	Gene expression, inflammation explained
NGS	Next Generation Sequencing
WDB	Water-damaged building
DI	Dominance Index – Actinobacteria
PI	Prevalence Index – Actinobacteria
MTB	Mycobacterium tuberculosis

Abstract

Recent publications [1,2] have presented the view that a wider variety of microbial contaminants are responsible for adverse human health effects in susceptible individuals exposed to the microbial "soup" that results in water-damaged buildings (WDB) than previously ascribed.
Those articles presented an in-depth understanding of the expanded use of Next Generation Sequencing (NGS) to detect the bacterial taxa present in these affected environments. When correlated with data from transcriptomic assays, these studies defined specific causation of innate immune activation by a growing list of microbial colonizers. Specifically, it was reported that a correlation existed between certain Actinobacteria as shown by NGS, with differential gene activation, from a transcriptomic assay (GENIE) detecting a defined clinical response.
This review seeks to assess the published literature to find and determine the potential for candidates that could be relied upon to act as a biomarker for *Actinobacteria* in dust and other samples in order to indicate the likelihood of *Actinobacteria* dominance or prevalence, sufficient to warrant progression to confirmation by NGS, much like endotoxins are broadly accepted as a biomarker for colonization by broad spectra of Gram-negative bacilli in WDB.

Background

As referenced in the abstract, recent publications have focused attention on exposure to *Actinobacteria*[1,2] and discussed the impact on those occupants who suffer from CIRS following exposure to WDB, confirmed by studying their genetic responses with GENIE.

In these recent publications, it is stated that CIRS is a chronic inflammatory response syndrome acquired following exposure to the interior environment of a WDB with resident microbes which include filamentous fungi, *Actinobacteria*, and other bacteria, including inflammagens and toxigens. The paper opines that little is known about the pathogenesis of *Actinobacteria* exposure for those with CIRS and proceeds to teach the relevant, emergent medical and scientific background.

By analyzing the immunoreactivity of the exposed and genetically predisposed occupants and determining concurrent exposure to *Actinobacteria* in CIRS cases without significant exposure to filamentous fungi or other bacteria, they have identified specific causation for *Actinobacteria*. For the first time, it was reported that clinical progression following exposure, as confirmed by NGS, to the development of elevated levels of mitogen-activated protein kinases (MAPKs) for those treatment naïve patients with elevated DI was demonstrated. This information was followed by enhanced levels of transforming growth factor beta receptors (TGFBR)[1,2] in those treatment naïve patients with elevated Actinobacterial Prevalence Index (PI).

As detailed below, this review found several approaches that are candidates for consideration in our search for screening biomarkers of Actinobacterial contamination. The first involved determining the amino acid sequences of the specific ribosomal proteins found in Actinobacteria[4], which examined the relationships among 30 mycolic acids producing Actinobacterial strains, plus 12 strains belonging to related taxa studied. By determining the 20 N-terminal amino acids of AT-L30 preparations, they concluded that the genera with mycolic acid-containing Actinobacteria could be sub-divided into the following clusters:

- The first group included *Nocardia*, *Rhodococcus*, *Gordona*, as well as *Tsukamurella*, and
- The second group included Corynebacterium and *Mycobacterium*.

The data presented indicated that *Tsukamurella paurometabolum* is closely related phylogenetically to *Gordona*. The phylogenetic groups identified were entirely consistent with the proposal that the family *Nocardiaceae* should encompass the mycolate-containing, cell wall type IV *Actinobacteria*, specifically *Nocardia, Rhodococcus, Gordona, and Tsukamurella*. *Actinomyces* and *Micrococcus* exhibited AT-L30 amino acid sequence characteristics between *Actinobacteria* and typical eubacteria. Thus, a phylogenetically coherent species included *Nocardia, Gordona, Mycobacterium, Actinoplanes, and Micromonospora*. In the obverse, *Rhodococcus* and *Corynebacterium* were only phylogenetically diversely related species. In general, the author's results were consistent with previous 16S rRNA sequencing results, but significant differences were also found. The published data, together with previous AT-L30 sequencing data, show that phylogenetic relationships among groups can be determined by using markers other than the ribosomal gene sequences.

However, this work was unlikely to be practicable in specifically detecting *Actinobacteria* in the dust matrix where we wish to locate these organisms.

Therefore, it was thought prudent to delve into the specific biochemistry at the molecular level possessed by these Actinobacteria focusing on some of their unique properties, particularly their enzymic distinctions that could be exploited for this study.

Synopsis of Unique Biochemical Properties of Actinobacteria

While in mycobacteria, these fatty acids have very long carbon chains (C60 - 90) and may contain various oxygen functions in addition to a specific hydroxyl group[5]. Mycolic acids found in other *Actinobacteria* consist of a mixture of saturated and unsaturated fatty acids with shorter carbon chains (C22 to 36) in corynomycolic acids[67]. Also, mycolic acids were reported to play a central role in forming a second permeability barrier functionally similar to the outer membrane in Gram-negative bacteria[8,9].

Besides mycolic acids, the outer membrane of mycobacteria also contains an array of very complex lipids relevant in the infection process of the pathogenic mycobacteria, such as dimycocerosate esters (DIMs), sulpholipids, and mycoketides[10,11,12]. In the last few years, cell-free reconstitution studies demonstrated that several of these compounds were synthesized from acyl-CoA molecules, most probably derived from the fatty acid biosynthesis pathway, which was further elongated by specific FAS related enzymes known as polyketide synthases[13,14,15].

Another remarkable feature of *Mycobacteria* and *Streptomycetes*, but not *Corynebacteria*, is that de novo synthesized fatty acids were not only dedicated to building membrane phospholipids but were also incorporated into neutral lipid storage compounds, the triacylglycerides[16,17].

The biosynthesis of fatty acids is the first step in forming membrane lipids. It is essential for all cells, except the Archaea. The membranes were composed of glycerol-ether lipids instead of glycerol-ester lipids and were based on isoprenoid side chains[18]. Thus, the basic features of fatty acid synthesis pathways, like the constituent chemical reactions and the identity of the component enzymes, were the focus of intensive studies more than four decades ago[19,20,21,22,23].

Both KasA and KasB catalyze the condensation of acyl-AcpM with malonyl-AcpM, elongating the growing meromycolate chain by a further two carbons[24,25]. In *M. tuberculosis*[26] the InhA protein is demonstrated to catalyze the 2-trans-enoyl-ACP reduction, with a preference for long-chain substrates[27].

Compared with other bacterial reductases, this may have unique functional and structural properties, such as a large hydrophobic substrate-binding pocket, which correlates with its preference for long-chain substrates consistent with its role in mycolic acid biosynthesis[28,29]. The biotin carboxylase component (BC) catalyzes the first half-reaction, which involves the phosphorylation of bicarbonate by ATP to form a carboxy phosphate intermediate, followed by transfer of the carboxyl group to biotin to form carboxybiotin[30,31,32,33,34].

In vivo, the cofactor biotin is attached to the biotin carboxyl carrier protein (BCCP) via a bond between biotin's valeric acid side chain and the amino group of a specific molecule Lysine residue[35,36 37,38,39]. This enzyme has long served as a model system for biochemical and mechanistic studies of biotin-dependent carboxylases[19,31]. In addition, a third subunit is identified as part of several ACCase complexes[40,41].

The increasing volume of sequence information revealed by the genome

sequencing projects facilitated the studies of the ACCases from several *Actinobacteria*, which rapidly revealed significant aspects of their biochemical, physiological, and structural properties[40,41,42,43]. *Streptomyces coelicolor*, for example, has four genes encoding putative subunits (accA1, accA2, pccA, SCO4381), four genes encoding subunits (accB, pccB, SCO2776, SCO4380), plus two genes encoding subunits (accE, pccE)[45,46,47,48].

MTB and other free-living mycobacteria also have an unusual number of genes related to putative ACCase complexes in their genomes; these genes encode various subunits as identified 49, and one other subunit was found [41,44]. Each of the six putative ACCases presumably serves a different physiological role, providing a wide variety of units for the biosynthesis of mycobacterial lipids in such rich diversity. MTB has been found with the three putatively essential ACCases (ACCase 4, ACCase 5, and ACCase 6) (see below), according to transposon site hybridization (TraSH) analysis[50].

The activity of the ACCase 5 complex, containing a specific subunit with different amino acids in its N-terminal sequence, resulted in an enzyme with a specific activity 300-fold lower than the enzyme complex when reconstituted[41]. Further, it has been recently demonstrated that members of this group were directly involved in mycolic acid biosynthesis [42,43,52]. In addition, a condensase and an acyl-AMP ligase are involved in the condensation reaction leading to the mycolic acid formation. Therefore, it has been suggested[53] that AccD4 should be the component of a long-chain acyl-CoA carboxylase. This generates the C26 carboxylic acid involved in the last condensation step of mycolic acid biosynthesis in *S. coelicolor*[46,47].

The ACC complex was reconstituted in vitro from their purified components, and their kinetic characterization was studied with several acyl-CoA substrates[46,47,48]). The individual analysis of each subunit shows that one adopts a stable hexameric conformation, while the specific subunit was found both as a trimer and as a hexamer[46,41].

As we mentioned above, some of these complexes need a specific subunit for total enzyme activity. However, the exact molar ratio between this subunit and other subunits has not been determined. At the same time, other CT domains from biotin-dependent carboxylases were also solved by X-ray crystallography, including the 1.3S transcarboxylase subunit[37] and the 12S carboxyltransferase for pyruvate carboxylation in *P. shermanii*[54].

Studies of the tetrameric pyruvate carboxylase[56] and the glutaconyl-CoA decarboxylase (for the sodium-ion pump) have also been reported[57]. Such a dimeric, di-domain active site arrangement is conserved across the biotin-dependent CTs, including PccB, 12S (a decarboxylase)[54], GCD (a sodium-transport decarboxylase)[57,] and yeast-CT (the specific subunit of ACC)[58].

Contrary to the previous proposal that the AccD4 complexes accept long-chain acyl-CoAs as their substrates, both crystal structure and kinetic assay consistently showed that AccD5 preferentially accepts propionyl-CoA as its substrate, producing methylmalonyl-CoA, the substrate for the biosynthesis of multi methyl-branched fatty acids such as mycocerosic, phthioceranic, hydroxyphthioceranic, mycosanoic and mycolipenic acids[59,60], many of which were essential factors for virulence, survival, multi-drug resistance, and latency development.

Excellent reviews on these topics can be found elsewhere[62,33,64,66]. The first antibacterial compounds targeting the ACC were natural products moiramide B and andrimid that selectively inhibit the ACC-carboxyltransferase reaction[67,68] and

synthetic variations of each moiety of the modularly composed pyrrolidinediones have revealed much improved activities against G+ve bacteria compared to those of previously reported variants. Also, in MTB, a structure-based in silico screening was conducted with the ChemDB database [69] and the crystal structure of AccD5[70]. E. coli[71,72,73,74]) and Bacillus subtilis[76,77] in *Strep. pneumoniae* were global transcriptional repressors that simultaneously control the expression of many genes involved in fatty acid and phospholipid metabolism[76,77,79]. Also, in MTB, this was again reported[80,81].

Methods for Consideration:

Traditional Methods for Detection of Mycobacteria

Before discussing methods used for detecting mycolic acids, it may be interesting to grasp traditional strategies employed to identify species of the *Mycobacterium* genus. These have included observations of staining properties of bacilli, cultural morphology, biochemical tests, and, rarely nowadays, the inoculation of susceptible animals with live bacilli for observation of animal pathogenicity. These tests were designed to discriminate among mycobacteria involved in disease and were directed toward detecting MTB, *Mycobacterium bovis*, *Mycobacterium avium*, or saprophytes[83,84,85]. However, when other Mycobacterium species were recognized as infectious agents, it was evident that additional differentiation criteria were needed. Thus, a classification system based on pigmentation and growth rate was introduced to define the occurrence of "atypical" (a term presently in disfavor in mycobacterial nomenclature) strains and their relationship to the *Mycobacterium* species perceived as pathogenic[86,87]. Thus, the "slow growers" were defined as having visible growth in >7 days and were categorized in the following groups: group I, photochromogens; group II, scotochromogens; and group III, non-photochromogens, while "rapid growers" were defined as having visible growth in <7 days, and they were designated group IV. Although this simple system is not used as extensively now, its longevity is demonstrated by references in publications and frequent communications between mycobacteriologists. However, these simple designations are not practical or sufficient for defining species within the *Mycobacterium* genus.

In related efforts, members of the International Working Group on Mycobacterial Taxonomy (IWGMT) made significant contributions to mycobacterial identification and taxonomy. Their collaborative studies evaluated various mycobacteria and related genera groups, defined variation in members of a given species, and proposed selected tests for routine species identification. These extensive studies, involving numeric taxonomy, clarified the phenetic integrity of the Mycobacterium genus[88,89] and provided a practical biochemical identification scheme for clinically essential species of Mycobacterium[90,91]. During this time, just over 20 of the known 54 species were regarded as potentially pathogenic, and the recommended tests appeared applicable to identify these species. Eventually, as the number of species increased, the resulting taxonomical complexity caused ambiguities in interpreting biochemical test results due to their reduced discrimination ability.

The synthesis of mycolic acids, which are long-chain hydroxy fatty acids and the major constituents of this protective layer, is critical

for the survival of enzymes involved in the mycolic acid synthesis, which also turn out to be the targets for front-line anti-tubercular drugs, such as isoniazid and ethionamide and may warrant further study as biomarkers.

Flux Balance Analysis - MAP

Flux balance analysis (FBA) has been performed on the MAP model[92], which has provided insights into the metabolic capabilities of the mycolic acid pathway (MAP) has been of great interest. As a result, a substantial amount of biochemical and genetic information is available in the published literature, in addition to the entire genome sequence of *M. tuberculosis*.

Given the biological importance of mycolic acids, it would be helpful to understand the behavior of the pathway as a whole and of its components for drug discovery and environmental biomarkers if the matrix issues from dust sampling can be overcome. Most of this method's work has focused upon MTB, a deadly human pathogen, and owes many of its unique qualities to its thick, waxy coat, containing fatty acids called. However, the MAP model was thought to be of value in drug design and for understanding mycolic acid synthesis in general[94]. This approach was focused on the comprehensive identification of the mycolic acid pathway components and represented in a mathematically generated schema based on reaction stoichiometry[94-98]. In addition, FBA is seen as a handy technique for analyzing the metabolic capabilities of cellular systems. Feasibility studies using sequence analysis of the MTB and human proteomes indicate proteins identified as essential by FBA correlate well with those previously identified experimentally through transposon site hybridization mutagenesis.

However, while it is of significant historical interest, this method will be unlikely to offer practicability for this study.

Molecular Genetic Methods:

MTB is the etiologic agent of tuberculosis and can be accurately detected by laboratories using commercial genetic tests. Non-tuberculosis mycobacteria (NTM) causing other *Mycobacteriodes* can be challenging to identify. The identification processes are confounded by an increasing diversity of newly characterized NTM species[99]. The ubiquitous nature of NTM, combined with their potential to be opportunistic pathogens in immunocompromised and non-immunodeficient patients, further complicates the problem of their identification. Since clinical case management varies depending on the etiologic agent, laboratories must identify the species promptly. However, only a few identification methods can detect the species diversity within the *Mycobacterium* genus. Over the last decade, high-performance liquid chromatography analysis of mycolic acids has become an accepted method for the identification of mycobacteria.

Over the years, the unequivocal identification of MTB and clinical interest species has dominated the mycobacteria's taxonomy[100]. This emphasis on identifying the most commonly recovered species by clinical laboratories prompted the development of genetic probes (e.g., Gen-Probe, San Diego, Calif.) for their detection.

Laboratories using these genetic tests were rarely reported to be misidentified species for which the tests were designed. However, tests were not developed for most lesser-known mycobacteria because they were not considered a severe threat to public health. In addition, most of the species were infrequently isolated and never transmitted from person to person. On occasion, these species produced severe (even fatal)

infections, especially in patients with a reduced immune response.

The genomic information available for several *Corynebacteria*, *Mycobacteria*, and *Streptomycetes* has expanded. However, it provides limited but specific opportunities for comparative genome analysis and the extrapolation of gene functions between these genera. For example, several genes involved in fatty acid metabolism can be identified and analyzed. When complemented with transcriptomic, proteomic, and metabolomic data, this information should be a significant step toward understanding this essential but poorly understood metabolic pathway in this group of organisms.

In these cases, rapid and accurate identification of the clinical species can benefit effective medical intervention, and much work has transpired to this end, as reviewed below.

HPLC Detection of Mycolic Acids

The development, introduction, and effectiveness of high-performance liquid chromatography (HPLC) analysis of the mycolic acids for chemotaxonomic classification and rapid identification of *Mycobacterium* species is discussed. However, it is not within the scope of this review to examine every method proposed or currently used to identify the mycobacteria.

HPLC principles and instrumentation originated in chemistry during the mid-1960s and widespread application in inorganic, organic, and biochemical areas, with clinical chemistry sections exploiting the method's versatility for rapid separation and identification of compounds for drug analysis[101-103]. In 1985, scientists at the Centers for Disease Control and Prevention (CDC) proposed using HPLC for mycobacterial classification. In 1989, the procedure was incorporated into the regiment of tests at the CDC Mycobacteriology Reference Laboratory[104-106].

HPLC analysis of mycolic acids for species identification offered a reprieve from the traditional time-consuming identification process[55,84]. An isolate submitted on culture could be analyzed in hours by HPLC, compared to weeks or longer for routine methods. However, HPLC is considered a sophisticated procedure compared to other laboratory methods, and a dedicated, highly trained operator is required (W. In addition, the instrumentation is costly compared to conventional methods. Laboratories that detect acid-fast bacilli only by smears may not have the capacity to develop proficiency with the HPLC method. However, many high-throughput laboratories that are proficient in all aspects of mycobacteriology have successfully incorporated HPLC into their methodologies[106-112]. A significant challenge for laboratory personnel is developing expertise in the visual interpretation of chromatographic patterns for species determination. This report presented 63 chromatographic patterns representing 73 known mycobacteria species, which may help with this difficulty.

The analysis of lipid fractions has contributed significantly to the knowledge of Mycobacterium species. The abundance of lipid constituents in mycobacterial cell walls made them classic candidates for early chemical investigations. Of intense interest to researchers was a difficult-to-purify wax fraction, initially termed “unsaponifiable wax.” Isolated after prolonged saponification and clarified as an alcohol-insoluble, hydroxy acid of high molecular weight. The saponified wax fraction was chemically stable and was named “mycolic acid” following its isolation from the original H37

strain of MTB. Complete saponification required 80 hours of reflux in methanolic potassium hydroxide and benzene. Chemically, the mycolic acid fraction stained acid-fast contained hydroxy and methoxy groups with 88-carbon chain lengths and displayed a pyrolysis product with 26 carbons when heated at 300 to 350°C.

Further structural studies confirmed that the hydroxy group of mycolic acid was in a position to the carboxyl group and thus defined the mycolic acids as high-molecular-weight hydroxy fatty acids with a long side chain. Additional studies demonstrated that the structural character of the mycolic acids was a mycolic acid-arabinogalactan-mucopeptide complex, an essential part of the cell wall whose abundance was not adversely affected by culture conditions[55,115]. This consistent production of mycolic acids was a stable phenotypic property for a species[116-119].

Early chemical investigators recognized the diagnostic value of the mycolic acids, which characterized the different mycolic acid-containing genera[120-122]. The chemical complexity of members of the different taxa was demonstrated by gas-liquid chromatography and single-dimension, thin-layer chromatography (1D-TLC) analysis of mycolic acid as methyl esters[121-123]. Methanolysates of mycobacterial mycolic acids were differentiated in 1D-TLC by producing multispot patterns, in contrast to single-spot patterns produced by species in related taxa [24,125]. *Mycobacterium* species were also chemically distinguished from those of other genera by the presence of C22 to C26 products by gas-liquid chromatography pyrolysis of mycolic acid methyl esters[121,122,125]. In addition, *Mycobacterium* species were rapidly distinguished from *Nocardia* or *Rhodococcus* species by precipitation of mycobacterial mycolic acids from ether solution by adding alcohol. In contrast, the mycolic acids from the other genera remained soluble.

In addition, it was shown that mycobacteria and other mycolic acid-containing taxa could be distinguished using 2D-TLC of mycolic esters as whole-organism methanolysates[122,125]. This method was combined with mass spectrometry (MS) and used to identify and chemically characterize the functional groups of the mycolic acids, thus demonstrating the species-specific nature of the mycolates. Extensive studies described mycobacterial mycolic acids as phenotypically stable chemical characters, having a discontinuous distribution with chemotaxonomic potential[124]. Because mycolic acids were confined in related taxa and not widely distributed in nature, they are ideal for discrimination studies. The impressive work done with 2D-TLC and MS advanced the knowledge of the structure and complexity of mycobacterial mycolic acids. Analysis of 50 species by 2D-TLC revealed that the mycobacteria were separated into 11 structural groups based on the composition of their mycolic acids. The analysis was suggested as applicable in clinical laboratories to identify mycobacteria.

These comprehensive studies defined the value of mycolic acid data in mycobacterial systematics. In addition, they led to the recommendation that minimal descriptions for a new *Mycobacterium* species include information on the chemical composition of mycolic acids.

Presently, all the mycolic acid-containing genera can be distinguished by the length of their mycolic acid carbon chains. They include *Corynebacterium*, C20 to C38; Dietzia, C34 to C38; *Rhodococcus*, C34 to C52; *Nocardia*, C40 to C60; *Gordonia*, C48

to C66; *Williamsia*, C50 to C56; *Skermania*, C50 to C64; *Tsukamurella*, C64 to C78; and *Mycobacterium*, C60 to C90.HPLC separation methods permitted researchers interested in the biosynthesis and biological properties of the mycolic acids to fractionate and collect "pure" compounds and confirmed earlier TLC and nuclear magnetic resonance data of chemical structure. In addition, the sensitivity of mycolic acid detection was increased by derivatization to UV-adsorbing p-bromophenacyl esters (PBPA)[126,127]. Subsequent studies used multiple-step methods with TLC, MS, and reverse-phase chromatography to achieve fractionation of discrete components of the mycolic acids[127,128].

These reverse-phase chromatography procedures utilized high-efficiency C18, microparticle-bonded stationary-phase columns with mixtures of organic solvents. Chromatographic methods combined reverse-phase HPLC, and MS analysis of cell wall extracts to demonstrate mycolic acid's chemical nature from MTB strain H37Ra[126,128,130]. Fractions were separated into chemically homologous mycolic acid series, consisting of 24 fractions from the MTB complex and *Mycobacterium smegmatis*. It was noted that the mycolic acids separated in the reverse-phase column by a combination of factors, including chemical functional groups, polarity, and hydrocarbon chain length[127,128,131]. In prior studies, similar results had been demonstrated with shorter-carbon-chain fatty acids, derivatized with chemically analogous phenacyl derivatives, which had demonstrated an increase in retention times with longer carbon chains but a decrease in retention times with greater unsaturation[130-135]. The separation properties of the mycolic acids were used to resolve an ongoing debate regarding the similarity of *Mycobacterium gordonae* and *Mycobacterium leprae*.

In an elaborate two-step HPLC procedure combined with TLC, the presence of specific methoxymycolates was demonstrated in *M. gordonae* and was absent in *M. leprae*; thus, the close relationship was not supported. It was reported that reverse-phase HPLC profiles for mycolate, methoxymycolate, and ketomycolate chemical functional groups demonstrated increasing retention times with increased carbon chain length. Moreover, it was noted that the mycolic acids were not completely chemically resolved by HPLC when subjected only to reverse-phase partition chromatography[126,127]. Consequently, chromatographic patterns were published for mycolic acids for mycobacteria that demonstrated extensive overlapping of the chemical fractions[126,127,128]. The recognition of pattern differences between the published chromatograms for members of the MTB complex (slow-grower) and *M. smegmatis* (rapid-grower) provided the stimulus for further research with the HPLC instrument as a tool for species detection. These and other chemical studies demonstrated the separation capabilities of the various methods and the discriminatory power of HPLC.

Phenacyl esters of fatty acids were separated with a single-step, C18 reverse-phase HPLC method utilizing UV detection (UV-HPLC). The procedure employed a dicyclohexyl-18-crown-6 ether (crown ether) catalyst for a rapid (30-min) derivatization of long-chain fatty acids detectable in the nanogram range. The use of this derivative provided a sensitive detection method for the complete separation of mycolic acids from mycobacteria into structural classes with silica and reverse-phase modes[126,128]. Moreover, complete chemical separation of the mycolic acids was

not necessary for species detection since the reverse-phase pattern with gradient elution appeared distinctive for MTB

The reverse-phase UV-HPLC method was used with a modified gradient elution system of chloroform-methanol to differentiate 13 Mycobacterium species into chromatographically related groups. Within each chromatographic group, distinctive mycolic acid patterns were used for species classification of mycobacteria. However, the mycolic acid isolation procedure was cumbersome and involved multiple chloroform extractions. In addition, the reflux-condensation process used for sample derivatization was time-consuming; consequently, only a few samples could be analyzed over several days.

Additional improvements to the method were made in a study with rapidly growing mycobacteria of clinical significance. It was shown that organisms could be killed, and fatty acids could be saponified in the autoclave for 1 hour at 121°C. Mycolic acids were extracted, derivatized, and processed in 3 hours. Under controlled growth conditions, similar but distinctive chromatographic patterns were developed for *M, chelonae*, and *M. abscessus*. On the other hand, 122 strains of the *M. fortuitum* complex and *M. smegmatis* could not be separated under these conditions. The stability of the mycolic acid patterns demonstrated by UV-HPLC for slow-growing mycobacteria was in contrast to that of rapidly growing mycobacteria, which were affected by culture conditions, especially temperature. Related studies of rapidly growing mycobacteria demonstrated either elongation or a shortening of mycolic acids as an adaptive response to changes in temperature. However, the effects appeared to be species-specific[136,137].

During the study with rapid-growing mycobacteria, a proprietary high-molecular-weight standard synthesized by Ribi ImmunoChem (now Corixa Corp.), Hamilton, Mont., was used for the first time as an internal standard to determine reproducible relative retention times (RRT) with a standard deviation of ±0.01 to 0.09 min for individual peaks.,In addition, this standard was used to devise an identification scheme to compare peak height ratios, calculated for peaks from different chromatograms with identical RRTs. The identification scheme required manual calculation of values and was time-consuming but correctly identified 36 isolates of *M. chelonae* and 24 of 25 isolates of other *Mycobacteria*. A subsequent 10-step dichotomous peak height differentiations scheme was reported for slow-growing species and tested at two laboratories. At one laboratory, 129 isolates of the following species were correctly identified: *Mycobacterium asiaticum, M. bovis*, BCG attenuated variants of MTB, *Mycobacterium gastri, M. kansasii, Mycobacterium marinum, and Mycobacterium szulgai.* A second laboratory identified 661 of 670 different strains of these same species. Overall, 790 strains (98.6%) were correctly identified by both laboratories.

The reproducibility of mycolic acid patterns from mycobacteria has been established by laboratory studies conducted under the auspices of the IWGMT. The first study employed molecular, biochemical, serologic, and other chemotaxonomic methods.

DISCUSSION:

The CIRS community is faced with a new syndrome, which devolves from data-gathering and responds to safe and effective treatment[2]. However, it remains to be

confirmed what role extracellular vesicles, TGFBR signaling, and inflammatory responses to mycolic acids play in the pathogenesis of HH-related CIRS, all of which will be subjects for further study. However, the following open discussion will include these elements, beginning with this targeted literature review.

Since it has been postulated that there is a role for HH-dwelling *Actinobacteria*, especially *Corynebacteria* in CIRS, it has been suggested[1,2] that a mechanism exists to link defective systemic immunity to inflammation caused by these skin organisms. Belkaid[137-140] from NIH has published a more in-depth treatise offering additional in-depth references on this issue. It demonstrates that *Corynebacteria* are the predominant skin microbes that promote a dramatic activation of a defined subset of gamma delta T cells enriched in mucosal and epithelial tissues. Furthermore, they mount a heightened cytokine response to transformed or infected cells. The remarkable feature is that this effect (i) is long-lasting, (ii) is independent of other microbes colonizing the skin, and (iii) is mediated by interleukin-23. In addition, the potential links among interleukin-23, TGFB1, and Th17 T reg imbalance are apparent. Of note is that the impact of *Corynebacteria* can be discrete and non-inflammatory under controlled steady-state conditions. However, when the host is fed a high-fat diet, *Corynebacteria accolens* promotes inflammation that is IL-23-dependent. This effect is conserved among *Corynebacteria* species and depends on the expression in the cell envelope of mycolic acids.

Dr. Belkaid's group has shown[137-140] that *C. accolens* strongly impacts the accumulation of IL-17A, producing gamma delta T-cell receptor T cells. These are migratory T-cells found in the dermis of a laboratory mouse. After *C. accolens* are introduced to a mouse free from *Corynebacteria*, the frequency and an absolute number of gamma delta T-cells and their potential to reduce IL-17A/Th17 cells were significantly increased. Their potential to reduce IL-17A/Th17 cells was significantly increased skin compared to controls. Thus, following the introduction of *C. accolens*, the gamma delta T-cells actively reduce 17A.

These results support the idea that predominant skin microbes evoked profound skin immunity and inflammation in response to its cell envelope compounds, representing a microbial skin signal differentiating HH from SH *Actinobacteria*. The authors also raised the additional tantalizing idea that a change in the skin that would follow a dietary alteration lends hope for a non-invasive correction of induced inflammation from *Corynebacteria*.

In the quest for suitable biomarkers for detection of the Actinobacteria in environmental dust samples, it is noted that there are differences in mycolic acid contents of cell walls between *Mycobacteria*, *Corynebacteria*, *Nocardia*, and other related genera. They have a thick wall of proteoglycan coupled to arabinogalactan, linked to long-chain fatty acids, forming the mycolic acids.

Corynebacteria have a mixture of saturated and unsaturated fatty acids up to 36 carbons long. In contrast, *Mycobacteria* have longer fatty acid chains, up to 90 carbons long, with oxygen molecules as part of their chemical structure). *Streptomyces*, common soil dwelling *Actinobacteria*, have a cell wall surrounded by a proteoglycan mesh, similar to Gram-negative organisms. Mycolic acids play a pivotal role in the formation of a secondary permeability barrier analogous to

the outer cell membrane of Gram-negative bacteria.

Conclusion

From this review, it is felt that there is ample encouragement that solutions can be found to achieve the aim, both from the extensive accumulation of biochemical literature and the rapidly expanding molecular genetic information being assembled to delineate the roles that *Actinobacteria* play in our envirobiome.

Thus, while extracellular vesicles could be the “missing link" tying local inflammation to adverse systemic and immune abnormalities in CIRS, we must solve the laboratory demands (i) to isolate intact extracellular vesicles and (ii) to confirm the prospective acquisition of adverse health effects following exposure to Actinobacteria vesicles and (iii) define and validate biomarkers so that screening can be undertaken to expand the scope of application within the relevant field.

Considering the role of mycolic acids and other Actinobacterial biomarkers is likely to be a fundamental part of unraveling the roles that vesicle release and other mechanisms play. In addition, biomarkers may allow widespread data and focus and further expansion, allowing transcriptomics and NGS to define the inflammatory and metabolic role of exposure to pathogenic Actinobacteria.

Then the mechanisms suggested herein will further aid assessment and proof of principle, leading the way by providing screening with appropriate and validated biomarker systems.

In addition, while DI and PI show tremendous promise as clinical and remediation tools, the molecular biology underlying these indices can be further understood with additional study.

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