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Gillis W. Long Hansen's Disease Center  
at Louisiana State University  
Baton Rouge, Louisiana 70894, U.S.A.

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## EDITORIAL

*Editorial opinions expressed are those of the writers.**Mycobacterium leprae* Iron Nutrition:  
Bacterioferritin, Mycobactin, Exochelin and  
Intracellular Growth

The discovery of membrane-bound 380 kilodalton bacterioferritin molecules<sup>1</sup> that store between 1000 to 4000 atoms of iron fundamentally changes the previous views on iron nutrition during intracellular-growth of *Mycobacterium leprae*.<sup>2-4</sup>

**Intracellular iron.** It is predictable that much will continue to be written about the growth of *M. leprae* under low-iron or iron-

deficient conditions inside host cells. This is a misnomer since, in reality, localized high-iron microenvironments are derived from monocyte-containing cellular infiltrates that enter the inflammatory lesions of leprosy to form epithelioid cell granulomas, the classic tissue response to intracellular mycobacterial infection.<sup>5-7</sup> Indeed, it is even possible to find stainable deposits of iron in chronic granulomatous lesions.<sup>8</sup> In the ongoing cellular turmoil inside inflammatory

<sup>1</sup> Pessolani, M. C. V., Smith, D. R., Rivoire, B., McCormick, J., Hefta, S. A., Cole, S. T. and Brennan, P. J. Purification, characterization, gene sequence and significance of a bacterioferritin from *Mycobacterium leprae*. *J. Exp. Med.* **180** (1994) 319-327.

<sup>2</sup> Wheeler, P. R. and Ratledge, C. Metabolism in *Mycobacterium leprae*, *M. tuberculosis* and other pathogenic mycobacteria. *Br. Med. Bull.* **44** (1988) 547-561.

<sup>3</sup> Barclay, R. and Wheeler, P. R. Metabolism of mycobacteria in tissues. In: *The Biology of the Mycobacteria. Vol. 3 Clinical Aspects of Mycobacterial Disease*. Ratledge, C., Stanford, J. and Grange, J. M., eds. New York: Academic Press, 1989, pp.37-106.

<sup>4</sup> Ratledge, C. Iron metabolism in mycobacteria. In: *Iron Transport in Microbes, Plants and Animals*. Winkelmann, G., van der Helm, D. and Neilands, J. B., eds. Weinheim: VCH Verlagsgesellschaft, 1987, pp. 207-233.

<sup>5</sup> Morrison, N. E. and Collins, F. M. Immunogenicity of an aerogenic BCG vaccine in T-cell-depleted and normal mice. *Infect. Immun.* **11** (1975) 1110-1121.

<sup>6</sup> Morrison, N. E. and Collins, F. M. Restoration of T-cell responsiveness by thymosin: development of antituberculous resistance in BCG-infected animals. *Infect. Immun.* **13** (1976) 554-563.

<sup>7</sup> Lucas, S. B. Mycobacteria and the tissues of man. In: *The Biology of the Mycobacteria. Vol. 3 Clinical Aspects of Mycobacterial Disease*. Ratledge, C., Stanford, J. and Grange, J. M., eds. New York: Academic Press, 1989, pp. 107-176.

<sup>8</sup> Momotani, E., Furugouri, K., Obara, Y., Miyata, Y., Ishikawa, Y. and Yoshino, T. Immunohistochemical distribution of ferritin, lactoferrin and transferrin in granulomas of bovine paratuberculosis. *Infect. Immun.* **52** (1986) 623-627.

lesions it is a simple matter for autodigestion or for *M. leprae*-secreted proteases to digest host iron-containing proteins and to release iron chelates, including iron peptides, that carry and donate their iron directly into the membrane-bound bacterioferritin.<sup>1</sup> The multiplication of *M. leprae* within cells of the hepatosplenomegaly of armadillo leprosy<sup>9</sup> can hardly be correlated with iron-deficient growth since these are the host organs of iron storage and turnover. In particular, presumptive iron-regulated envelope proteins isolated from *M. leprae* cannot be correlated with any state of iron deficiency for liver-grown *M. leprae*.<sup>10</sup> This implies that the bacterioferritins must be loaded with liver iron, as has been reported.<sup>1</sup>

**Iron movement.** A fundamental intracellular scavenging role for iron-binding peptide exochelins<sup>11-12</sup> that are released from mycobacteria growing in low-iron laboratory cultures appears unlikely. This is due to the time-curve release of exochelins during the growth cycle. Exochelins are secondary metabolites produced from secondary metabolism since they are without an intracellular pool and are released abruptly toward the end of logarithmic growth when the resting phase is becoming established under iron-stress limitation.<sup>13-14</sup> This means that exochelins have no iron scavenging role

during critical lag and logarithmic phases when mycobacterial growth demand for iron is high. This demand is satisfied by bacterioferritin storage iron.<sup>1</sup> If exochelins were fundamental to the transport of iron they would have been released during the lag phase. Undoubtedly the highly localized level of host iron inside inflammatory lesions would prevent formation of exochelins. Given the high turnover of mononuclear infiltrates entering inflammatory lesions,<sup>15</sup> it is more likely that endogenous formation of host-originated iron chelates, with appropriate partition coefficients, carry iron through the mycobacterial cell wall to the bacterioferritin membrane site of iron storage and utilization.

There is no evidence for active membrane transport of iron in slowly growing mycobacteria.<sup>16</sup> The movement of iron is by "random-walk"<sup>17</sup> of the iron chelate through the cell wall to the outer membrane bacterioferritin site. This is not facilitated diffusion as argued for exochelin-transport of iron into *M. leprae*<sup>18-19</sup> since the host iron chelate never crosses the bilayer cytoplasmic membrane. The partition coefficient of the iron chelate thus becomes the dominant parameter to effect "random-walk" across the envelope cell-wall fraction. The "random-walk" mechanism occurs as a result of free-energy change as the iron-chelate partitions between the aqueous extracellular phase and the lipid cell-wall phase. A partition coefficient that will move a neutral iron chelate such as ferrimycobactin P<sup>20</sup> into a myco-

<sup>9</sup> Job, C. K., McCormick, G. T. and Hastings, R. C. Intracellular parasitism of parenchymal cells by *Mycobacterium leprae*. *Int. J. Lepr.* **57** (1989) 659-670.

<sup>10</sup> Sritharan, M. and Ratledge, C. Iron-regulated envelope proteins of mycobacteria grown *in vitro* and their occurrence in *Mycobacterium avium* and *Mycobacterium leprae* grown *in vivo*. *Biol. Metals* **2** (1990) 203-208.

<sup>11</sup> Macham, L. P. and Ratledge, C. A new group of water-soluble iron-binding compounds from mycobacteria: the exochelins. *J. Gen. Microbiol.* **89** (1975) 379-382.

<sup>12</sup> Macham, L. P., Stephenson, M. C. and Ratledge, C. Iron transport in *Mycobacterium smegmatis*: the isolation, purification and function of exochelin-MS. *J. Gen. Microbiol.* **101** (1977) 41-49.

<sup>13</sup> Macham, L. P., Ratledge, C. and Nocton, J. C. Extracellular iron acquisition by mycobacteria: role of exochelins and evidence against the participation of mycobactin. *Infect. Immun.* **12** (1975) 1242-1251.

<sup>14</sup> Barclay, R. and Ratledge, C. Iron-binding compounds of *Mycobacterium avium*, *M. intracellulare*, *M. scrofulaceum*, and mycobactin-dependent *M. paratuberculosis* and *M. avium*. *J. Bacteriol.* **153** (1983) 1138-1146.

<sup>15</sup> Collins, F. M. Mechanisms of antimicrobial immunity. *J. Reticuloendothel. Soc.* **10** (1971) 58-90.

<sup>16</sup> Stephenson, M. C. and Ratledge, C. Specificity of exochelins for iron transport in three species of mycobacteria. *J. Gen. Microbiol.* **116** (1980) 521-523.

<sup>17</sup> Fujita, T. and Hansch, C. Analysis of the structure-activity relationships of the sulfonamide drugs using substituent constants. *J. Med. Chem.* **10** (1967) 991-1000.

<sup>18</sup> Hall, R. M., Wheeler, P. R. and Ratledge, C. Exochelin-mediated iron uptake into *Mycobacterium leprae*. *Int. J. Lepr.* **51** (1983) 490-494.

<sup>19</sup> Hall, R. M. and Ratledge, C. Exochelin-mediated iron acquisition by the leprosy bacillus, *Mycobacterium leprae*. *J. Gen. Microbiol.* **133** (1987) 193-199.

<sup>20</sup> Snow, G. A. Mycobactins: iron-chelating growth factors from mycobacteria. *Bacteriol. Rev.* **34** (1970) 99-125.

bacterial cell-wall "random-walk" will not move the same iron chelate into a cytoplasmic membrane "random-walk." The partitioning differential is far too great in the uphill direction. This means that the iron chelate will not now cross the outer boundary of the cytoplasmic membrane.

Partition theory calculations<sup>21</sup> do not support current views of ferrimycoactin movements across bilayer membranes.<sup>4, 22</sup> Such calculations indicate that ferrimycoactin P will remain stuck on the outer cytoplasmic membrane surface because of the lipophilic drag imposed by the C<sub>18</sub> alkyl side chain which is precisely where excess mycoactin molecules become localized.<sup>23</sup> This also means that the enzyme machinery that assembles the final mycoactin structure also is located at the outer membrane surface.

The apparent overproduction of mycoactin, particularly in *M. smegmatis*,<sup>4</sup> at first sight suggests that mycoactin is an end product from an unregulated pathway. The counter argument defines mycoactin as a product from a regulated pathway. The counter argument becomes intriguing because unlike the exochelins, the mycoactins are initially formed during the lag phase.<sup>24</sup> Therefore, they are self-defining as primary metabolites. If this is so then it becomes necessary to look for mycoactin function outside of chelation theory, a point made long ago by Snow.<sup>20</sup>

**Salicylic acid dual function.** While chelation theory associates mycoactin and iron in function, partition theory associates mycoactin and salicylic acid in function since mycoactin and salicylic acid are primary metabolites produced in synchrony during the lag phase<sup>4, 24</sup> in a regulated pathway. Be-

cause salicylic acid is an intermediate<sup>4</sup> in mycoactin synthesis and mycoactin is assembled at the outer membrane surface, the mycoactin overproduction is, in reality, a "sink" for the overall control of the intracellular level of salicylic acid since once the salicylate membrane intermediate is incorporated into the mycoactin structure it cannot return to the cell interior. The mycoactin "sink" argument requires that intracellular synthesis of salicylic acid not be subjected to control by feed-back inhibition. The mycoactin "sink" function does not relate to salicylate function as a mycoactin pathway intermediate but, rather, to a second intracellular function for salicylic acid that has been reasoned for by Ratledge.<sup>4</sup> The nature of this function is unknown, but it is well to recall the early observations of Brodie and Gray<sup>25</sup> that uncoupling agents like salicylic acid were able to uncouple energy-transducing functions from the oxygen uptake of respiration that are carried out by iron-dependent membrane-bound systems.<sup>26</sup> Therefore, salicylic acid occurrence in mycobacteria relates, in part, to its natural function as a regulator of energy-transduction. The mycoactin "sink" function maintains intracellular levels of salicylate for control of energy-yielding reactions. There is a further subtle caveat to this argument in that salicylic acid itself does not cross bilayer membranes;<sup>27</sup> thus a membrane salicylate carrier structure that is, at the same time, a mycoactin pathway intermediate as well as an energy-transducing regulator must be present in the cytoplasmic membrane. In effect the mycoactin "sink" directly controls the membrane concentration of this salicylate-containing structure through the mycoactin assembly rate. It is possible that partition theory will be helpful in formulating a

<sup>21</sup> Hansch, C. and Leo, A. J. *Substituent Constants for Correlation Analysis in Chemistry and Biology*. New York: John Wiley and Sons, 1979.

<sup>22</sup> Ratledge, C. Metabolism of iron and other metals by mycobacteria. In: *The Mycobacteria—A Sourcebook*. Kubica, G. A. and Wayne, L. G., eds. New York: Marcel Dekker, 1984, pp. 603–627.

<sup>23</sup> Ratledge, C., Patel, P. V. and Mundy, J. Iron transport in *Mycobacterium smegmatis*: the location of mycoactin by electron microscopy. *J. Gen. Microbiol.* **128** (1982) 1559–1565.

<sup>24</sup> Antoine, A. D. and Morrison, N. E. Effect of iron nutrition on the bound hydroxylamine content of *Mycobacterium phlei*. *J. Bacteriol.* **95** (1968) 245–246.

<sup>25</sup> Brodie, A. F. and Gray, C. T. Bacterial particles in oxidative phosphorylation. *Science* **125** (1957) 534–537.

<sup>26</sup> Brodie, A. F. Microbial phosphorylating preparations: *Mycobacterium*. In: *Methods in Enzymology*. Colowick, S. P. and Kaplan, N. O., eds. New York: Academic Press, 1967, vol. 10, pp. 157–169.

<sup>27</sup> Tute, M. S. Principles and practice of Hansch analysis: a guide to structure-activity correlations for the medicinal chemist. *Adv. Drug Res.* **6** (1971) 1–77.

membrane-compatible structure for this salicylated compound.

**Iron-stress exhaustion.** It is apparent that exochelins belong to a class of secondary metabolites that are re-utilized as secondary sources of nitrogen to maintain viability during the resting phase of nonmultiplication prior to the logarithmic death phase.<sup>13-14</sup> If this did not occur, the resting phase would be short-circuited into a death phase. The exochelins are, in fact, substituted peptides<sup>4, 28</sup> that are produced from iron-stress exhaustion at the end of low-iron-phenotypic growth.<sup>29</sup> Not all of these peptides chelate iron.<sup>30</sup> Thus, the exochelins belong to a class of extracellular iron-stress metabolites, many of which are re-utilized during the resting phase. Iron-stress exhaustion must occur only after the bacterioferritin compartment becomes completely denuded of iron atoms. This is dependent upon the stored iron load. For *M. leprae* it can be calculated to occur after 10 generations or three logs of growth in the absence of external iron supply. Iron-stress exhaustion is on the approach curve to chaotic metabolic conditions that spread throughout the metabolic landscape, the end result of which is growth cessation and death of a major portion of the bacterial population. In addition to the release of peptides, an abrupt release of acids, including ketonic acids,<sup>24</sup> occurs during iron-stress exhaustion. Many other changes undoubtedly occur.<sup>31</sup>

**Chelate structure.** Just as there is remarkably little structural specificity in released exochelins<sup>14, 32</sup> there is, likewise, little

structural specificity for iron chelates to carry iron into bacterial cells<sup>33-36</sup> consistent with "random-walk" movement. The higher order of structural specificity required for hexadentate mycobactin homologs<sup>20</sup> to grow *M. paratuberculosis* in the laboratory may be argued against on theoretical grounds.

It is predictable that naturally occurring hexadentate mycobactins can be replaced by synthetic bidentate iron chelators,<sup>36</sup> provided such chelators possess structures that are in the same range of partition coefficient as are the mycobactins in order to penetrate the hydrophobic mycobacterial cell wall. Since such synthetic chelators do not exist, the prediction cannot be tested. However, chemical conversion of the hexadentate mycobactin iron-binding center<sup>37</sup> into a bidentate diacetylmycobactin results in an iron chelate with much improved growth efficiency.<sup>34</sup> There is one caveat to the bidentate/hexadentate chelate argument in that growth-active species of bidentate chelate must remain in a 1:1 equilibrium of ferric iron to chelator concentration. Ferric iron to chelator concentrations in the 1:3 complexed state are invariably inhibitory to growth<sup>36</sup> unless steric hindrance is present to prevent 1:3 complexation. Thus, there is a distinct selective advantage to hexadentatism versus bidentatism in the biological effects of iron chelation.

It is of interest that one *M. tuberculosis* exochelin appears to form a quadridentate complex with ferric iron<sup>28</sup> in contrast to hexadentate mycobactin complexes. Quadridentate complexes will have lower binding

<sup>28</sup> Hanberg, F. B., Gobin, J., Reeve, J. R., Jr., Gibson, B. W., Tang, D. and Horwitz, M. A. Exochelins of *Mycobacterium tuberculosis*. Twenty-Eighth Joint Research Conference. U.S.-Japan Cooperative Medical Science Program, 1993, pp. 24-25.

<sup>29</sup> Ratledge, C. Nutrition, growth and metabolism. In: *The Biology of the Mycobacteria. Vol. 1 Physiology, Identification, and Classification*. Ratledge, C. and Stanford, J., eds. New York: Academic Press, 1982, pp. 185-271.

<sup>30</sup> Morrison, N. E. Unpublished results, 1976.

<sup>31</sup> Winder, F. G. and Coughlan, M. P. Comparison of the effects of carbon, nitrogen and iron limitation on the growth and on the RNA and DNA content of *Mycobacterium smegmatis*. *Irish J. Med. Sci.* 140 (1971) 16-25.

<sup>32</sup> Barclay, R. and Ratledge, C. Mycobactins and exochelins of *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum* and other related species. *J. Gen. Microbiol.* 134 (1988) 771-776.

<sup>33</sup> Burnham, B. F. Bacterial iron metabolism: investigations on the mechanism of ferrichrome function. *Arch. Biochem. Biophys.* 97 (1962) 329-335.

<sup>34</sup> Antoine, A. D., Morrison, N. E. and Hanks, J. H. Specificity of improved methods for mycobactin bioassay by *Arthrobacter terregens*. *J. Bacteriol.* 88 (1964) 1672-1677.

<sup>35</sup> Morrison, N. E. and Dewbrey, E. E. Growth factor activity of mycobactin for *Arthrobacter* species. *J. Bacteriol.* 92 (1966) 1848-1849.

<sup>36</sup> Morrison, N. E., Antoine, A. D. and Dewbrey, F. E. Synthetic metal chelators which replace the natural growth factor requirements of *Arthrobacter terregens*. *J. Bacteriol.* 89 (1965) 1630.

<sup>37</sup> Francis, J., MacTurk, H. M., Madinaveitia, J. and Snow, G. A. Mycobactin, a growth factor for *Mycobacterium johnei*. 1. Isolation from *Mycobacterium phlei*. *Biochem. J.* 55 (1953) 596-607.

affinities than hexadentate complexes and retain a net positive charge. The bidentate/quadridentate/hexadentate complexes follow a pattern of 1:1 ferric iron-ligand complexing for growth effects to emerge. Unless mixed iron chelates are formed, the quadridentate chelate would not be anticipated to be growth inhibitory. It is, of course, possible that the exochelins are mixtures of bidentate and quadridentate chelators of iron.

**Iron atoms.** The total number of iron atoms required to run the metabolic machinery of a single leprosy bacillus is estimated to be extremely small, less than 1000 atoms, somewhere in the range of 100 to 400 atoms. Therefore, a fully loaded, single bacterioferritin molecule would provide enough iron atoms to run all of the iron-based metabolism inside the bacillus. Based on the literature<sup>1, 38</sup> it is possible to estimate the number of bacterioferritin molecules per single leprosy bacillus. This figure calculates at 60 molecules of bacterioferritin per cell. Therefore, there is a 600- to 2400-fold excess of iron storage capacity in a single cell within which the bacterioferritin compartment occupies up to 0.1% of the individual cell mass.<sup>38</sup> Given the prolonged nature of leprosy infection, there is ample residency time for *M. leprae* to maintain 60 bacterioferritins per bacillus in a fully loaded state from host-derived sources of iron. Thus, it is the bacterioferritin storage of iron that assumes the primacy of iron source in order to run iron-based mycobacterial metabolism during divisional periods of high metabolic demand. The bacterioferritin compartment is, in effect, conserving iron so that the future progeny of dividing cells have adequate iron-based metabolisms. Thus, the principle of iron conservation overdominates the principle of iron chelation in the supply of iron for intracellular growth.

**Mycobactin conservation function.**

Clearly iron conservation had its origins in prokaryotic evolution.<sup>39</sup> The iron conser-

vation principle is likely to be the true function of the mycobactins situated inside hydrophobic waxy colonies and mats of mycobacteria where they form linear connecting arrays to exchange ferric iron between bacterioferritins of adjacent cells lying side by side. This cell-to-cell reversible exchange of ferric iron by the "bucket-brigade principle" requires the mycobactins to form linear stationary arrays through hydrophobic interaction of the mycobactin alkyl side chain with mycolate alkyl side chains<sup>40</sup> across the cell-wall envelope. The mycobactins, therefore, represent altruistic molecules<sup>41</sup> engaged in iron exchange and conservation between mycobacterial cells in a nonaqueous environment. The mycobactin arrays form interlocking networks for the movement of iron atoms. A self-organizing colony sharing of essential iron atoms can thus be brought about by colony networks of mycobactin-bacterioferritin operating exchange pathways for iron atoms within the hydrophobic environment of adjoining cell-wall envelopes. The few species of mycobacteria that do not utilize mycobactin chelate structure *in situ* clearly must rely on alternative chelate mechanisms to move iron atoms for iron conservation.<sup>42</sup> *M. leprae* and *M. paratuberculosis* are likely to fall into this class of species<sup>42-43</sup> because they have co-evolved with their hosts to rely on host iron chelates for iron movement in model systems.

**Epilogue.** Mycobacterial bacterioferritins are the missing link to our understanding of the iron nutrition of slowly growing, endoparasitic mycobacteria which have co-evolved their mechanisms of iron nutrition along with those of their hosts. It is clear that iron nutrition of mycobacterial endoparasites has to be viewed through the win-

<sup>38</sup> Draper, P. and Misell, D. L. Determination of the mass of *Mycobacterium leprae* by electron microscopy. *J. Gen. Microbiol.* **101** (1977) 207-209.

<sup>39</sup> Andrews, S. C., Smith, J. M. A., Yewdall, S. J., Guest, J. R. and Harrison, P. M. Bacterioferritins and ferritins are distantly related in evolution. Conservation of ferroxidase-centre residues. *FEBS Lett.* **293** (1991) 164-168.

<sup>40</sup> Draper, P. Wall biosynthesis: a possible site of action for new antimycobacterial drugs. *Int. J. Lepr.* **52** (1984) 527-532.

<sup>41</sup> Dawkins, R. *The Extended Phenotype*. New York: Oxford University Press, 1982.

<sup>42</sup> Lambrecht, R. S. and Collins, M. T. Inability to detect mycobactin in mycobacteria-infected tissues suggests an alternative iron acquisition mechanism by mycobacteria *in vivo*. *Microb. Pathogen.* **14** (1993) 229-238.

<sup>43</sup> Morrison, N. E. Circumvention of the mycobactin requirement of *Mycobacterium paratuberculosis*. *J. Bacteriol.* **89** (1965) 762-767.



dow of co-evolved iron conservation theory. Iron chelation theory is but a moving reflection in this window of coupled evolution<sup>44</sup> which requires the endoparasite to utilize host iron chelates to move iron.

The concept is advanced that exochelins belong to a class of iron-stress exhaustion peptides formed as secondary metabolites by an aerobic metabolism that iron stress has poised at the edge of chaos. The exochelins are re-utilized as sources of nitrogen to delay the chaos of cell death. Theoretical reasoning indicates that exochelins can be replaced by synthetic metal chelators,<sup>36</sup> with appropriate partition coefficients, for the movement of iron.

The concept is advanced that the dualistic function of salicylic acid involves both a mycobactin pathway intermediate and an energy-transducing regulatory function within the cytoplasmic membrane. The mycobactins likewise have dualistic regulatory functions: they are formed as "sinks" in reg-

ulating overall salicylic-acid levels; secondly, they have an altruistic function in forming cell-to-cell interlocking networks to exchange iron between bacterioferritins. Mycobactin networks form the basis of colony conservation of iron between mycobacterial cells, an essential fitness characteristic for low-iron phenotypic growth in the laboratory. High-iron phenotypic growth occurs in the host because the mycobacterial endoparasite provokes a granulomatous type of inflammation, a consequence of which carries host blood-borne cellular iron into the endoparasitic growth site. This forms the basis of coupled iron conservation mechanisms that arise from Dawkins' co-evolutionary principle of genetically shared agendas between the host and the endoparasite.<sup>45</sup>

—Norman E. Morrison, M.Sc. , Ph.D.  
*317 Woodlawn Road*  
*Baltimore, Maryland 21210, U.S.A.*

<sup>44</sup> Hennig, W. *Phylogenetic Systematics*. Urbana: University of Illinois Press, 1966.

<sup>45</sup> Mitchison, A. Will we survive? *Sci. Am.* **269** (1993) 136–144.