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A New Mycobactin, Mycobactin J, from *Mycobacterium paratuberculosis*

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Abstract. This paper reports the production, purification, and biological assay of a new mycobactin from *Mycobacterium paratuberculosis*. This new mycobactin, designated mycobactin J, is produced by a strain of *M. paratuberculosis* adapted to growth on synthetic medium without exogenous mycobactin. Mycobactin J reduces the incubation period of *M. paratuberculosis* in complex medium by 3 weeks when compared with medium containing mycobactin P, and a higher proportion of the organisms, in inocula from infected tissues or fecal specimens, produce colonies. Also, some strains of *M. paratuberculosis* will grow on medium containing mycobactin J that will not grow on medium containing mycobactin P.

One of the distinguishing characteristics of the Johne's bacillus, *Mycobacterium paratuberculosis,* is its requirement for the incorporation of mycobactin in the complex medium used for primary cultivation [1]. After their activities have been determined, the mycobactins from any species of mycobacteria usually can be used in the medium for cultivation of *M. paratuberculosis,* as can the nocobactins produced by nocardia. The amount of these compounds required for the growth of *M. paratuberculosis* is used as a measure of their activity. The standard ferric mycobactin that we produce for cultivation of *M. paratuberculosis* from field specimens is made from *Mycobacterium phlei* and contains mainly ferric mycobactin P. The amount of mycobactin produced by a given species is an inducible characteristic determined by the level of ferric ion in the medium. Very little mycobactin is produced by any mycobacterial species in high-iron medium; when iron is the growth-limiting factor, mycobactin production is maximal [4].

After isolants of *M. paratuberculosis* have been adapted to growth on laboratory media, they can be cultured on media without exogenous mycobactin if enough ferric ion is incorporated [3]. Also, they multiply in tissues of susceptible animals without an exogenous mycobactin source. Either they need less iron during in vivo growth, or possibly high enough levels of iron are available in the macrophages so that the organisms are not in an induced state and their mycobactin production is extremely low.

We have isolated, from goats, some strains of *M. paratuberculosis* which were very difficult to culture on standard media containing ferric mycobactin P. It seemed that if a strain of *M. paratuberculosis* adapted to in vitro growth without exogenous mycobactin were to be grown on the standard low-iron medium used for mycobactin P production, the strain might produce an extractable quantity of mycobactin. A mycobactin produced by *M. paratuberculosis* could be better able to provide the necessary ferric ion to mycobactin-dependent strains of *M. paratuberculosis* and increase the probability of successful cultivation. Moreover, such a homologous mycobactin should support the maximum growth rate.

This paper reports the production, partial purification, and biological evaluation of a mycobactin from *M. paratuberculosis*. Most mycobacteria previously reported have been named by the initial letter of the producing species. However, because the name mycobactin P has been assigned to the principal mycobactin from *M. phlei,* we chose the name mycobactin J for the principal mycobactin from the Johne's bacillus. Analysis of the chemical structure of mycobactin J is reported separately [2].

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Materials and Methods

Cultivation. Seed cultures of laboratory-adapted *Mycobacterium paratuberculosis* (NADC St. 18) were grown as surface pellicles in Roux bottles containing 110 ml of high-iron liquid medium. This medium consisted of 0.9 g sodium citrate · $2H_2O$, 0.3 g ferric citrate, 1.42 g anhydrous dibasic sodium phosphate, 1.5 g magnesium sulfate · $7H_2O$, 0.027 g zinc sulfate, 0.004 g copper sulfate, 0.067 g calcium chloride, 0.0013 g cobalt nitrate, 14.0 g asparagine, 10.0 g glucose, and 63.0 g glycerol per liter.

When the pellicles of seed cultures were well developed (ca 3 weeks), the culture fluid was aspirated and the organisms (15 g wet wt) were suspended in 200 ml of pentane. A Cornwall automatic pipette was used to inoculate 1 ml of the suspension onto the surface of low-iron liquid medium in each of 200 Roux bottles. The low-iron medium was identical to the high-iron medium except that the ferric citrate was replaced by 0.001 g ferric chloride per liter.

The cells were harvested on coarse filter paper after 3 weeks of incubation at 38°C. Each batch of 200 Roux bottles of the low-iron medium yielded approximately 3 kg of wet cells (470 g dry wt). This contrasts with an average yield of 5 kg of wet cells when these organisms are grown on the same amount of high-iron medium.

Extraction. The wet cells were suspended in 2.8 liters of 95% ethanol per kg and were stirred 2 day at 4°C. The cell mass was allowed to settle, then the alcoholic extract was filtered through coarse filter paper, and 30 ml of saturated ferric chloride in 70% ethanol was added per liter of alcoholic extract. Ferric mycobactin was extracted into chloroform by shaking each volume of the extract with one volume of distilled water and one volume of chloroform and removing the chloroform layer with a separatory funnel. The chloroform was removed by evaporation in vacuo at 60°C, leaving 0.3 g of residue per kg wet cells.

Purification. In stage 1, the chloroform residue was dissolved in methanol and centrifuged at 2,100 × *g* at 6 to 8°C to sediment the major part of nonmycobactin lipids. Since the mycobactin could not be crystallized from the methanol solution, the methanol was removed by evaporation in vacuo at 60°C and the residue was taken up in benzene. The benzene solution of mycobactin was applied to a 3-cm diameter, 25-cm long preparatory column of aluminum oxide in benzene. The mycobactin solution was added until one-fourth to one-third of the column was saturated with mycobactin as evidenced by the dark purplish red color of the mycobactin. The column then was flushed with 2 liters of benzene to remove additional lipids. Ferric mycobactin J then was washed through with 2% methanol in benzene. The yield of ferric mycobactin J was 9.5 mg per g of dry organisms. The extract was used at this level of purity for microbiological assays.

In the second stage of purification for structural analysis, the preparation was dissolved in cyclohexane and applied to a column of aluminum oxide slurried with cyclohexane. The chromatogram was developed by the successive application of 1 liter of cyclohexane, 1 liter of benzene, and a discontinuous gradient of methanol-benzene in which the methanol concentration was varied from 0.5% to 1.0% to 2.0% and to 5.0%. The most mobile fraction contained the major concentration of material followed by three smaller fractions displaying extensive tailing. The most mobile fraction was again carried through this procedure. The most mobile fraction contained more than 80% of the material from stage 1, as estimated by optical density measurements at 450 nm. This fraction was dried at 40°C in vacuo and used for chemical structure determination [2].

Assay. Ferric mycobactin J was incorporated into egg-yolk agar medium, 7H10 agar medium, and Dubos broth medium [1] at levels of 0.0, 0.1, 0.5, 1.0, 2.0, and 5.0 mg/liter dispensed in 7-ml portions in 25-cm^2 tissue culture flasks. Egg-yolk agar medium containing 2.0 mg/liter of ferric mycobactin P was used as a standard control. In tests to determine the effect on growth of a standard strain, 0.25 ml of a suspension of ATCC 19698 *M. paratuberculosis* was inoculated into each flask and incubated at 38°C.

Fecal samples from 3 paratuberculous cows were used to determine cultural efficacy for primary isolation of *M. paratuberculosis*. A 20-g fecal sample from each cow was placed in 180 ml of distilled water and shaken 10 min. After standing 30 min to settle the heavy particles, the top 20 ml of each sample was shaken with 20 ml of pentane. After separation, the interface of each was collected, the rest of the pentane was removed by evaporation, and the aqueous part was decontaminated with 0.3% benzalkonium chloride for 18 h at room temperature. Tissue culture flasks containing egg-yolk agar with each of the above concentrations of mycobactins were inoculated with the treated specimens and incubated as before.

In tests to determine the effect of mycobactin J on previously noncultivable strains of *M. paratuberculosis*, tissue specimens from paratuberculous goats that had failed to yield cultures of *M. paratuberculosis* on medium containing ferric mycobactin P were ground in 0.3% benzalkonium chloride and held for one-half, 1, or 18 h at room temperature. The treated specimens were inoculated on egg-yolk agar containing 2 mg/liter ferric mycobactin P or ferric mycobactin J.

Results

Growth of ATCC 19698 *Mycobacterium paratuberculosis* was apparent in 3 weeks on egg-yolk medium containing 0.5 mg/liter or more of ferric mycobactin J, and on 7H10 agar, and in Dubos broth containing 0.1 mg/liter or more. No growth was evident at 3 weeks in the flasks without mycobactin or in the flasks with ferric mycobactin P. At 6 weeks, the growth in flasks with mycobactin P was equal to the 3-week growth with mycobactin J.

In flasks inoculated with fecal specimens from paratuberculous cows, no colonies developed on any medium without mycobactin. Minute colonies observable at 30× were present at 6 weeks in the flasks containing from 0.5 to 5.0 mg/liter of ferric mycobactin J. These colonies were visible without magnification at 8 weeks. Approximately 10% as many colonies were visible at 30× on the medium containing 2.0 mg/liter ferric mycobactin P at 9 weeks. These colonies were visible without magnification at 11 weeks.

The tissues from previously noncultivable paratuberculous goats yielded colonies observable at 30× at 6 weeks and unaided at 10 weeks on the

medium containing ferric mycobactin J which had been inoculated with specimens treated for either one-half or 1 h with benzalkonium chloride. No growth was found after the 18-h treatment with benzalkonium chloride on either medium or after any treatment on medium containing ferric mycobactin P even after 6 months of incubation.

Discussion

Most of the mycobactin used in this country and in many other countries is ferric mycobactin P produced at this laboratory. The media, inoculation, extraction, and chelation methods described for the production of ferric mycobactin P are those that have been in routine use at this laboratory for many years. Ferric mycobactin P crystallizes readily from methanol solution so that it can be purified without column chromatography; however, no other mycobactin described has been crystallized. We also were unable to induce crystallization of ferric mycobactin J from methanol or from any other solvent; therefore, column chromatography was necessary to separate it from other lipids.

Culture of fecal specimens from animals in paratuberculous herds has been the most efficient method of culling infected animals. The most serious disadvantage of culturing as a diagnostic tool has been the relatively long incubation period required. When mycobactin P is used as the iron source, 12 weeks of incubation has been considered the minimum time necessary to assure development of all possible colonies. In all comparisons examined here, the time required for equivalent colony development was reduced by 3 weeks. This difference in incubation period is important both to the producer who wishes to remove shedders from the herd as quickly as possible and to the diagnostic laboratories that must ration incubator space. The increased number of colonies that develop from each inoculum on medium containing ferric mycobactin J implies that a higher percentage of shedder animals will be detected and that they will be detected earlier.

The use of the homologous mycobactin J should greatly enhance the usefulness of fecal culture as a diagnostic tool for detecting *Mycobacterium paratuberculosis* infected animals. An additional benefit from the use of ferric mycobactin J is the capability of culturing the strains of *M. paratuberculosis* found in a few goat herds.

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