Research Paper

Volume : 3 | Issue : 7 | July 2014 • ISSN No 2277 - 8179

Study of Mycobactin From Different Mycobacterial Species by its Effect on Growth of *Mycobacterium Avium Subsp Paratuberculosis*

Medical Science

KEYWORDS : Mycobacteria. Mycobacterium avium subsp. paratuberculosis, mycobactin, growth assay

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ABSTRACT

Genus Mycobacterium consists of pathogenic and saprophytic organisms who meet the demand of iron by synthesizing mycobactins and exochelins. Mycobacterium avium subsp paratuberculosis (MAP) is unable to synthesize required amount of mycobactin and require exogenous mycobactin. The mycobactins extracted from various mycobacterial species by ethanol and chloroform extraction and purified by aluminium oxide chromatography. M phlei and M tuberculosis gave maximum growth in varying iron concentrations indicating its ability to utilize iron efficiently. Among all the mycobacterial species studied, highest mycobactin production was noticed in M fortuitum. Out of all extracted mycobactins used for the growth assay, mycobactins extracted from M tuberculosis, M phlei and MAP had produced best growth of MAP.

INTRODUCTION

Mycobacteria are a large, varied group of organisms, which include some significant pathogens and many free-living non-pathogens. Iron plays a central role in enzymatic reaction involved in electron transport, nucleic acid synthesis and oxidative stress defense (Jones & Niederweis, 2011). To meet the demand of iron, mycobacteria synthesize and utilize specific high affinity iron binding compounds called mycobactin and grow in iron restricted conditions. One of the distinguishing characteristics of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is its requirement of mycobactin in the complex medium used for primary cultivation. The mycobactins are a group of closely related cell associated, iron-binding compounds which act as specific growth factors. The mycobactins from any species of mycobacteria can be used in the medium for cultivation of MAP. When iron is the growth-limiting factor, mycobactin production is maximal (Morrison, 1965).

The isolation of MAP in culture takes more than 16 weeks as they are unable to synthesize their growth factor, mycobactin. Addition of mycobactin into media of MAP is essential for ample growth. It is considered necessary for large scale culture of MAP during production of Johnin PPD (purified protein derivative). Conventional method is the use of dried *M. phlei* cultural as the source of mycobactin. But this will contribute proteins of *M. phlei* in addition to Johnin PPD which may impart erroneous result in intradermal test. This can be rectified by adding purified mycobactin to the culture media. In this study, mycobactins were extracted from cultures of some species of mycobacteria grown in iron deficient media by ethanol and chloroform extraction. These crude mycobactins can be used in media for MAP for primary isolation and for enhancing growth of these organisms for Johnin PPD production.

Materials and methods

Fast growers such as *Mycobacterium fortuitum* subsp *fortuitum* MTCC929, *M phlei* MTCC 1724, *M smegmatis* MTCC 940, *M vaccae* and slow growers such as *M bovis* AN5, *M avium* D4, *M avium* 1723, *M microti* MTCC 1727, *M tuberculosis* H37Rv and *M.avium* subsp *paratuberculosis* ATCC 19698 obtained from Biological products division, IVRI were used for study. For maintenance of cultures, LJ medium slants were used and for studying mycobactin production Dorset and Henley's media was prepared. Dorset and Henley's media was divided in to three groups, with different iron concentration. The concentrations of iron used were 200µg/liter, 35mg/liter and 75mg/liter for low iron media, medium iron media and high iron media, respectively.

Both slow and fast growers were cultured first on LJ slant with antibiotics by incubating at 37^0c for 7 - 21 days. After attaining sufficient growth, cultures were inoculated in to broth media and incubated until sufficient surface growths were attained. Fast growers were incubated for 3 weeks and slow growers were incubated for 8 weeks at 37^0c. All cultures were harvested and mycobactins were extracted by chloroform- ethanol- ferric chloride extraction and purified by aluminium oxide gel chromatography (Merkal & McCcullough, 1982)

For growth assay, Dorset and Henley's media with 200µg iron per liter was prepared. Each mycobactin (4µg/ml and 40µg/ml) was added in to the media separetely and sterilized by autoclaving. Medium with dried *M. phlei* and medium without mycobactin were used as controls. All media were inoculated with same amount of MAP culture. Cultures were incubated at 37^0C for 2 months. Cultures were centrifuged after 2 months and the cell mass were quantified. These were then compared and analyzed

Results

All the mycobacterial species showed good growth in Lowenstein–Jensen slant at 37^0C within 3-4 weeks time. MAP showed no growth in media with medium and low iron content even after 4 months. Crude mycobactins were extracted from all organisms grown in high, medium and low iron concentration. Production of mycobactin is illustrated graphically in figure 1. This was then purified by column chromatography on neutral aluminium oxide column and used for growth assay.

Media with mycobactins were incubated for 2 months. Negative control showed visible growth only after 7 months and media with *M. phlei* showed growth after 3 months. Growth analysis of MAP is illustrated graphically in figure2. Cells after pelleting were used for DNA isolation and were confirmed as MAP with IS 900 PCR. MAP in low iron media yielded 290 mg cell mass after 7 months and MAP in media with *M phlei* extract yielded 400 mg cell mass after 3 months of incubation.

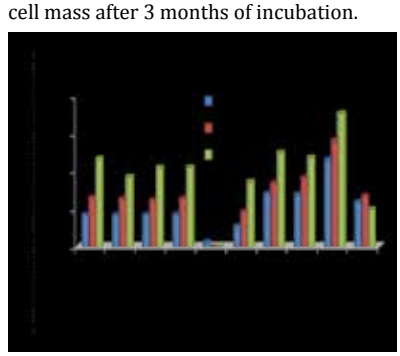

Figure 1: Mycobactin production per gram of cells

Table 1: Cell masses of MAP culture after growth on media with different mycobactins

Source of mycobactin	Cell mass of MAP in media with 4µg mycobactin	Cell mass of MAP in media with 40µg mycobactin
M. tuberculosis	980 mg	1580 mg
M. bovis	570 mg	810 mg
M. avium subsp paratuberculosis	910mg	1320 mg
M. avium 1723	880 mg	1280 mg
M .avium D4	770 mg	990 mg
M. microti	420 mg	580 mg
M. phlei	970 mg	1370 mg
M. fortuitum	803 mg	1280 mg
M. vaccae	550 mg	800 mg

Discussion

Availability of iron regulates synthesis of these iron binding compounds in Mycobacteria. MAP is unable to synthesize mycobactin in required amount and so it requires an external source of mycobactin to promote its growth. The mycobactins produced by all mycobacterial species can be extracted from their cell by ethanol and chloroform extraction. These were further purified by adsorption chromatography and can incorporate in media for growth of MAP (Francis, Macturk, Madinaveitia & Snow, 1953). Extract at this level of purity can be used for microbiological studies. All organisms showed maximum growth in high iron media indicating that iron is an essential factor for their growth.

Fast growing mycobacterial species were incubated at 37^0C for 3 weeks (Snow, 1970). Of the fast growers maximum amount of growth was seen in *M. fortuitum* in high iron media followed by *M phlei*, *M smegmatis* and *M vaccae*. In medium and low iron media, *M phlei* showed more rate of growth than other bacteria followed by *M fortuitum* and *M smegmatis*. Growth of *M vaccae* was less efficient compared to other three organisms. This shows the ability of *M phlei*, *M fortuitum* and *M smegmatis* for better iron utilization and mycobactin production than *M vaccae*. Messenger, Hall, and Ratledge (1985) found out that some strains of *M vaccae* do not produce mycobactin and hence *M vaccae* showed decreased growth in iron deficient media. White and Snow in 1968 reported that rate of growth of *M smegmatis* is similar or greater than *M phlei* which is in par with this study. They also reported that the rate of growth of *M fortuitum* and *M phlei* are more or less equal, same pattern also is observed in this study.

Among slow growers, *M tuberculosis* showed maximum growth in high iron media, followed by *M avium* 1723, *M avium* D4, *M bovis, M microti* and MAP. MAP showed no significant growth in medium or low iron media after 2 months of incubation indicating its inability to synthesize sufficient iron binding compounds. Barclay and Ratledge (1988) reported that in iron deficient media, *M tuberculosis* showed enhanced growth than *M bovis* and *M microti* as observed in this study.

As far as mycobactin synthesis is concerned, low iron media favored more synthesis, as iron is the limiting factor for mycobactin production (Wheater & Snow, 1966). Of all the mycobacterial species studied, highest mycobactin production was noticed in *M fortuitum* and least production was noticed in *M vaccae.* When production of mycobactin was analyzed per gram of cells, efficient mycobactin producer was *M smegmatis.*

Assay of mycobactins of different mycobacterial species was done by analyzing growth rate of MAP in iron deficient media for 2 months. Out of all extracted mycobactins used, mycobactins extracted from *M tuberculosis, M phlei and* MAP had promoted best growth of MAP. Mycobactin extracted from *M microti,* proved least efficient MAP growth promoter. From this study it is revealed that for mass production of MAP, mycobactins from M *tuberculosis, M phlei* and MAP are suggested. Media without mycobactin was unable to produce no or detectable growth of MAP in 2 months incubation. In comparison with MAP grown in mycobactin added media, visible growth of MAP was delayed in media with dried *M phlei.* Merkal and McCullough (1982) found that mycobactins from MAP promoted growth of MAP, than mycobactins of *M phlei*. But this study revealed that *M tuberculosis* and *M phlei* mycobactins also promoted growth of MAP more than MAP mycobactin.

Conclusion

All mycobacterial species showing less growth in low iron media revealed the necessity of iron for multiplication of mycobacteria. *M phlei* and *M tuberculosis* gave maximum growth in varying iron concentrations indicating its ability to utilize iron efficiently through enough mycobactin production. Among all the mycobacterial species studied, highest mycobactin production was noticed in *M fortuitum*. When production of mycobactin is analyzed per g of cells, efficient mycobactin producer was *M smegmatis.* MAP was unable to grow in medium and low iron medium due to insufficient mycobactin production. Out of all extracted mycobactins used for the growth assay, mycobactins extracted from *M tuberculosis, M phlei* and MAP had produced best growth of MAP. Mycobactin of *M tuberculosis, M phlei and MAP* is to be used for large scale production and for primary culturing of MAP.

REFERENCE

1. Barclay, R., and Ratledge, C. (1988). Mycobactins and exochelins of Mycobacterium tuberculosis, M. bovis, M. africanum and other related species. Journal of General Microbiology, 134, 771-776. | 2. Francis, J., Macturk, H. M., Madinaveitia, J., and Snow, G. A. (1953). Mycobactin, a growth factor for Mycobacterium johnei. I. Isolation from Mycobacterium phlei. Biochemical Journal, 55,596-607. | 3. Jones, C. M., & Niederweis, M. (2011). Mycobacterium tuberculosis Can Utilize Heme as an Iron Source. Journal of Bacteriology, 193(7), 1767–1770. doi: 10.1128/JB.01312-10. | 4. Merkal, R. S., and McCullough, W. G. (1982). A new mycobactin, mycobactin J, from Mycobacterium paratuberculosis. Current Microbiology, 7, 333-335 | 5. Messenger, A., Hall, M. J., and Ratledge, C. (1985). Iron Uptake Processes in Mycobacterium vaccae R877R, a Mycobacterium Lacking Mycobactin. Journal of General Microbiology, 132, 845-852. | 6. Morrison, N. E. (1965). Circumvention of the mycobactin requirement of Mycobacterium paratuberculosis. Journal of Bacteriology, 89,762-767. | 7. Snow, G.A., (1970). Mycobactins, iron-chelating growth factors from mycobacteria. Bacteriol Rev. 34, 99-125. | 8. Wheater, D. W. F., & Snow, G. A., (1966). Assay of the Mycobactins by Measurement of the Growth of Mycobacterium johnei. Biochemical Journal, 100, 47-51 | 9. White, A. J., and Snow, G. A., (1968). Methods for the separation and identification of mycobactins from various species of mycobacteria. Biochemical Journal, 108, 593-597. |