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Identification of Factors that Promote the Growth of Dormant Mycobacterium avium subsp. paratuberculosis

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Identification of Factors that Promote the Growth of Dormant *Mycobacterium avium* subsp. *paratuberculosis*

By

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A Thesis Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

In

Biological Science Department

Minnesota State University, Mankato

Mankato, Minnesota

November, 2012

Acknowledgements

First of all, I would like to express my deep gratitude to my advisor, Dr. Timothy Secott. His patient guidance, enthusiastic encouragement and continuous support are indispensable to my thesis as well as my growth in the past three years. His willingness to give his time so generously has also been very much appreciated. I am especially grateful to Secott for his devotion to his students' education and success. In addition, I would like to thank the committee members, Dr. Dorothy Wrigley and Dr. James Rife, for their insightful comments, questions as well as all the help for HPLC. A special thank of mine goes to my colleague, Ekta Ghimire and Kristen Curry, who helped me in completing the project, giving suggestions on my thesis writing and helping me with my presentation and making this project less hard and more accurate. Special thanks to the Department of Biological Sciences who provided all the support I needed to complete my thesis and for funding my study.

Last but not the least, I would like to thank my family, my parents giving birth to me at the first place and supporting me spiritually throughout my life. Thanks my brother for being a great brother and always be.

Abstract

Johne's disease, which is caused by the acid-fast bacterium *Mycobacterium avium* subspecies *paratuberculosis* (Mpt), is a chronic, inflammatory intestinal disease that primarily affects ruminants. This disease has a significant effect on the economics of dairy farming. Mpt has a remarkable ability to survive in host tissues for 2-6 years without producing any signs of infection. However, the reliability of diagnostic techniques is limited only to those animals with clinical disease. This may be due to the entry of the organism into a dormant state, which has been reported for other mycobacteria. Reversion to the actively growing state (resuscitation) would improve the sensitivity of diagnostic culture; however, the substances effecting resuscitation have not been defined. Previous work in our laboratory showed that conditioned medium obtained from starvation-dormant Mpt (SDCM) enhanced the resuscitation of dormant Mpt. Research by others demonstrated that resuscitation of other dormant mycobacterial pathogens was enhanced by resuscitation promoting factors (Rpf), a class of 17-19 kDa proteins that are secreted by these organisms. We therefore hypothesized that one or more Rpf was responsible for the resuscitative activity in SDCM. In this study, we sought to identify and characterize fractions of SDCM that contain resuscitative activity. SDCM was fractionated into 6 molecular weight classes, and each fraction was tested for the ability to promote the resuscitation of anaerobically dormant Mpt responder cells. Most (if not all) of this activity was confined to a fraction containing molecules with a molecular weight of 5 kDa or less (F5). To characterize F5, this fraction was subjected to

heat, protease treatment, and combinations of the two. The resuscitative activity of F5 was stable at high temperatures (65°C and 80°C), resistant to trypsin and thermolysin, and was not impaired by a combination of heat and protease treatment. Growth factors in F5 were concluded to be non-protein in nature, thereby refuting our hypothesis. F5 prepared from 5-, 6, and 8-month-old cultures were analyzed by reversed-phase high performance liquid chromatography to compare peptide and acyl homoserine lactone (AHL) components in F5. AHL peaks steadily accumulated with time, whereas peptide analysis identified hydrophobic peaks that generally diminished over time, and hydrophilic peaks that increased with time. Further characterization and identification of this Mpt-resuscitative growth factor will be necessary in order to test its ability to enhance the recovery of dormant Mpt in clinical specimens.

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Chapter 1: Introduction

Johne's disease, which is caused by the acid-fast bacterium *Mycobacterium avium* subsp. *paratuberculosis* (Mpt), is a chronic, inflammatory intestinal disease that primarily affects ruminants. In the clinical phase of the disease, infected animals lose weight despite having a good appetite, and develop profuse diarrhea (11). These animals become weakened and eventually die. Animals are usually infected within the first few weeks following birth, but clinical signs typically are not apparent until 2-5 years post-infection (12). Mpt is transmitted mainly by the fecal-oral route, in which healthy animals ingest food or water contaminated by feces from infected animals (74).

Johne's disease has a significant effect on the economics of dairy farming. In the United States, yearly losses have been estimated to be up to $1.5 billion, and its impact on the world economy is remarkably immense (79, 80). The average cost of Johne's disease per cow is about $245, which is due to reduced milk production, early culling, and poor body condition at culling (79). Although it is still a controversial issue, the presence of Mpt in intestinal tissues of patients with Crohn's disease, a chronic, inflammatory intestinal disease in humans, has led some to believe that this organism may cause Crohn's disease (20). Recent reports about the presence of Mpt in milk from infected cows have therefore raised concern about the safety of dairy products (24, 25).

At present, there is no effective treatment for Johne's disease. Although vaccine development is being actively pursued, no currently available vaccine is able to prevent infection (41). Therefore, early Mpt diagnosis is essential in order to manage Johne's

disease. There are many tests available to diagnose Johne's disease. Among these, fecal culture is considered the gold standard for detecting Mpt infection.

Fecal culture has been used to support a diagnosis of Johne's disease for almost 100 years. This method requires little equipment and is technically simple to perform. Moreover, the analytical specificity of fecal culture approaches 99.1%-100% (60, 88). Unfortunately, the sensitivity of fecal culture ranges from 96% in clinically diseased animals to 30%-50% when asymptomatically infected animals are included in the testing population (10, 60, 88). It is generally believed that animals in the early stages of disease pose a low risk to the herd with respect to disease transmission, i.e a sample from early-stage animal can be easily detected as positively infected and the animal would be culled out of the herd before it spreads Mpt to others (66). That belief is based on an assumption that, all Mpt are able to survive the sample decontamination process that precedes fecal culture and will be able to grow on nutrient-rich culture medium. However, there have been mounting evidences that pathogenic mycobacteria enter a dormant state that enables them to persist for years inside the host, and these dormant mycobacteria cannot be easily recovered in culture (2, 33, 84). When conditions again become favorable in the host, dormant mycobacteria can resume growth, leading to the progression of disease.

Given these observations for other pathogenic mycobacteria, it is possible that the poor sensitivity of fecal culture from animals in the early stages of Johne's disease may be due to the presence in feces of dormant Mpt which cannot be recovered using the current fecal culture protocols (84). It is therefore important to ensure that dormant cells in the samples have been resuscitated. This may be accomplished by augmenting culture media with growth factors demonstrated to facilitate the recovery of dormant organisms.

Some gram-positive organisms produce a protein called resuscitation-promoting factor (Rpf) (33, 97). This protein was first found in conditioned medium (cell-free medium from a previous culture) from *Micrococcus luteus,* which can form dormant cells (33). The addition of Rpf to the dormant *M. luteus* can enhance the growth and restore the ability of this organism to replicate. Rpfs are small (15-17 kDa), heat labile, and trypsin-sensitive (33, 45). Several mycobacteria possess Rpf homologues, and the proteins they encode possess the ability to resuscitate *Mycobacterium tuberculosis, Mycobacterium bovis and Mycobacterium smegmatis* (97). It is reasonable to assume that the protein may have similar effect on Mpt. However, factors accelerating the growth of Mpt dormant cells have not yet been identified.

Research in our laboratory has shown that the recovery of Mpt rendered dormant by slow transition to anaerobiosis (anaerobic dormancy) was enhanced when these cells were cultured in conditioned medium obtained from Mpt rendered dormant by nutrient deficiency (starvation dormancy). It was also observed that the factor responsible for resuscitation was a secreted product (18). It indicated that in starvation-dormant conditioned medium there are one or more specific factors that can improve the recovery of dormant Mpt. While *M. tuberculosis* contains five homologues of Rpf, four of them were identified in the genome of Mpt (27). Therefore**, the hypothesis of this research was that the recovery of Mpt from anaerobic dormancy is mediated by resuscitation-promotion factors (Rpf) present in conditioned medium obtained from starvation-dormant Mpt.**

The objectives chosen to generate data to fulfill the above hypothesis were to i) partition of conditioned medium from starvation dormant Mpt (SDCM) into different

molecular weight fractions and test these fractions individually for their ability to promote the resuscitation of anaerobic dormant Mpt; ii) to subject resuscitation promoting fractions to heat and protease treatment to determine the effect of these treatments on resuscitative activity; iii) use reversed-phase high-performance liquid chromatography (HPLC) to characterize the components of the resuscitation-promoting fractions.

Characterization of the factors affecting the resuscitation of dormant Mpt is expected to lead to changes in protocols and media used in fecal culture that will improve the sensitivity of early detection of Mpt. This in turn will lead to improved diagnosis and keep dairy products safe and affordable for consumers.

Chapter 2: Literature review

2.1 Johne's disease

2.1.1 History

Johne's disease (also known as paratuberculosis) was first described by H. Johne and L. Frothingham in 1895 (12). Shortly after their published description, the disease was also noticed in the Netherlands and the United Kingdom (6). Johne's disease was subsequently reported in several other European countries including Switzerland in 1905, Denmark in 1906, and France in 1907 (41). The first documented case of Johne's disease in US was reported in 1908 (52). By the 1920s cases of Johne's disease was observed in Africa and Asia, and cases in India and South America were reported in the 1930s (41). Australia reported the presence of Johne's disease in 1936, and shortly thereafter the disease was considered to be endemic in South Australia (41). Johne's disease had been identified on all continents except Antarctica.

2.1.2 Economic impact of Johne's disease

Mycobacterium avium subsp. *paratuberculosis* (Mpt), the causative agent of Johne's disease, can infect a wide range of animals including wild and domestic animals. Among them, domestics are the most commonly infected animals (3, 5, 6). Infections in domestic ruminant usually result in economic losses. In US the effect of Johne's disease is particularly severe in dairy cattle. The disease causes losses in milk production, premature culling and reduction in slaughter value. Although dairy animals are typically infected early in life, clinical sign usually don't appear until 2-5 years after infection,

which causes prolonged exposure of herd to Mpt **(**12**)**. The first study of Johne's disease in the US was performed at University of Pennsylvania in 1908 and the resulting report warned about the serious economic loss for paratuberculosis (52).

A study performed by The National Animal Health Monitoring System (NAHMS) in 1996 estimated that the average cost for producers for a Johne's disease clinical cow in infected herds is $245, mostly due to reduced milk production (79). Economic loss due to paratuberculosis in US dairies was estimated to be at least $250 million annually. The National Animal Health Monitoring System Dairy 2007 study estimated that 61.8% of US dairy operations were infected with Mpt and at least 25% US dairy operations had high percentage of infected cows in the herd (80**)**.

2.1.3 Symptoms and stages of Johne's disease

The incubation period for Johne's disease is prolonged. The mean incubation has been estimated to be 5 years in cattle. The disease progresses through 4 stages (87). In stage I, also referred to as the silent stage, young animals (from birth to a few months old) get infected by ingesting Mpt in milk or from the environment. These animals can get infected with a low dose of organisms (about 10^3 Mpt) (41). During stage I, shedding of Mpt is not detected, and the animal shows no clinical signs. This stage can last from a few months to years and slowly progresses to the stage II (87).

In stage II, subclinical infection stage, the animals begin to shed Mpt in their feces. The shedding contaminates the environment and serves as source of future infections. Animals in this stage may show no sign of infection but may be susceptible to reproductive problems. These animals nevertheless have a high chance of remaining in

the herd and continuing the spread of infection. Only 15%-25% cases at this stage of disease can be detected as positive by fecal culture method (87).

Stage III, the clinical stage, is characterized by chronic diarrhea. Signs can include emaciation, decline in milk production, and proteinemia. Other signs include abortion and alopecia. Animals lose weight despite having a good appetite. Large numbers of Mpt are shed in feces, with more than 10^{10} colony-forming units per gram of feces. This dramatically increases the level of environmental contamination in the area. Infections in stage III animals are easily detected by most diagnostic methods (87).

In stage IV, the advanced stage, the animal has persistent diarrhea. This leads to death of the animal due to dehydration and wasting (87).

The dynamics of Johne's disease in dairy herds has been described as "the iceberg phenomenon". For every case of clinical Johne's disease on the farm, there may be 6-8 animals in stage II and 10-15 animals in stage I (80).

2.2. *Mycobacterium avium* subsp. *paratuberculosis*

2.2.1 Characteristics

Mpt was identified as the causative agent of Johne's disease by Twort in 1910 (78). It belongs to the genus *Mycobacterium*, which includes gram positive, non-motile, obligately aerobic, acid-fast bacilli. Mpt is a small bacillus, approximately 0.5 μm in diameter by 1.5 μm in length. The cell wall contains 60% lipid, primarily mycolic acids, which makes the organism able to resist decolorization by acidified alcohol. The thick, waxy cell wall protects Mpt from chemical and physical factors, thus giving the organism a survival advantage (12, 87). However, this cell wall also restricts the uptake of nutrients.

Among the mycobacteria, Mpt possesses two intriguing characteristics: extremely

slow growth and the inability to produce the siderophore mycobactin. Mpt is the slowest growing species of mycobacteria, with the generation time over 20 hours under optimal conditions (35). When growing on solid agar under optimal conditions, Mpt takes 3-6 weeks to produce colonies. The colonies are small, white and have a complete margin (41). Mpt grows best at 37°C (12). Early attempts to culture Mpt from cases of Johne's disease were initially unsuccessful. In order to recover Mpt, Twort and colleagues added a hot ethanol extract of *Mycobacterium phlei* to their culture medium and suggested that something present in *M. phlei* supported the recovery of Mpt (78). Further characterization identified mycobactin as the necessary ingredient. Mycobactin belongs to a group of organic compounds known as siderophores, which are used by most bacteria and fungi to obtain iron from the environment. Mycobactin dependency was originally used as a distinguishing characteristic for Mpt; however, some strains of *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *silvaticum* also require mycobactin (96). Therefore, mycobactin dependency is no longer used as the sole criterion for identifying Mpt.

2.2.2 Classification

Mpt belongs to the *Mycobacterium avium* complex (MAC). This complex includes *Mycobacterium intracellulare, M. avium* subsp. *avium, M. avium* subsp. *hominissuis* and *M. avium* subsp. *paratuberculosis*. Members of this complex share 90% genomic similarity. MAC species can be distinguished based on the 16S-23S internal transcribed spacer (ITS1) and 16S rRNA sequences (22). Subspecies are distinguished based on the insertion sequences (IS) present in their respective genomes. Mpt alone

contains IS*900,* and its genome does not contain IS*1245,* while all of the other MAC species have the latter insertion sequence in their genome (89).

Two Mpt strains have been initially recognized based on host species of origin. These are designated as S (sheep) and C (cattle) strains. Molecular typing techniques have been used to further discriminate among Mpt isolates (70). Because Mpt has a fairly broad host range, the S and C strain type designations may cause confusion. Therefore these strain types were then designated as type I (formerly S type) and type II (formerly C type).

There are differences between these two strains based on their host preferences, phenotypic characteristics, virulence, and genotypes. Type I strains are reported to be sensitive to ampicillin and vancomycin hydrochloride. These antibiotics are used in specimen decontamination procedures, making culture of type I strains more difficult (55). Type I strains are found in sheep and goats. They produce pigmented colonies on solid medium. Type II strains produce non-pigmented colonies and are resistant to vancomycin and ampicillin (70). Type II strains have a wide range of hosts. They can be isolated from both domesticated and wild animals such as cattle, foxes, stoats, and rabbits (3). Type II strains have also been isolated from Crohn's disease patients (89).

2.2.3 Genome

The complete genome sequence of Mpt (bovine isolate K-10) was published in 2005. The Mpt genome is a single circular chromosome containing 4,829,781 base pairs. It has a G + C content of about 67% and encodes 4,350 predicted ORFs and one rRNA operon. More than 3,000 genes of Mpt are homologous to genes of *M. tuberculosis*, the organism that causes tuberculosis in humans (39)
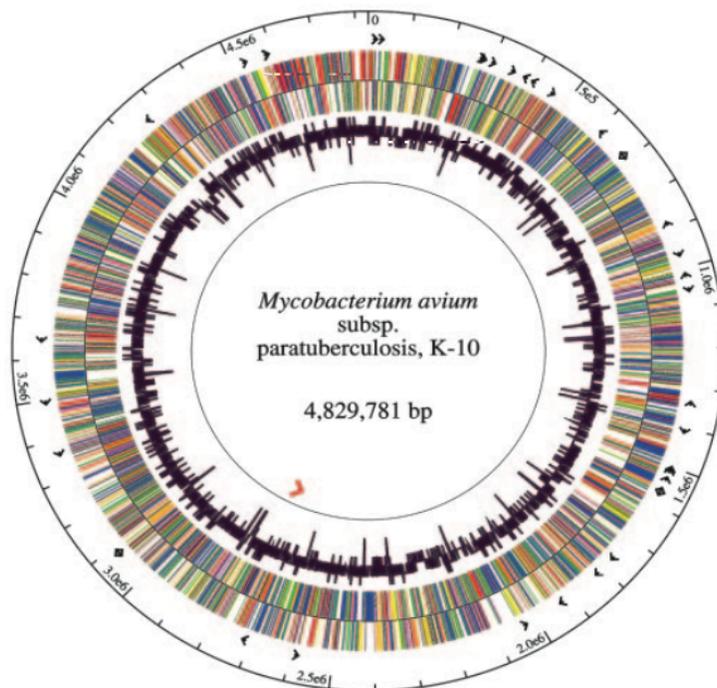
Fig 1. The complete genome map of *Mycobacterium avium* subsp.

***paratuberculosis* strain K-10** (39)

The genome sequence explains the mycobactin dependency of Mpt. Mpt has a deletion in *mbtA-J*, the genes necessary for mycobactin synthesis. Mpt also lacks genes for *frdBCD*, which encodes fumarate reductase; *narX* which encodes a positive regulator of nitrate reductase, and *ureABC* and *ureDFG*, which encode urease. All of these genes are present in other MAC organisms (39).

Genes associated with virulence in *M. tuberculosis* were also detected in the genome of Mpt. The PE/PPE family of proteins in *M. tuberculosis* is believed to be responsible for host interaction, inhibition of antigen progressing, and survival in macrophages. This gene family was also found in Mpt, with 10 PE and 37 PPE homologues present (39). One of the *mce* gene clusters in *M. tuberculosis*, which are

believed to be responsible for mammalian cell entry, was found in Mpt but is absent from the genomes of other MAC. It may play an important role in the virulence of Mpt (39).

The Mpt genome was found to contain a high number of repeat sequences and insertion sequences (IS). IS are short DNA that functions as transposable elements. The genome of Mpt K10 contains 58 IS, including 17 copies of *IS900,* 7 copies of *IS1311* and 3 copies of *ISMav2,* along with 185 mono- to trinucleotide repeats. These sequences may be useful as molecular tools for the detection or identification of Mpt (39).

2.2.4 Transmission and spread of Mpt

Mpt is transmitted mainly through the fecal-oral route. Infected animals shed Mpt in their feces, contaminating the livestock environment (74). Animal can get infection by grazing in the contaminated area. Mpt can be absorbed into the soil and contaminate water sources. The agent can then be spread when the water flows to another location. Mpt is also found in raw cow's milk and colostrum, meaning that nursing can directly result in transmission (74). There is also evidence that Mpt can be transmitted to offspring *in utero* (82). Because farmed animals are maintained in fairly restricted spaces, transmission can occur easily, placing the entire herds at risk once Mpt has been introduced into the herd.

2.2.5 Immunology of Mpt infections

After Mpt enters the host by ingestion of contaminated food or water, it invades the host intestine by entering microfold cells (M cells) covering Peyer's patches in the small intestine (46). The function of M cells is to transport foreign particles or organisms across the epithelial layer to underlying antigen presenting cells, T cells, and B cells of the Peyer's patches, so that the foreign particle can be recognized and destroyed by the

immune system. Subepithelial macrophages phagocytose the bacteria and may present Mpt antigens to T lymphocytes (46). Like other mycobacteria, Mpt is able to survive inside macrophages (44). The organism prevents macrophage apoptosis. Further, Mpt-containing-phagosomes fail to become acidified and do not fuse with lysosomes. Infected macrophages produce cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-∝, and IL-12. These cytokines activate another macrophages as well as T cells and B cells. Stabel et al. studied the effects of TNF-∝ on the recovery of Mpt. They concluded that if the host cells were treated with high doses (4,000 IU) of TNF-∝ before infection, the number of viable Mpt recovered in culture was reduced (66). CD4+ T cells were reported as the main source for TNF-∝, but other immune cells also produce this cytokines during Mpt infection (11).

In the early stages of infection, type I T helper cells (Th1) are dominant in the immune response to Mpt. As the disease progresses to later stages, the Th1 response wanes, and Th2 response begin to dominate. As a result, anti-Mpt antibody production increases; however, these antibodies are largely ineffective against an intracellular pathogen such as Mpt.

γδ T cells may also play a role in the protection of the host. In a study by Tanaka about the role of γδ T cells, TCR-γ/δ deficient mice showed more Mpt colonization than did TCR-∝ or TCR-β deficient mice (75). Others have suggested that γδ T cells play a role in granuloma formation (41).

Granulomas, which are characteristic lesions of mycobacterioses, are formed when macrophages, T cells, and B cells accumulate around infected macrophages in order to minimize the spread of the pathogens beyond the site of infection. Macrophages

within granulomas often fuse together to form multinucleate giant cells. The formation of granulomas and the accumulation of immune cells in infected tissues result in the thickening of the intestinal wall, which causes a reduction in the surface area available for nutrient absorption in the intestine (41). This eventually leads to malnutrition and the diarrhea that are observed in the latter stages of Johne's disease.

2.2.6 Survival of Mpt in the environment

Mpt has the ability to survive outside the host for extended periods of time. Depending on environmental physical and chemical factors such as heat, organic matter, pH, sunlight, and water availability, Mpt can survive for a year or longer in the livestock environment. In dry, fully shaded locations, Mpt in fecal pellets could survive up to 55 weeks (90). Mpt can survive in dam water in the shade up to 48 weeks and Mpt can survive in sediment an additional 12 -26 weeks (91). The ability of Mpt to resist heat was demonstrated in a report about the presence of Mpt in commercially pasteurized milk (26, 27). This finding suggested that Mpt-contaminated milk might be responsible for causing Crohn's disease. Mpt is also resistant to chlorine, the disinfectant used in water treatment (86). Exposure of Mpt to 2 μg of chlorine for 30 minutes resulted in a less than 2,000-fold reduction in the population of Mpt. Therefore Mpt is likely to be able to contaminate water systems as well as water reservoirs. In a survey of US water systems in 2008, Mpt was found in 80% of the tap water samples, with concentrations of 1 to 29,000 CFU/l, although most samples had less than 500 CFU/l (4).

2.3 Management of Johne's disease

Because of the increasing worldwide burden of Johne's disease, control programs are needed to manage the spread of the disease. Since there is no cure for Johne's disease,

the best ways to control infection are vaccination and early detection of infected animals in the herd.

2.3.1 Vaccination

Vaccines for Johne's disease have been available for many years, and many iterations of vaccine are being developed. Some commercial vaccines are prepared as heat killed whole cell vaccines such as Mpt strain 316F (Gudair[TM] and Mycopar, Neoparasec[TM]), and the heat-killed 5889 Bergey strain. Gudair[TM] is the only vaccine approved for use in Australia (Pfizer, NSW, Australia), and it is also available in some European countries (51). The vaccine is used for sheep and goats, and the animal requires only one dose during its lifetime. The age for vaccination should be before the exposure to Mpt (4-16 weeks after birth) (51). In US the only approved vaccine is Mycopar (Boehringer Ingelheim, Ridgefield, CT, USA) (28). The age of vaccination is between 7 to 35 days old (28). Both of these vaccines, however, produce non-specific cross reactivity in serology diagnostic tests due to high persistent level of antibodies (69). It is hard to distinguish between vaccinated animals and infected animals, thus leading to confusion when people use serologic testing to cull infected animals.

Next generation vaccines are subunit-based vaccines, including protein subunit vaccines and DNA vaccines. The antigens for these proposed vaccines have been shown to induce strong immune responses in animals with stage I Johne's disease (Table 1). Genes of the candidate protein are being used to produce DNA vaccines. Advantages of DNA vaccines include their stability and their easy purification.

Table 1: Protein vaccines for paratuberculosis

Protein vaccines	Subunit function	Reference number
Ag85 complex (Ag85A, Ag85B, and Ag85C)	Mycolyl transferase activity- responsible for the synthesis of cell wall components	58
Hsp65 (GroEL) and Hsp70 (DnaK)	Chaperonin	34
P22 (22KDa)	Secreted protein- immune target	17
MAP1518 and MAP3184	Virulence factors	57

Even though these subunit vaccines are promising, none of these is yet approved. Most importantly, while the approved and proposed vaccines may help to reduce the shedding of Mpt in feces, none have been shown to prevent infection.

2.3.2 Diagnosis

Diagnosis of Johne's disease includes detection of the etiologic agent (Mpt) or detection of the immune response against the agent. The application of a combination of tests and the quantitative interpretation of test results provide valuable tools for diagnosis of Johne's disease in a herd. In addition to culture, polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA) are used to help diagnose Johne's disease.

2.3.2.1 Culture

The gold standard for the detection of Mpt is fecal culture. The specificity of fecal culture is near absolute, but the sensitivity is heavily influenced by the stage of disease in the host animal (10, 60, 88). Isolation of Mpt in liquid or on solid medium offers the ability to complete molecular studies that increase our understanding of the epidemiology, physiology and virulence of this pathogen.

The first effective culture procedure was first developed in 1912 by Twort and Ingram (78). The general composition of medium for Mpt growth includes nutrients essential for supporting Mpt growth, antimicrobials and/or dyes to eliminate contaminants and select for Mpt, and mycobactin. Many mycobacterial media can be used to culture Mpt so long as they are supplemented with mycobactin J, but Lowenstein-Jensen medium (LJ), Herrold's egg yolk medium (HEYM) or Middlebrook media are the most common used (41). Because most bacteria can be detected in broth sooner than in solid media, new systems have been developed that use broth media and indicators that signal Mpt growth. Most of these media are based on Middlebrook broth formulations.

The BACTEC 460TB system is a radioisotope-based detection method. In addition to selective antibiotics and mycobactin J, Middlebrook 7H12-based BACTEC broth includes ^{14}C-labeled palmitic acid as a carbon source. When mycobacteria grow, they produce $^{14}CO_2$, which is detected and measured by BACTEC 460TB instrument. The sample is considered positive or negative based on a growth index (36). The main advantage of this method is that it can detect Mpt faster than conventional culture method (4 weeks instead of 10 weeks for conventional culture) (13). Disadvantages include the expense of the BACTEC instrument and the handling and disposal of radioactive waste (7).

The Mycobacteria Growth Indicator Tube (MGIT) system contains 7 mL of modified Middlebrook 7H9 broth base, enrichment and an antibiotic mixture. At the bottom of the test tube, silicone beads are embedded with an oxygen-sensitive fluorochrome. At the time the medium is inoculated, there is an abundance of free oxygen in the medium, thus quenching fluorescence (62). As Mpt grows, the oxygen is consumed.

Fluorescence increases with decreasing oxygen levels, and cultures are flagged as positive when fluorescence reaches a set intensity. Fluorescence can be assessed visually, or it can be measured using the BACTEC MGIT 960 instrument (62).

In a comparison of the MGIT and BACTEC systems for the recovery of Mpt from milk, both media were able to detect 10 organisms per mL of milk (25). However, while the liquid media are better at supporting the growth of mycobacteria, they also support the growth of unwanted organisms. Mpt is a very slow growing bacterium, so culture contamination by much more rapidly growing organisms (such as those commonly present in feces) can prevent the detection of Mpt.

2.3.2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) methods have made possible the rapid detection of Mpt in clinical samples, by reducing the time of detection time from weeks to within a couple of hours. In most studies, the DNA target has been IS*900*, initially considered to be an Mpt-specific marker (26). The specificity of the IS*900* PCR is about 83%; the sensitivity depends on the load of Mpt in samples, with 81% sensitivity if the sample concentration is over 70 CFU/g of feces and 45% sensitivity if the concentration is lower than 1 CFU/g of feces (68). However the IS*900* target has been found in other mycobacteria such as *Mycobacteria intracellulare*, *Mycobacterium scrofulaceum*, and *Mycobacterium* sp. strain IWGMT 90236 (19). Therefore, more PCR targets specific for Mpt have been identified such as F57, ISMap02, and ISMav2 (53, 67, 72). New PCR assays for Mpt have been developed, such as the triplex real-time PCR (TRT-PCR) assay (29), and a quantitative TaqMan PCR assay (85). TRT-PCR is designed to co-amplify IS900, F57, and ISMap02. In a study using TRT-PCR to detect Mpt, Irenge et. al.

concluded that TRT-PCR is specific and sensitive when compared with culture methods

(29). The TaqMan PCR assay, which uses fluorescent probes, has a higher specificity

(99.7%) than conventional IS*900* PCR; however, test sensitivity varied from 4% in light

and moderate fecal shedders to 76% in heavy fecal shedders (85). Although PCR assays

offer the advantage of speed, they cannot distinguish between viable and nonviable Mpt

cells. Direct testing of feces may lead to false-negative PCR results due to PCR inhibitors

present in fecal samples or in sample decontamination solutions.

2.3.2.3 Enzyme-linked immunosorbent assays (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a technology that has been used

for detection of immune responses to Mpt. Commercially-available ELISA kits for Mpt

have been designed to detect antibodies or the cytokine interferon gamma (IFN-γ) in

serum or milk of an animal (41). These assays enable the rapid, inexpensive testing of

large numbers of samples. Commercially-available antibody ELISA kits have a high

specificity and, when animals with clinical Johne's disease are tested, high sensitivity. In

one study, the sensitivity of ELISA on serum and milk relative to fecal culture was 74.3%.

The corresponding values for ELISA-specificity based on the percentage of non-*M.*

avium subsp. *paratuberculosis*–infected goats testing ELISA-negative were 98.6% and

99.3% on serum and milk, respectively (30). However, ELISA is relatively insensitive

when subclinically infected animals are in the testing population. In an experimental

comparison of PCR, ELISA and fecal culture for detecting infection in subclinically

diseased animals, culture and PCR were able to detect more infected animals than ELISA.

The sensitivity and specificity of ELISA were 4.8 % and 97.8%, respectively while those

of PCR were 57% and 85.3% (88). Disadvantages of the current ELISA are that it

requires skilled laboratory technicians. In addition, the specificity of ELISA is dependent on the antigen used in the assay. If the antigen in ELISA is not specific for paratuberculosis, it will lead to false positive results.

2.4 Dormancy

2.4.1 Dormancy definition and models

The ability of Mpt to survive in the macrophages for extended period of time is believed to be associated with the dormant state (84). Dormancy has been defined as " a reversible state of low metabolic activity, in which cells can persist for extended periods without division" (32). Dormancy is considered a viable but not culturable state because dormant Mpt cannot be easily re-cultured. The anaerobic dormancy model for mycobacteria was developed by Wayne (84). In this model, *M. tuberculosis* was cultured in the presence of oxygen then oxygen was suddenly and gradually depleted. Growth and survival were measured by turbidity, numbers of colony forming unit on the solid agar, ATP concentration, and glycine dehydrogenase (GDH) activity. When oxygen was depleted rapidly, the organism died shortly thereafter. However, when oxygen was gradually depleted, the organism remained alive but had low metabolic activity. In this stage, no growth was observed, and GDH activity was reduced. When the surviving organisms were diluted into fresh and oxygen-rich medium, the number of viable count (CFU) doubled within 32 hours after dilution (84). Other mycobacteria such as *Mycobacterium smegmatis* and *Mycobacterium bovis* also show the ability to transform from an actively growing state to dormant state (32). Mycobacteria can also enter dormancy as the result of starvation. In a study with the organism, authors observed that

the metabolic rate dropped by 60% at day 10 and by 80% at day 53, then the viability was about 92% at day 25 (2).

2.4.2 Dormancy characteristics

The physiological state of Mpt changes during adaptation to starvation. Total lipid content per cell declines in the beginning of the starvation period, then recovers to the original level after a month (day 32) of starvation (2). Lipid was hypothesized as the energy source during starvation. Archuleta et. al. explored lipid consumption and concluded that the bacteria may have stored the lipid as triglyceride globules (2).

Mycolate synthesis also decreases during starvation. As a major component of the mycobacterial cell wall, mycolic acids provide a protective thick layer. There are three types of mycolic acid in mycobacteria: type alpha- , type beta- (methoxyl-), and type gamma (keto). Among them, α-mycolic acid is the most abundant (at least 70% of the mycolic acids), folllowed by then keto-mycolic acid (approximately15%), and methoxyl-mycolic acid (approcimately 10%). During starvation, the total synthesized mycolate diminished, with a small decline in methoxyl-mycolic acids (responsible for wax ester). However synthesis of alpha-mycolic acids increases slightly, and the synthesis of keto-mycolic acid increases considerably (2).

2.4.3 Resuscitation

Resuscitation refers to the transition from dormancy to culturable state. The mechanism underlying resuscitation has been the subject of investigation by several laboratories (33, 45, 61, 83). Several factors that enhance the reactivation of dormant cells have been reported.

The most frequently cited factor is a family of small (15-17 kDa) secreted proteins known as resuscitation promoting factors (Rpf). Rpf was first isolated from the conditioned medium (CM) of *Micrococcus luteus* (33). CM is cell-free medium from a previous culture. The addition of Rpf-containing CM induced the resuscitation of dormant *M. luteus*, resulting in the forming of colonies on agar plates (33). Rpf proteins exhibit activity at low picomolar concentrations (31). The proteins contain a lytic transglycosylase domain similar to that of lysozyme (45). The lytic activity has been demonstrated to have an effect on peptidoglycan substrates. However the relationship between peptidoglycan hydrolysis and the resuscitation is not clear (76). Rpf genes are also present in other gram-positive high G+ C organisms such as *Rhodococcus rhodochrous, Mycobacterium smegmatis, Mycobacterium tuberculosis* and *Mycobacterium paratuberculosis* (27, 97). Five homologues of Rpf (rpfA-rpfE) are present in the genome of *M. tuberculosis*. Inactivation of individual Rpf genes showed no effect on resuscitation, which suggested the functional redundancy of these genes (77). However, if three of these genes were disrupted, resuscitation was reduced. This indicated a potential synergy of Rpf genes in the resuscitation of dormant cells. It was predicted that two of the Rpf proteins function to break down peptidoglycan and produce second messengers that stimulate the bacterial growth (61). This second messenger was proposed to be a phospholipid or a specific peptide (Rv1174c). Based on HPLC and MALDI mass spectra, the fatty acid was identified as 10-methyloctadecanoic acid or arachidonic acid (95).

Peptide Rv1174c is an 8kDa peptide isolated from the culture supernates of *M. tuberculosis* (95). This peptide was expressed in *E. coli* and demonstrated resuscitative

activity when tested on *Mycobacterium tuberculosis* H37Ra. Peptides (22 to 25 amino acid residues in length) derived from the Rv1174c also showed resuscitative effects separately and in combination. Zhang and colleagues suggested that Rv1174c may be subjected to proteolysis to form smaller signaling peptides (95).

In previous research in our lab, it was shown that CM from nutrient depleted dormant Mpt (starvation-dormant CM, or SDCM) was able to enhance the resuscitation of both starvation- dormant Mpt and cultures rendered dormant by gradual oxygen depletion (anaerobically-dormant Mpt) (18). The ability to enhance the recovery of organisms that are not otherwise likely to grow in culture would be expected to have strong potential for improving the sensitivity of diagnostic culture for Mpt. To that end, characterization of the resuscitative factors in Mpt SDCM merits further study.

Chapter 3: Materials And Methods

3.1 Preparation and collection of conditioned medium from starvation-dormant Mpt

Mycobacterium avium subsp. *paratuberculosis* (Mpt) PAMSUM8 (Minnesota State University, Mankato culture collection) was grown in M7H9C [Middlebrook 7H9 broth (Becton Dickinson, Sparks, Md.) supplemented with 10% oleic acid-albumin-dextrose-catalase (Becton Dickinson), 0.05% Tween 80 (Sigma, St. Louis, Mo.), and 2 µg of mycobactin J (Allied Monitor, Fayetteville, Mo.) per ml]. Mpt (1 ml) was grown in 25 ml M7H9C at 37^0C in sealed 160ml tissue culture flasks. Acid fast staining was done to check for contamination. After at least 5 months, Mpt cultures were harvested by centrifugation at 3000rpm at 4^0C for 30 minutes. The supernate was collected and filtered through a 0.2 µm filter. This filtrate was used as starvation-dormant conditioned medium (SDCM).

3.2 Preparation of anaerobically-dormant responder Mpt

Anaerobically dormant Mpt responder cells were prepared by inoculating 100 µl of a mid- to late-exponential phase Mpt into a 10 ml serum vial containing 7 ml M7H9C. The vial was closed with a rubber septum, sealed with an aluminum cap, and incubated at 37^0C for at least 4 months.

Before Mpt responder cells were collected (after at least 4 months in culture), the viability of the culture was measured using the LIVE/DEAD *Bac*Light™ - Bacterial Viability Kit (Invitrogen**)**. The kit contains SYTO 9, which fluorescent green when bound to DNA and is membrane-permeant (staining DNA in all cells); and propidium iodide

(PI), which fluorescent red when bound to DNA and is membrane-impermeant (displacing SYTO 9 in dead and dying cells).

To collect cells for the viability assay, 100 μl of the dormant culture was withdrawn from the culture vial with needles fitted to 3 ml syringes then put in an eppendorf. The eppendorf was centrifuged at 1817 x g for 30 minutes. The pellet was collected and washed in 1 ml filtered phosphate buffered saline (PBS). The suspension was centrifuged again at 1817 x g for 30 minutes, and the dormant cell pellet was resuspended in 1 mL PBS. One hundred microliters of the bacterial suspension was transferred to a clean 1.5ml microcentrifuge tube and mixed with an equal volume of 1:1 SYTO 9:PI. The cell-dye suspension was incubated for 15 minutes and then analyzed by flow cytometry (Guava Technologies, Millipore). The percentage of cells fluorescing green only was recorded as the percent viability of the dormant Mpt responder cells.

To collect anaerobically dormant Mpt responder cells, cultures were centrifuged at 1200 x g for 30 minutes at 4°C. Cell pellets were collected and suspended in 3 ml sterile PBS with Tween-20 (PBST). Cell suspensions were adjusted to 30% transmittance at 490 nm, which is equivalent to 2×10^8 cfu/ml. Tenfold serial dilutions of the adjusted bacterial suspensions were used to obtain dormant cell concentrations of 2×10^5 cfu/ml in 10 ml of M7H9C.

3.3 Fractionation of conditioned medium

SDCM was fractionated by sequential ultrafiltration (Vivaspin 20, Sartorius Stedium Biotech GmbH, Germany) to yield fractions with nominal cut-off values of 5 kDa, 10 kDa, 30 kDa, 50 kDa, and 100 kDa. SDCM was added to ultra-filters and centrifuged at 1200 x g at 4^0C for 30 minutes. The eluate, containing substances with

molecular weights lower than 5 kDa, was collected as fraction 5 (F5). The retentate
(greater than 5 kDa) was resuspended in PBS pH 6.8 and centrifuged as before, using a
10 kDa filter. This second eluate, containing substances with molecular weights between
5 kDa and 10 kDa, was designated fraction 10 (F10). The procedure was repeated to
collect fraction 30 (F30), fraction 50 (F50), fraction 100L (F100L; molecular weights
between 50 kDa and 100 kDa) and fraction 100U (F100U; molecular weights > 100 kDa).

3.4 Preparation of incubation chambers

Two pieces of benzylkonium chloride-soaked paper were placed inside a plastic
sandwich container. The container was then autoclaved at 121^0C, 15 psi for 30 minutes
and kept closed in a biosafety hood until use. To avoid contamination, the incubation
chamber was exposed to UV light in the hood for 15 minutes prior to resuscitation assay
setup or measurement of optical density (OD).

3.5 Assay for resuscitative effect of SDCM and SDCM fractions

Three twofold serial dilutions of SDCM or SDCM fractions were prepared in
M7H9C, and 100μl of each dilution was added to quadruplicate wells of a 96-well
microtiter plate. Anaerobically dormant responder cells (100μl) were inoculated into all
wells to a final concentration of 2000 cfu/well. Control well contained only M7H9C and
dormant responder cells (100μl of each), and blank wells contained only 200 μl M7H9C.
Microtiter plates were covered with a thin transparent plastic seal (Whatman, Piscastaway,
NJ) to minimize evaporation and contamination. The plate was then placed in the sterile
plastic container and incubated at 37^0C for about 15 days. Every two days, plates were
allowed to equilibrate to room temperature, plastic seals were replaced to remove
condensation, and OD_{590nm} readings were obtained. All of the set up for resuscitation

assays and preparation for OD measurements were performed aseptically in a biosafety hood.

3.6 Heat denaturation of fraction 5

Aliquots of fraction 5 were incubated at 65^0C for 20 minutes or 80^0C for 10 minutes in a water bath. After cooling to ambient temperature, F5 was used in resuscitation assays.

3.7 Protease treatment of F5

Trypsin (Sigma-Aldrich) and thermolysin (Sigma-Aldrich) were used separately at 2.5 g/l, 0.25 g/l and 0.025 g/l concentrations to treat SDCM. Following protease treatment for 30 minutes at 37^oC, SDCM was ultrafiltered as described above to collect F5 and remove the protease. Protease-treated F5 was used in resuscitation assays immediately after preparation.

3.8 Combined effect of heat denaturation and protease treatment on F5

The combination of heat and protease treatment was performed two ways: protease treatment followed by heat treatment, and heat denaturation followed by protease treatment. For the former combination, SDCM was treated with trypsin (0.25 g/l) or thermolysin (0.25 g/l). F5 was then prepared from protease-treated SDCM and incubated at 80^oC for 10 minutes. The F5 thus prepared was immediately used in resuscitation assays.

For the latter combination, F5 was prepared as described above and incubated at for 10 minutes at 80^0C. Heat-denatured F5 was cooled to ambient temperature, then incubated at 37^oC with trypsin (0.25 g.l) or thermolysin (0.25 g/ml) for 30 minutes. The

F5 was then filtered through a 5kDa untrafilter to remove the protease. F5 was then used immediately for resuscitation assays.

3.9 High performance liquid chromatography (HPLC)

Reversed-phase high performance liquid chromatography (HPLC) using a C18 column was used to characterize the components of F5. Three F5 preparations of different ages (obtained from five-month old, six-month old and eight-month old cultures) were analyzed. A 1 ml sample of each F5 preparation was subjected to reversed-phase HPLC with a C18 column in a buffer with 0.1 % formic acid (buffer A) and formic acid and acetonitrile (buffer B) to characterize the peptide components. M7H9C was used as a blank and was also characterized for peptide components. To characterize the acyl homoserine lactone (AHL) components in the F5 preparations, the same column was used but with 50% and 90% methanol (buffer A and B, respectively). Again, M7H9C was used as a blank. The results from HPLC were read at 254 nm and at 220 nm (for peptides and AHLs). In the HPLC for peptides, results at both wavelengths showed no difference, thus data at 254nm was chosen for results and data at 220 was placed in Appendix. In the HPLC for AHL results, the peaks appearing after 18 minutes reflected the gradient of the buffer used for HPLC therefore they were not included in the result.

3.10 Data analysis

All results were entered into GraphPad Prism statistical software (La Jolla, CA) for data analysis. Each treatment, including serial dilutions, was graphed and compared to the control. One-way analysis of variance (ANOVA) was used to look for the significant difference among the means of different dilutions in a fraction. Two-way ANOVA was

used to look for significant differences among the means of different dilutions of all

fractions.

Chapter 4: Results

4.1 The effect of SDCM fractions on dormant responder cells

Two-fold serial dilutions of SDCM obtained from cultures aged from 5 months up to 1 year old were tested for the ability to resuscitate anaerobically-dormant Mpt. In the absence of CM, little to no resuscitation was detected in our assay. The optical densities (590 nm) of responder cells incubated with 1:2 dilutions of SDCM increased with increasing time of exposure, and this effect was significant by day 10 ($p < 0.05$ on day 10). Higher dilutions of SDCM yielded results that were not significantly different from control ($p > 0.05$) (Fig 4.1).

In order to isolate the factor responsible for resuscitative activity, SCDM was partitioned by differential centrifugal filtration into six unit molecular weight fractions. Two-fold serial dilution of each fraction was tested for resuscitative activity using dormant responder cells, with unfractionated SDCM used as a positive control. Unfractionated SDCM enhanced the growth rate of responder cells ($p < 0.01$ on day 8, 10, 14 and 16) (Fig. 4.2). Treatment with F5 demonstrated a trend towards improved resuscitation; however, this effect was not statistically significant from that seen for untreated controls ($p > 0.05$). No clear trend could be observed for any of the other SDCM fractions ($p > 0.05$ for all of other fractions).

Because the resuscitative effect of F5 on dormant responder cells was not clearly established in our fraction-screening assay, two-fold serial dilutions of this fraction was again used to treat dormant responder cells. A clear, statistically significant enhancement of resuscitation by F5 was observed ($p < 0.01$ relative to untreated controls). In addition,

the resuscitative effect was greater than that seen for unfractionated SDCM and this effect was statistically significant ($p < 0.05$) (Fig 4.3).

4.2 Testing the effect of F5 in combination with other fractions

Because centrifugal filtration of complex protein solution into discrete molecular weight fractions can be confounded by protein conformation, F5 was combined with F10 or F30 and tested on anaerobically-dormant responder cells. Neither F10 nor F30 alone enhanced the recovery of Mpt (Fig 4.2). However, combination of either of these fractions with F5 showed the resuscitative effect that appeared better with F5 alone (Fig 4.4). This effect was significant ($p < 0.05$ day 8 for the combination of F5 and F10, $p < 0.05$ day 6 for the combination of F5 and F30).

4.3 Effect of heat denaturation on resuscitation enhancement by F5

In order to test the heat stability of the resuscitative factor in F5, aliquots of F5 were incubated at $65^{o}C$ for 20 minutes or $80^{o}C$ for 10 minutes prior to addition to dormant responder cells. Heat treatment of F5 did not appear to affect the resuscitation of dormant Mpt regardless of temperatures as compared with untreated F5 or CM (Figs 4.5A and B). Two-way ANOVA revealed significant ($p < 0.05$) differences among the treatment groups on day 14,, although Bonferroni post-tests did not identify any particular treatment group ($p > 0.05$).

4.4 Effect of protease digestion on resuscitation enhancement by F5

To determine if the resuscitative activity of F5 was susceptible to protease treatment aliquots of F5 were treated with trypsin or thermolysin. SDCM was first treated with either trypsin or thermolysin for 30min at $37^{o}C$ and fractionated through a 5 kDa-cutoff

filter to collect F5. Untreated and protease-treated F5 enhanced the recovery of dormant

Mpt ($p < 0.01$ relative to untreated controls) equally well (Figs. 4.6A and B).

4.5 Effect of both heat and protease treatment on resuscitation activity of F5

Following digestion with trypsin or thermolysin, SDCM was fractionated, and F5

was then heated at $80^{\circ}C$ for 10 minutes. Trypsin-digested, heat-denatured F5 was as

effective as untreated F5 in promoting the resuscitation of anaerobically-dormant Mpt

(Fig 4.7A). Similarly, heat denaturation following thermolysin digestion did not affect the

resuscitative ability of F5 (Fig 4.7B).

Because conformation can influence the access of proteases to cleavage site, we

then heat-denatured F5 prior to digestion with either trypsin or thermolysin. It was

observed that heat denaturation followed by trypsin digestion impaired the ability of F5

to promote resuscitation (Fig 4.7A). However, heat denaturation followed by thermolysin

digestion had no effect on the resuscitative ability of F5 (Fig. 4.7B).

4.6 Reversed-phase HPLC analysis of F5

F5 was prepared from SDCM obtained from 5-month-old, 6-month-old, and 8-

month-old dormant cultures and analyzed by reversed–phase HPLC. These F5 and

SDCM preparations were chosen on the results of earlier resuscitation tests.

4.6.1 Resuscitation assay for CM and F5

SCDM and F5 prepared from 5-month-old, 6-month-old, and 8-month-old

cultures were tested using two responder cells populations, the first containing 2% viable

cells (low viability) and the second containing 42% viable cells (high viability). When

tested using low viability responder cells, SDCM and F5 from 5- and 6-month-old

cultures did not enhance the recovery of dormant responder cells ($p > 0.05$ for both as

compared with untreated control) (Fig. 4.8A and B). In contrast, SDCM and F5 from 8-month old-cultures demonstrated significant resuscitative activity ($p < 0.0001$ for both when compared with untreated control) (Fig. 4.8C). When tested using high viability responder cells, all of the SDCM preparations promoted the recovery of the dormant Mpt ($p < 0.0001$), but only F5 from the 8-month-old culture had a significant resuscitative effect ($p < 0.0001$) (Fig 4.8E, D, and F).

4.6.2 Reversed-phase HPLC analysis of F5 peptides

All three F5 preparations yielded numerous peaks that were not present in the medium blank. These peaks were divided into four areas of interest: area 1 included peaks collected within 6 minutes; area 2 included peaks collected between 12.03- 14 minutes and 16.483-16.492 minutes; area 3 included peaks collected at 29 minutes; and area 4 included peaks collected at 39.542-42.608 minutes. Increasing time of elution represented increased hydrophobicity.

Area 1: All F5 samples had peaks with different profiles, with older sample yielding higher peaks, with the exception of the peak eluted at 4.3. These peptides were considered hydrophilic.

Area 2: Peaks in area 2 had the same pattern as that seen in area 1, in that the peaks increased in size with increasing age of source culture. However, peaks from F5 prepared from the 5-month-old culture were barely noticeable. The peak height from F5 from 8-month-old cells was at least twice that of F5 obtained from 6-month old cells, and those peaks were 25 times higher than area 2 peaks seen in F5 from the 5-month-old cells.

Area 3: A single peak in area 3 (at 29 minutes) clearly decreased with age of source cells.

Area 4: Peaks in area 4 (39.542-42.608 minutes) were hydrophobic. The 39.5 peak was smallest in F5 prepared from 5-month-old cultures and very prominent in F5 from 6-month-old cultures By 8 months, this peak was much smaller, but still greater that that observed from 5-month-old F5. Peaks at 41.8 and 42.6 minutes were present only in F5 from 5-month-old and 8-month-old cultures. The 41.8 minute peak in F5 prepared from 5-month-old cultures was greater than that seen in F5 from 8-month-old cultures. The peaks heights of the 42.6 minute peak were the same from both samples

4.6.3 Reversed-phase HPLC analysis of F5 for AHLs

Since AHLs are variable in structure, two wavelengths (220 nm and 254 nm) were used to measure the eluted HPLC peaks. Peaks were grouped in two areas (area 1-2). Area 1 included peaks collected within 5 minutes. Area 2 included peaks collected at 8.5-13.2 minutes. There was no difference in absorbance between 220nm and 254 nm for all three F5 samples (Fig. 4.10).

Area 1: The sample peak heights increased with increasing age of the source culture; however F5 obtained from 5-month-old and 6-month-old cultures were similar with respect to pattern and height of peaks. The heights of the peaks from F5 prepared from 8-month-old cultures were 2-6 times higher than those of the other F5 preparations.

Area 2: All three F5 preparations were similar with respect to peak high and peak pattern, and the peak heights were compared to those of area 1.
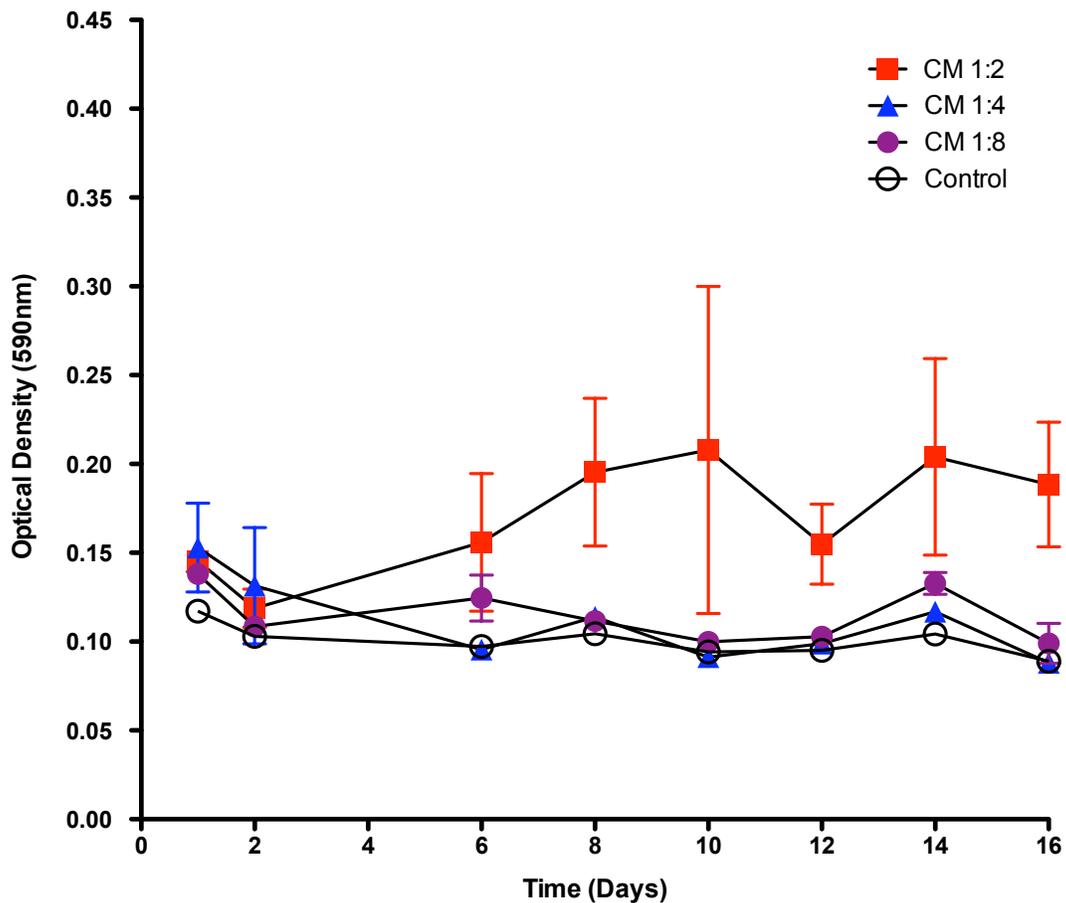
Fig 4.1 Cell-free conditioned medium from starvation-dormant *Mycobacterium avium* subsp. *paratuberculosis* (SDCM) promoted the recovery of anaerobically-dormant responder cells. Twofold serial dilutions of SDCM (100 μl) were added to microplate wells containing 100 μl (2000CFU/ml) of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis*. Plates were incubated for 16 days and growth was assessed by optical density measurements (590 nm) every 48 hours at 590 nm. Control wells contained medium and dormant responder cells only.
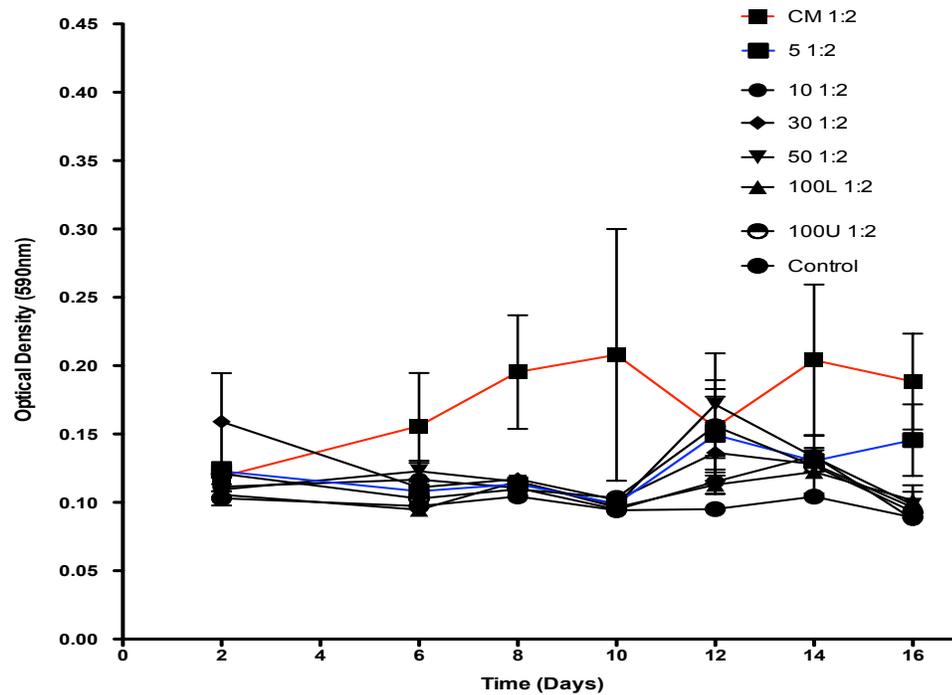
Fig 4.2 The fraction of starvation-dormant conditioned medium (SDCM) containing molecules < 5kDa (F5) best promoted the resuscitation of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis* cells. SDCM was fractionated using ultrafilters with MW cut-offs of 5 kDa, 10 kDa, 30 kDa, 50 kDa, and 100kDa. Fractions were labeled according to the cut-off. F5 contained all molecules with mass lower than 5kDa, F10 all molecules with mass from 5k- 10k, F100L contained all molecules with mass from 50k-100kDa and F100U contained all molecules with mass over 100kDa. Twofold serial dilutions of SDCM (100 μl) were added to microplate wells containing 100 μl (2000CFU/ml) of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis*. Plates were incubated for 16 days and growth was assessed by optical density measurements (590 nm) every 48 hours at 590 nm. Control wells contained medium and dormant responder cells only.
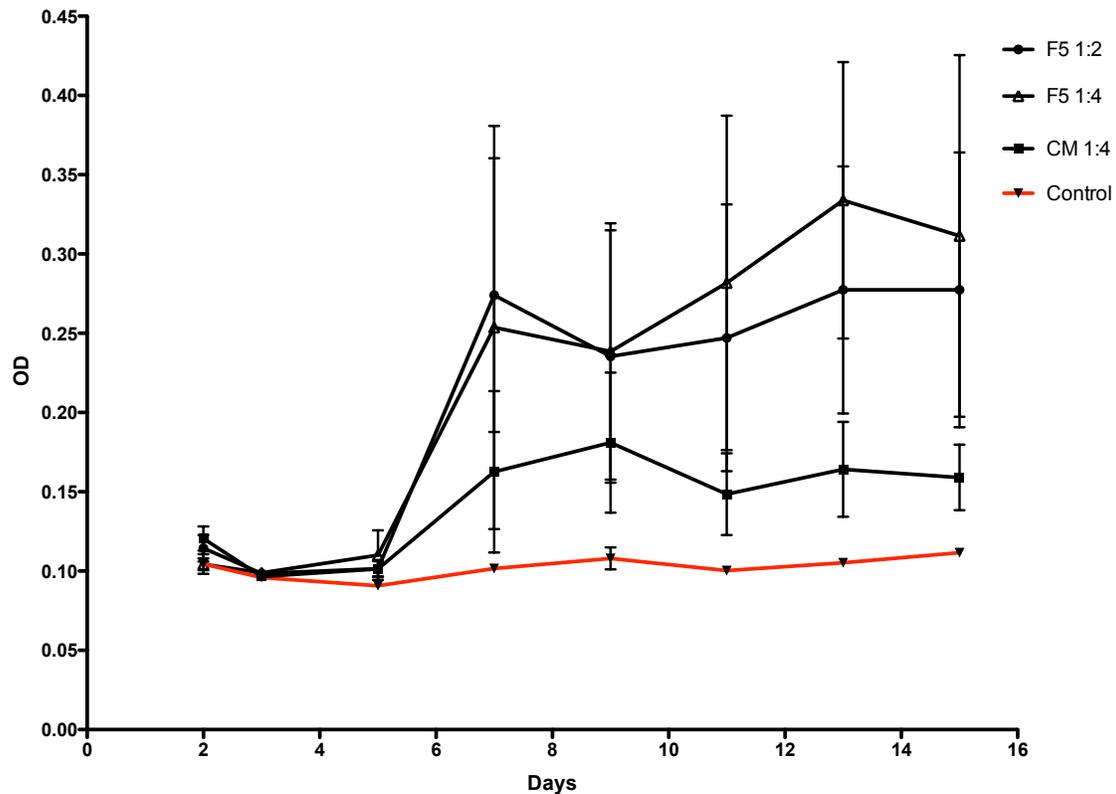
Fig 4.3 Fraction 5 (F5) enhanced the resuscitation of anaerobically dormant

Mycobacterium avium **subsp.** *paratuberculosis* **(Mpt) in a dose-dependent manner.**

Twofold serial dilutions of F5 (100 μl) were added to microplate wells containing 100 μl

(2000CFU/ml) of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis*.

Plates were incubated for 15 days and growth was assessed by optical density

measurements (590 nm) every 48 hours at 590 nm. Negative control wells contained

medium and dormant responder cells only. Inverted triangles - negative control; squares –

SDCM (positive control); solid circles – F5, 1:8 dilution; triangles - F5, 1:4 dilutions
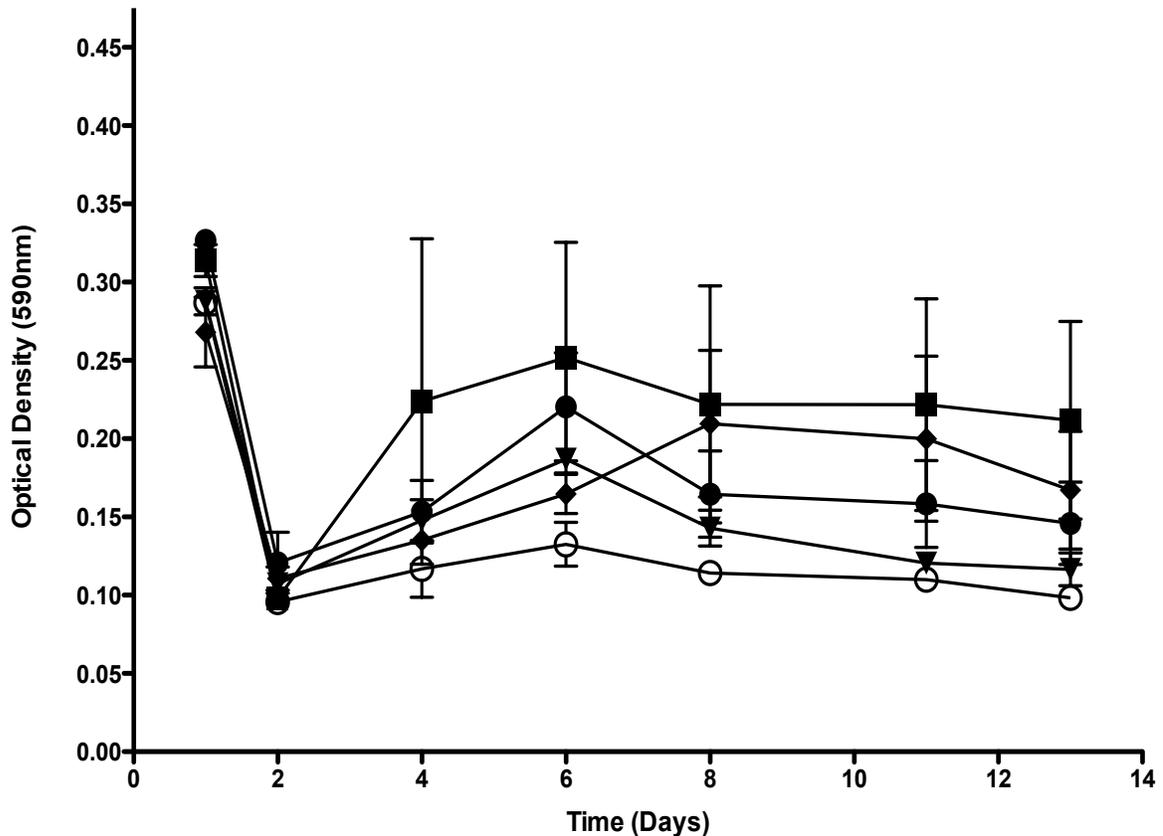
Fig 4.4 Combining SDCM fractions 5 and 10 (F5 and F10, respectively) or F5 and F30 enhanced the recovery of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis* (Mpt). Twofold serial dilutions of F10 or F30 (50 µl) were mixed with an equal volume of twofold serially diluted F5 and added to microplate wells containing 100 µl (2000CFU/ml) of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis*. Plates were incubated for 13 days and growth was assessed by optical density measurements (590 nm) every 48 hours at 590 nm. Negative control wells contained medium and dormant responder cells only. ■ SDCM (positive control) 1:2 dilution; ● Combination of F5 and F30 1:2 dilution; ▼ F5 1:2 dilution; ◆ Combination of F5 and F10 1:2 dilution; O Negative control
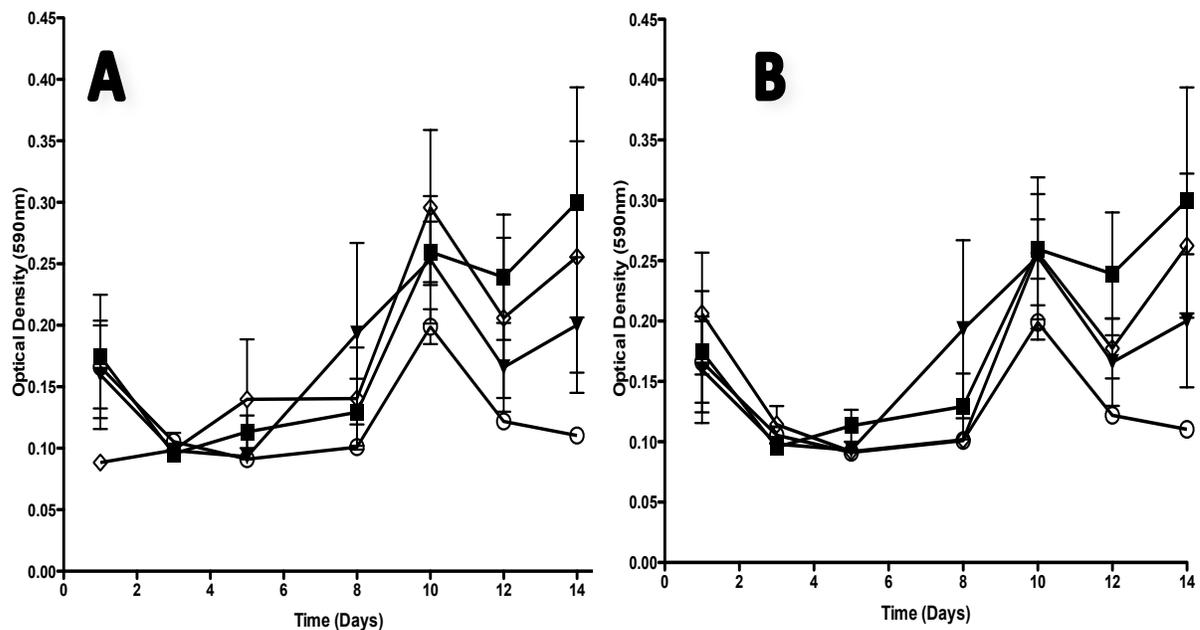
Fig 4.5: Heat treatment alone did not affect the resuscitative activity of Fraction 5 (F5). Fraction 5 was incubated at 65^0C for 20 minutes (A) or at 80^oC for 10 minutes (B). Heat-treated F5s were added (100 μl) microplate wells containing 100 μl (2000CFU/ml) of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis*. Plates were incubated for 15 days and growth was assessed by optical density measurements (590 nm) every 48 hours at 590 nm. Negative control wells contained medium and dormant responder cells only. ■ SDCM (positive control) 1:2 dilution; ◇ F5 treated at high temperature, dilution 1:2; ▼ F5 1:2 dilution; O Negative control
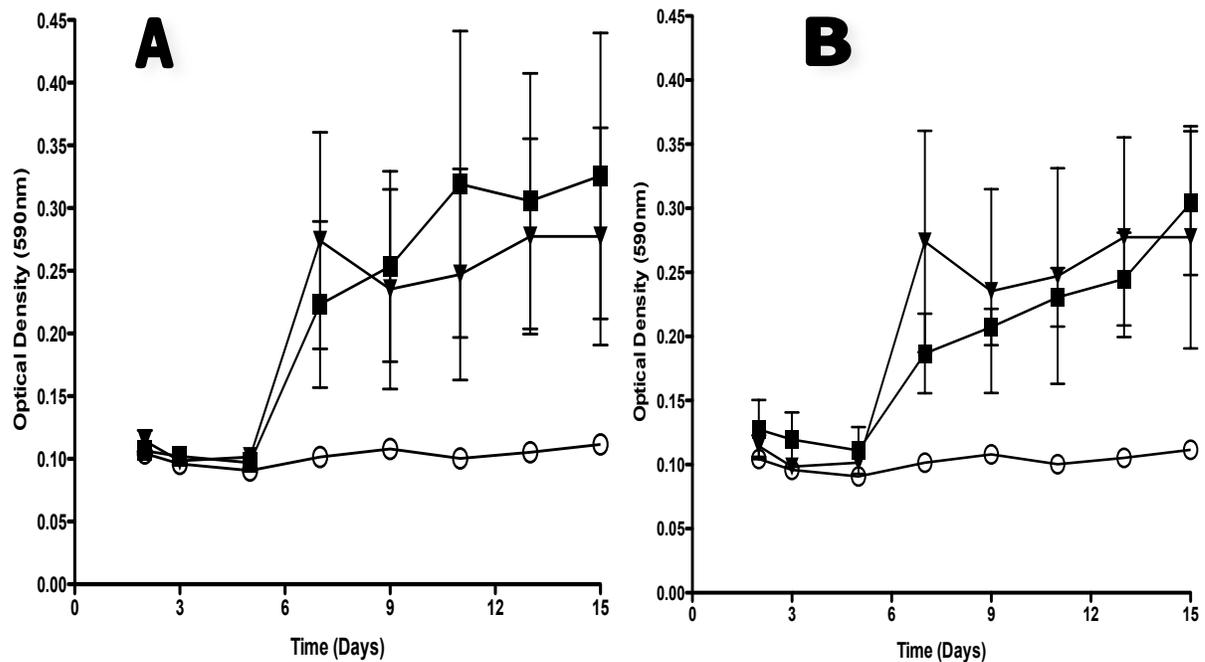
44

Fig 4.6: Protease treatment did not affect the resuscitative activity of fraction 5 (F5).

SDCM was incubated with trypsin (0.25 mg/ml, A) or thermolysin (0.25 mg/ml, B) at 37^0C for 30 minutes. Treated SDCM was filtered through a 5kDa-cutoff filter to collect F5. Protease-treated F5s (100 μl) were added to microplate wells containing 100 μl (2000CFU/ml) of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis*. Plates were incubated for 14 days and growth was assessed by optical density measurements (590 nm) every 48 hours at 590 nm. Negative control wells contained medium and dormant responder cells only. ▼ F5 (no treatment) 1:2 dilution; ■ F5 treated with protease; ○ Negative control
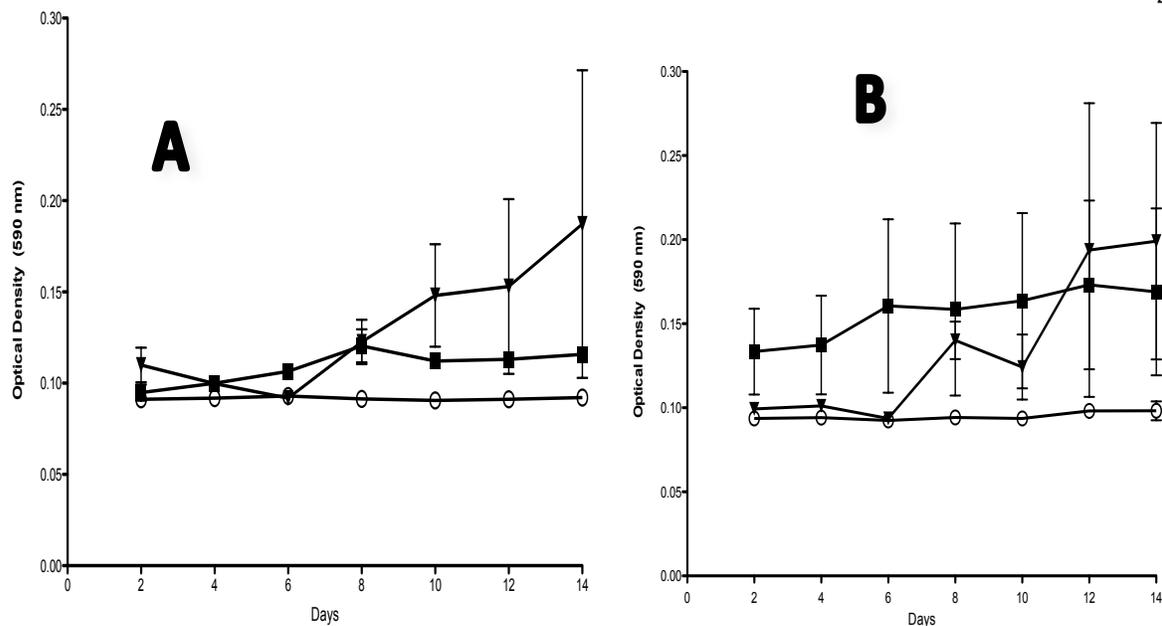
Fig 4.7: Resuscitative activity of fraction 5 (F5) was diminished by heat treatment followed by trypsin digestion but not by trypsin digestion followed by heat treatment (A), but was not affected by the order of heat treatment and thermolysin digestion (B). For protease digestion followed by heat treatment, SDCM was incubated with either protease (0.25g/l) at 37^0C for 30 minutes, filtered through a 5kDa-cutoff filter, and incubated at 80^oC for 10 minutes. For heat treatment followed by protease digestion, F5 was incubated at 80^oC for 10 minutes. After cooling to ambient temperature, F5 was then incubated with either protease (0.25 g/l) at 37^0C for 30 minutes and then filtered through a 5kDa-cutoff filter. Treated F5s (100 μl) were added to microplate wells containing 100 μl (2000CFU/well) of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis*. Plates were incubated for 14 days and growth was assessed by optical density measurements (590 nm) every 48 hours at 590 nm. Negative control wells contained medium and dormant responder cells only; ○ Negative control; ■ F5treated at 80^oC for 10 min followed by protease digestion; and ▼F5 treated with the protease, then incubated at 80^oC for 10 min.

2% viability responder cells
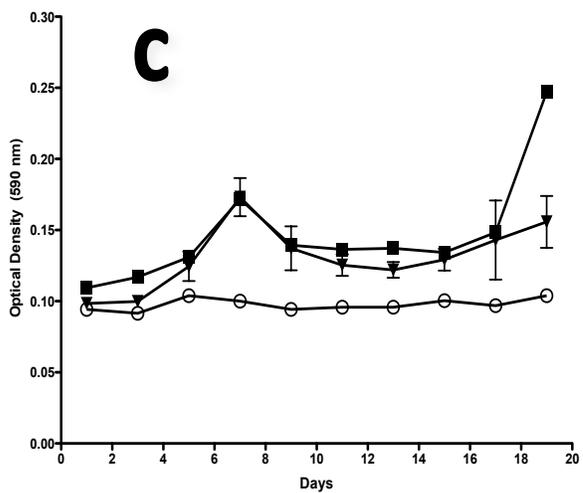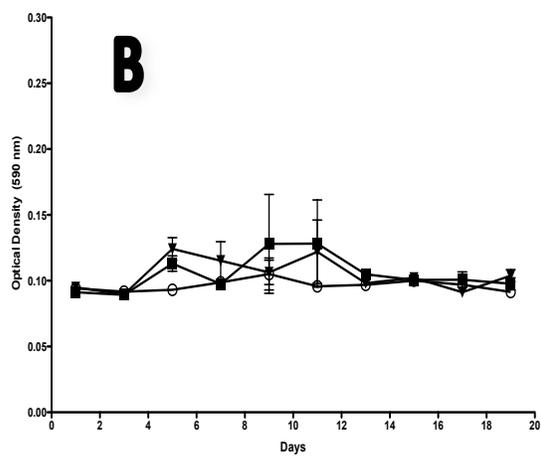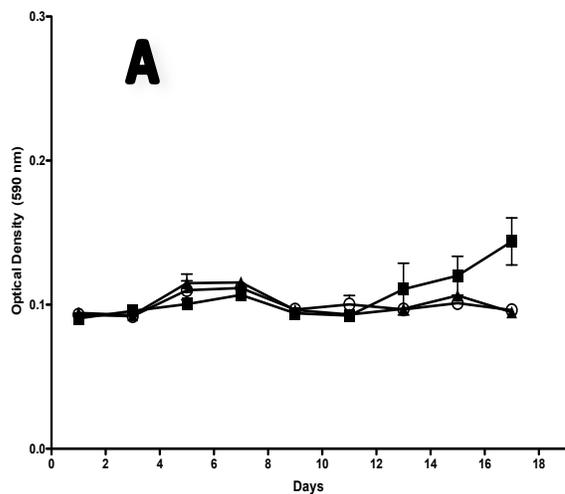
42% viability responder cells
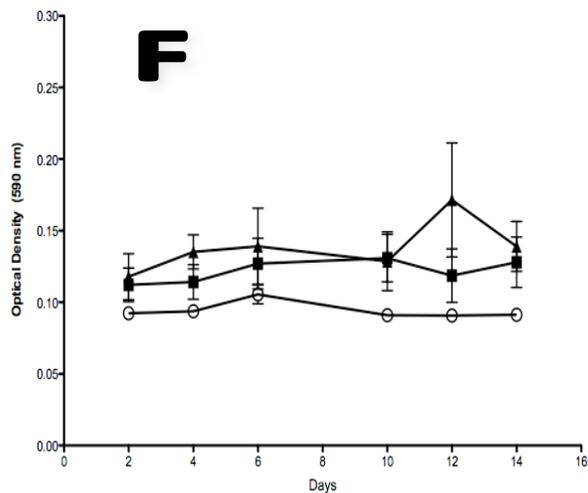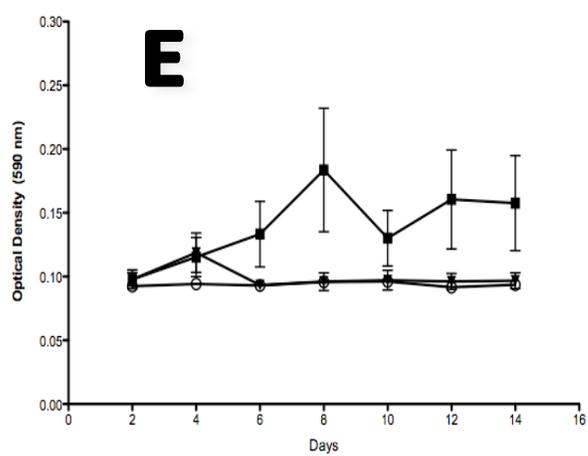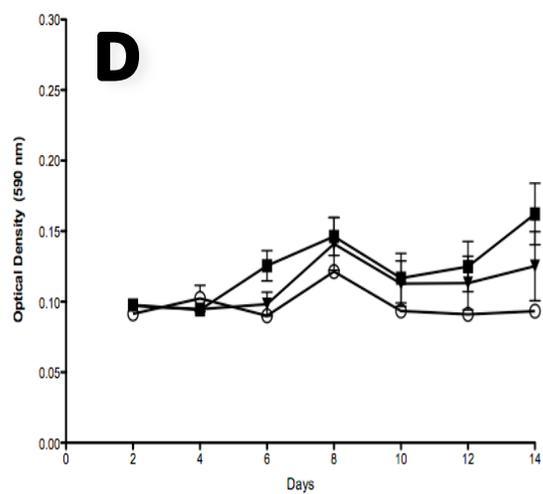
Fig 4.8: The resuscitative effect of SDCM and F5 depends on the age of the source culture and the viability of responder cells. SDCM was collected from 5- (A and D), 6- (B and E), and 8-month-old cultures (C and F), and each of these was used to prepare F5 SDCM and F5 prepared from each source (100 μl) were added to microplate wells containing 100 μl (2000CFU/ml) of anaerobically-dormant *Mycobacterium avium* subsp. *paratuberculosis* that contained 2% viable responder cells (A-C) or 42% viable responder cells (D-F). Plates were incubated for 14-19 days, and growth was assessed by optical density measurements (590 nm) every 48 hours at 590 nm. Negative control wells contained medium and dormant responder cells only. ■ SDCM 1:2 dilution; ▼ F5 1:2 dilution; ○ Control
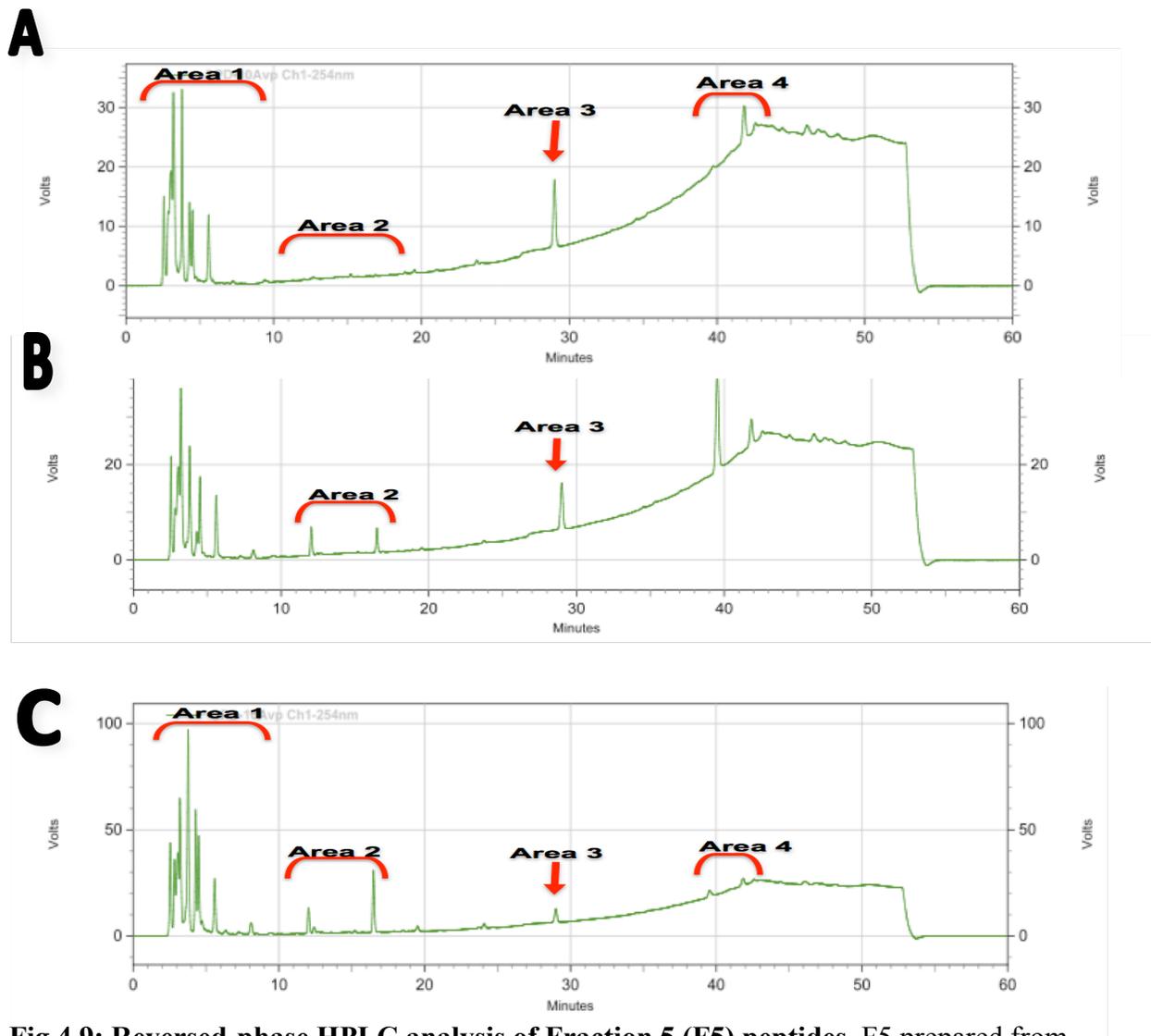
Fig 4.9: Reversed-phase HPLC analysis of Fraction 5 (F5) peptides. F5 prepared from 5-month old cultures (A), 6-month-old cultures (B), and 8-month-old cultures (C) were compared with each other and with the blank (fresh medium). Differences from the medium blank were observed in four areas: Area 1- 0.0-6.0 min, area 2 - 12.03-12.04 min and 16.483-16.492 min, area 3 - 29 min, and area 4 - 39.542-42 min.

Fig 4.10: Reversed-phase HPLC analysis of possible acyl homoserine lactones in Fraction 5. F5 prepared from 5-month old cultures (A), 6-month-o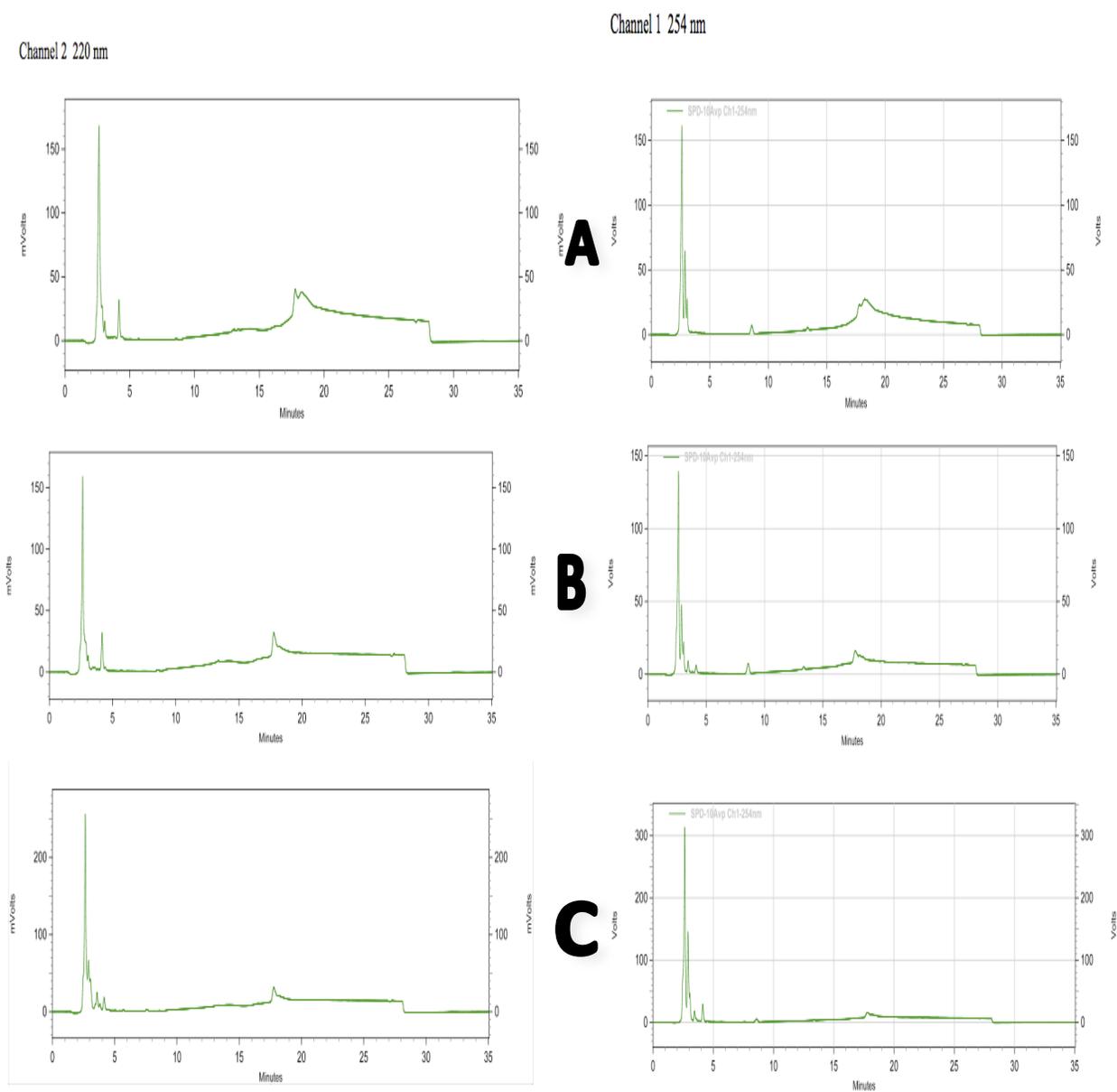ld cultures (B), and 8-month-old cultures (C) were compared with each other and with the blank (fresh medium). Fractions were read at 220 nm (left panels) and 254 nm (right panels).

Chapter 5: Discussion

Fecal culture, because of its relatively high sensitivity and specificity, is considered as the best means for ante mortem diagnosis of Johne's disease. However, because the sensitivity of fecal culture is approximately 45% when animals in all stage of disease are considered, it is a flawed standard. It seems likely that the low sensitivity of fecal culture – particularly with samples from sub-clinically infected animals – reflects the inability to reliably culture dormant organisms. It is therefore important to define the conditions necessary to allow the resuscitation of the organism in order to improve the sensitivity of fecal culture, improve the success of disease management efforts, and allow the development and implement of new testing procedures.

Previous work by Ghimire demonstrated that Mpt secreted factors that promoted the recovery of dormant Mpt (18). The present work sought to further characterize the resuscitative factor present in SDCM. We observed the resuscitative activity was present only in F5; however resuscitation appeared to be enhanced when F5 and F10 or F5 and F30 were used in combination.

Secreted proteins from culture filtrate of Mpt have been subjects of several investigations; however most of them were not well characterized. Cho et. al. found that Mpt cellular extract rich in molecules with molecular weight lower than 30 kDa (9). This suggested that most of the Mpt proteins in SDCM would be present in F5, F10 and F30. Of all the fractions from SDCM, only fraction 5 (F5), which contained molecules with molecular weights lower than 5 kDa, consistently supported the resuscitation of dormant Mpt. Because F5 was necessary and sufficient for resuscitation and because F10 and F30

alone had no resuscitative effect, we believed F5 merits further characterization for identification of resuscitative factors.

The positive effect of CM was also observed in other mycobacteria and gram-positive bacteria (47). The protein identified as Rpf is also a secreted product. Rpf was first found in the culture supernates from *Micrococcus luteus* (31). Several mycobacteria also contain multiple homologues of *M. luteus rpf*. Hedlund identified four *rpf* homologues in the genome of Mpt (27). Therefore, the possibility that the resuscitation of dormant Mpt by Rpf in SDCM was considered. However, because the molecular weights of Rpf are approximately 15-17 kDa (46), those proteins would have been present in F30. Because F30 alone had no resuscitative activity, it is not likely that Rpf plays a role in the resuscitation of dormant Mpt. This is consistent with Hedlund's observation that purified recombinant RpfB did not improved the recovery of dormant Mpt (27)

It is interesting that while F5 alone was sufficient for resuscitation of dormant Mpt, this effect was apparently enhanced when F5 was used in combination with F10 or F30. If the resuscitative factor has a molecular weight near the 5kDa cut-off or had a conformation that limited its passage through the filter, one could expected that both F5 and F10 would have similar activities when used alone. This reasoning would be insufficient to explain the enhanced resuscitation seen when F5 and F30 were combined. F10 and F30 may contain molecules that are precursors of those in F5 or act synergistically with molecules in F5 (95). Alternatively, F10 and F30 might contain enzymes turning precursors in these fractions into the growth factor in F5 or require a factor in F5 for resuscitative activity.

In our investigation we saw a clear dose-dependent effect both with CM and F5; that is, the highest concentration of SCDM and F5 components promoted resuscitation best. In an earlier investigation by Ghimire, this was not the case - lower concentrations of SDCM were required in order for resuscitative activity to be observed (18). It was suggested that the observed effect reflected a conflict between resuscitative factors and inhibitory factors in SDCM. The reason for the difference in observations is not immediately clear and awaits explanation.

The combination of heat treatment followed by trypsinization appeared to have a negative effect on resuscitation. This may indicate that either the resuscitative factor was cleaved, or that inhibitory factor was released as the result of trypsin digestion. Because thermolysin cleaved at more sites (cleavage following isoleucine, leucine, valine, alanine, methionine, and phenylalanine residues) than does trypsin (cleavage following arginine or lysine residues), it would be expected that the similar loss of activity would have been observed for thermolysin digestion. Because thermolysin treatment did not reduce the resuscitative activity of F5, it seems likely that trypsin digestion may have resulted in the release of a factor that inhibited resuscitation rather than degrading a resuscitative factor.

Ying et al. identified Rv1174c (GenBank accession number NP215690) as potential resuscitative factor for *M. tuberculosis*. This 8 kDa protein and three synthetic peptides containing 22-25 amino acids from the Rv1174c sequence appeared to enhance the colony-forming activity of the old H37Ra cells (95). Based on the published sequence from *Mycobacterium tuberculosis*, Rv1174c protein was predicted to have a mass of 10 kDa; however, MALDI mass spectrophotometry revealed the protein with molecular weight as 8.3 kDa. This discrepancy was explained by the observation that the

first 28 amino acids present in the sequence, which represented the signal sequence was cleaved during the secretion process. The finding that 22-25-mer peptides representing Rv1174c had resuscitative activity suggested that the protein might be broken down by proteolysis without the loss of activity.

MAP2609 (GenBank accession number NP9615430), a protein predicted from the genomic sequence of Mpt, is a homologue of Rv1174c. Comparison of amino acid sequences of MAP2609 and Rv1174c by BLAST revealed 78% amino acid similarity between the proteins (40). Because of its homology with Rv1174c, the amino acid sequence of MAP2609 was analyzed using SignalP (http://www.cbs.dtu.dk/ services/ SignalP/) to predict if the gene encoded a potential signal sequence. As with Rv1174c, SignalP predicted a signal peptide with the cleavage site between amino acid residue 28 and 29. It was therefore possible that a peptide from MAP2609 may have been present in F5. However, *in silico* digestion of either Rv1174c or MAP2609 with thermolysin (PeptideCutter, http://web.expasy.org/peptide_cutter/) yielded individual amino acid and short peptides no longer than 8 residues. Because our observations showed that the growth factor in F5 was protease-stable, this indicated that MAP2609 was not likely to be responsible for the resuscitation of Mpt, unless the 8-residue peptides resulting from thermolysin treatment were sufficient for resuscitation.

The characteristics of the unknown growth factor in F5 were similar to that observed for a growth promoting factors from *Mycobacterium tuberculosis*. Four-week-old Mtb H37Ra conditioned medium had a resuscitative effect on dormant (at least 8 month old) Mtb H37Ra (73). The resuscitative phenomenon was found not only in Middlebrook 7H9 medium (relatively rich medium) but also in Sauton's simple salt

medium. The growth factor was characterized as both heat- and trypsin-stable. Resuscitative activity was not affected by RNase, DNaseI, or exonuclease III treatment. The authors concluded that the growth factor was neither protein nor polypeptide. Size exclusion chromatography revealed the mass of this factor to be less than 1.4 kDa (73). This non-protein factor may correspond to the growth factor present in F5. However, information regarding Zhang's non-protein factor, beyond its molecular weight, is presently very limited.

Since Mpt appears capable of making substances necessary for resuscitation from the dormant state, those factors can be considered autoinducers. Peptide and acyl homoserine lactone (AHL) are frequently cited as autoinducers in bacteria (43, 54). However the involvement of these substances in stimulating cell division, a necessary part of resuscitation from dormancy, is not commonly described in literature.

AHL are associated with quorum sensing in gram-negative bacteria. Gram-positive organisms are generally unresponsive to AHLs, and the presence of and the production of acyl homoserine lactonases by *Rhodococcus erythropolis* (which, like Mpt is a high G+C Gram-positive organism) (81) may suggest that AHLs are not likely to be involved in the resuscitation of dormant Mpt. However, overexpression of a LuxR homologue (which binds AHLs) in Mpt led to an increase in virulence in a cell culture assay (1). Therefore, the increases observed over time in area 1 peaks in our AHL screen indicates that further characterization of this region should be considered, regardless of their involvement in resuscitation.

Several Gram-positive bacteria, including Mpt, encode homologues of LuxS, the enzyme that synthesizes 4,5-dihydroxy-2,3-pentanedione (DPD), which acts as a second

autoinducer (AI-2) in *Vibrio harveyi*. While AI-2 plays a role in quorum sensing in Gram-negative bacteria, AI-2-like molecules may act in a quorum sensing-independent manner. For example, biofilm formation can be a quorum sensing-regulated process in bacteria. However, microarray analysis of AI-2-treated *Mycobacterium avium* subsp. *avium* (Maa) and subsequent treatment of Maa cultures separately with AI-2 and H_2O_2 led Geier et al. to conclude that formation of biofilms by this organism was related to environmental stress rather than quorum sensing (23). The observation that dormant *Listeria innocua* cultures did not respond to AI-2 from *Escherichia coli* suggests that AI-2 may not cause resuscitation (94). Because we were unable to obtain information that would allow us to screen for F5 for AI-2-like molecules, it remains unknown whether this class of molecule plays a role in the resuscitation of Mpt.

In considering the possibility of resuscitation by peptides present in areas 1 and 2 of our peptide screening may, at first glance, hold interesting possibilities. The peaks in these areas accumulated with time, whereas peaks in area 3 and 4 either decreased or did not have clear time-dependent relationship. The observation that only F5 obtained from 8-month-old cells resulted in significant resuscitation would indicate that peaks in area 1 or 2 must accumulate to a threshold value before resuscitation can occur. However, it may also be possible that resuscitation occurs as a result of the loss of a repressor rather than the appearance of an inducer. In this case, peaks in areas 3 and 4 would be of interest. A third possibility is that peaks in area 3 and 4 may be hydrolyzed to yield peaks in area 1 and 2. If this is true, it would be necessary to characterize all the peaks in all four areas. What is known is if the resuscitative factor is a peptide, it would likely be a small peptide

(approximately 15 amino acids in the length or smaller) in order to be resistant to thermolysin.

In the low viability culture, we saw resuscitative effect only when F5 and SDCM obtained from 8 month-old cells were used. In our high viability responder cells, SDCM worked well regardless of the age of the source cultures, but F5 promoted resuscitation only when it was obtained from 8 month-old cells. From these observations several explanations can be formulated. First, SDCM may contain both inhibitors and effectors of resuscitation. Since SDCM may contain waste products that can stop the activity of growth factor, resuscitative factors must accumulate to a particular level if resuscitation is to be initiated exclusively exogenously, i.e., if dormant cells have an absolute requirement for resuscitative factor, they need to produce and secret it in the environment until the concentration of factors can overcome the inhibitory effect of the waste products. Sun et al. (1999) reported that dormant Mtb H37Ra could be resuscitated by cell-free supernates from 3-4 month Mtb H37Ra cultures (20-fold better than with fresh medium), but resuscitation was impaired if supernates from 8 month-old cultures were used (1000-fold lower than with fresh medium) (73). They concluded that the increased inhibition resulted from the accumulation of waste products in the supernates. In contrast, we saw an increase in resuscitative potential with increasing age of the source culture. Thus, it appears that time is required for resuscitative factors to accumulate and/or inhibitory factors to be inactivated. The fact that, in high-viability responder cells, CM promoted the resuscitation but F5 did not may indicate that removal of inhibitor molecules was controlled by processing factors present in un-fractionated CM, and a certain ratio of these factors allowed resuscitation to proceed. Separating components in the CM to

collect F5 may have excluded these processing factors, preventing resuscitation. Longer exposure of CM to the source cells would have allowed processing of the inhibitors by extracellular enzymes, explaining the successful enhancement of resuscitation by F5 obtained from 8-month-old cultures.

Second, resuscitation may be achieved with a lower level of promoting factors as initiator if the viability of the dormant culture is above particular threshold. Stated differently, a subpopulation of responder cells may have the ability to amplify the resuscitative effect. In a study of the resuscitation of *Micrococcus luteus*, Votyakova et al. noted that a very small proportion of actively growing cells is required to be present in a dormant culture in order to achieve recovery from dormancy. In the absence of the active responder cells, they were unable to observe Rpf-mediated, i.e even though there was Rpf presence, resuscitation did not occur (83). Although we did not attempt to distinguish active Mpt within the dormant responder cell population, it is possible that they were present. This may be an academic consideration, since it is impractical to distinguish among these Mpt types in diagnostic samples. Thus, the identification of factors that allow resuscitation of low numbers of dormant but viable organisms present in a fecal sample is likely to have greater impact in improving the sensitivity of diagnosis culture.

Third, the resuscitation may require the processing of a precursor in order to release the active resuscitative factor. In the present study, we observed that SDCM from 5-month-old, 6-month-old, and 8-month-old cultures promoted the resuscitation of dormant Mpt, whereas only F5 obtained from 8-month-old cultures did. Removal of molecules with a molecular weight greater than 5 kDa would have likely excluded enzymes that were necessary to cleave the precursor molecule into its active state, and

only after 6 months had enough processed product accumulated to the threshold required for resuscitation.

There were two major problems that require resolution in order for this line of investigation to proceed. The first of these is the "spikes" that appeared in some growth curves in this study. The reason for the sudden, sharp increases in optical density is not clear. However we feel confident that the spikes reflect something other than an increase in bacterial number. If indeed there was a sudden increase in a number of bacteria, the optical density would not have returned to baseline values in the brief period observed. Supporting this conclusion was the fact that these spikes occurred within experiments and were not consistent for any given treatment across experiments.

The second problem was the high degree of variability within and among experiments. Part of this can be attributed to the tendency of mycobacteria to clump together. As such it is difficult to ensure that responder cells get evenly distributed in each experimental setup. Another factor that is likely to contribute to experimental noise is the current lack of a culture system that will yield dormant cultures with consistent levels of viability. This problem has been encountered by others (92), and will require continued effort to refine the experimental system.

Conclusions

Growth factors promoting the resuscitation from dormant state are found in CM of starvation culture of Mpt. These factors as characterized in the present investigation were revealed to be low molecular weight (less than 5 kDa), heat- and protease (trypsin and thermolysin) -stable factors. Since Rpf proteins are 15-17 kDa and trypsin-sensitive, the hypothesis that Rpf is responsible for the resuscitation of dormant Mpt responder cells was rejected. The initial determination that the resuscitative factor was present in F5 suggested that another potential resuscitative factor, MAP2609, may be involved. MAP2609 is an Mpt gene encoding a homologue of Rv1174c from Mtb, which was demonstrated by others to have resuscitative activity in Mtb. However, thermolysin digestion of both MAP2609 and Rv1174c *in silico* indicated that participation of MAP2609 in the resuscitation of Mpt was unlikely, unless octopeptides or smaller peptides from MAP2609 were sufficient for resuscitation. Reversed-phase HPLC was used to analyze the peptides and AHLs in F5 prepared from cultures of various age. AHL peaks steadily accumulated with time, whereas peptide analysis identified hydrophobic peaks that generally diminished overtime, and hydrophilic peaks that increased with time. Resuscitation tests with F5 and SDCM from different age cultures suggested that a threshold of resuscitative factors is required to promote resuscitation. These factors may be needed to remove resuscitative inhibitors or cleave resuscitative precursors. Thus all peaks identified by HPLC in this study should be further characterized, particularly those in area 1 from the AHL analysis and areas 1 and 2 from the peptide analysis, since they accumulated over time.

There were problems in this study that caused a high level of experimental noise as well as unexplainable spikes. First, sudden spikes appeared and subsequently disappeared in many of the time course experiments. The quick disappearance of these spikes strongly suggests that they were not a result of Mpt growth. Nevertheless, they may have had an effect on statistical analyses. Second, the well-documented tendency of mycobacterial cells to clump together likely interfered with the even distribution of dormant responder cells into wells, leading to high standard deviations. Third, the viability of responder cells was not able to controlled. This problem has been observed by others (92). This may explain at least some of the variability in results between similar experiments. Finally, in this study, resuscitation was best when the lowest dilution of SDCM (1:2) was used. In a previous investigation by Ghimire (18), which used the same experimental system, the optimal concentration of SDCM was approximately fourfold lower. The explanation for this discrepancy is as yet unknown, but it may relate to the balance of growth factors versus inhibitors present in SDCM. Therefore it will be necessary to modify the experimental system to avoid or account for these issues.

The results revealed some of the characteristics of the resuscitative factors in SDCM. However, there are many questions remaining. What concentration of the growth factor is necessary for resuscitation? Is there a requirement for actively growing cells in the dormant responder population? Are there factors in other fractions that are necessary for processing so that factors that accumulate in F5 can promote resuscitation? Preparative HPLC and mass spectrometry would enable better characterization of the component present in the peaks identified here, and each of the isolated compounds could

then be used in a resuscitation assay (modified to address the problems mentioned above) to answer some of these questions.

Further analysis is necessary to clarify whether AHLs or AI-2-like molecules play a role in the resuscitation of Mpt from dormancy. A reversed-phase HPLC analysis of commercially available AHLs may help to determine the nature of the peaks present in our AHL screening, and a screening for AI-2-like molecules should be implemented.

Once identified and fully characterized, the factors present in F5 can be purified from cultures or synthesized in the laboratory for use as culture medium supplements. It is therefore hoped that by localizing the resuscitative factor in SDCM to F5, the present study has contributed to the developments that will improve the sensitivity of fecal culture for Mpt, the "gold standard" for the diagnosis of Johne's disease. This will ultimately translate into the improved profitability of dairy farming and provide consumers with safe, affordable dairy products.

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Appendix

**Appendix 1: Screening results for resuscitative factors from SCDM**
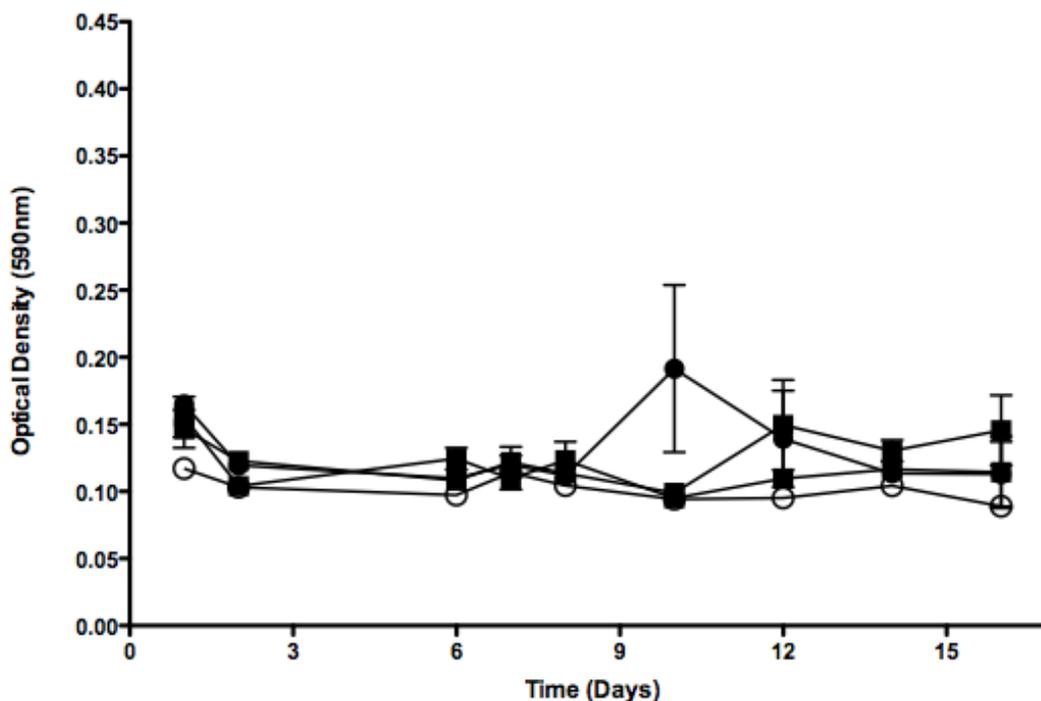
A1. Effect of fraction 5 on the recovery of anaerobically dormant Mpt: Conditioned medium was filtered through 5,000 Da filtered to obtain fraction 5. Two fold serial dilutions of fraction 5 in Middlebrook 7H9 broth were added (100 μl) to the wells of microtiter plate, then 100 μl of anaerobically Mpt dormant cells were added to all the wells. Plate was incubated for 16 days and optical density monitored every 48 hours at 590 nm. The control well contained Middlebrook 7H9 broth and Mpt dormant cells only. Open circle- Control, Square- 1:2 dilutions, Triangle- 1:4 dilutions, and Solid circle- 1:8 dilutions
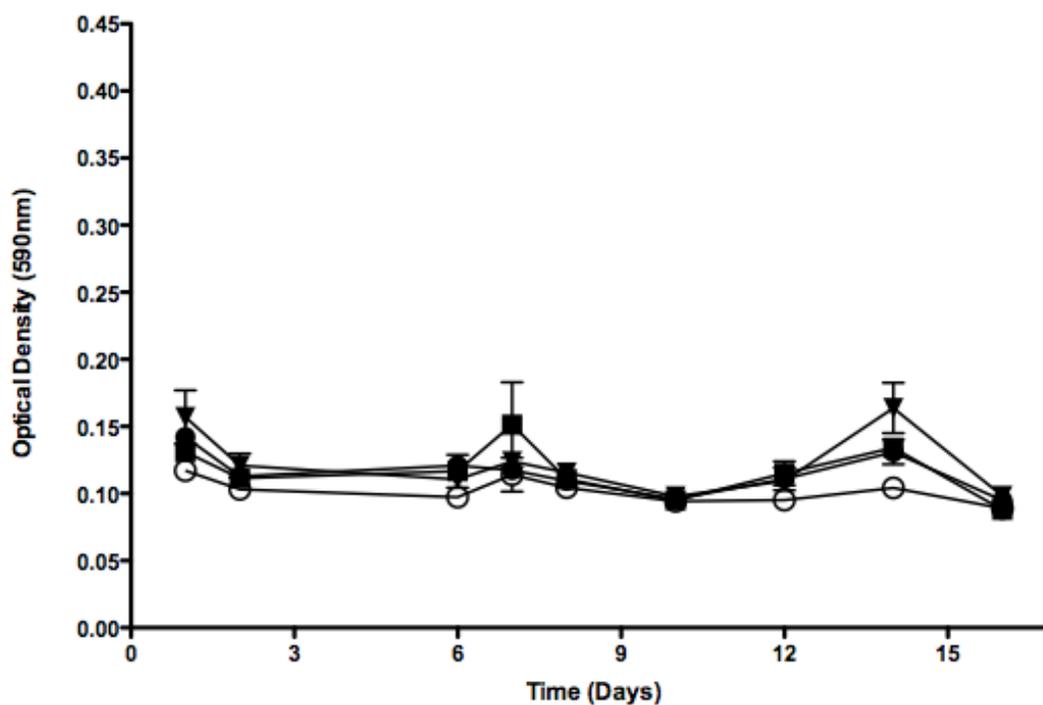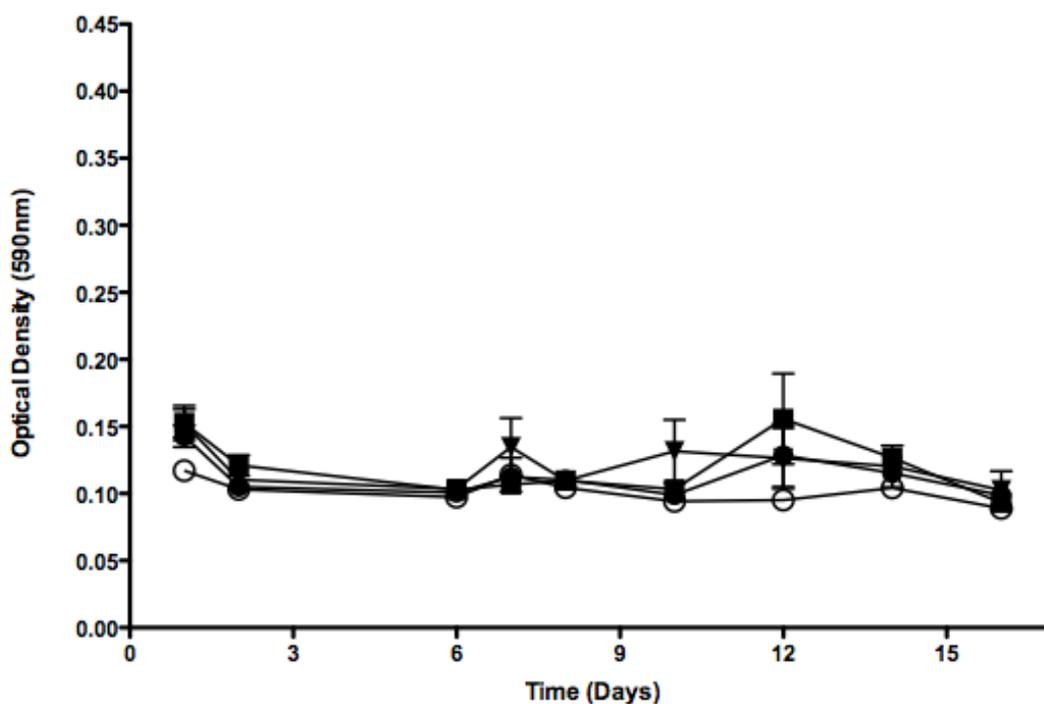
A2. Effect of fraction 10 on the recovery of anaerobically dormant Mpt: The supernatant, which molecular weight was greater than 5,000 Da, was filtered through 10,000 Da filtered to obtain fraction 10. Two fold serial dilutions of fraction 10 in Middlebrook 7H9 broth were added (100 μl) to the wells of microtiter plate, then 100 μl of anaerobically Mpt dormant cells were added to all the wells. Plate was incubated for 16 days and optical density monitored every 48 hours at 590 nm. The control well contained Middlebrook 7H9 broth and Mpt dormant cells only. Open circle- Control, Square- 1:2 dilutions, Triangle- 1:4 dilutions, and Solid circle- 1:8 dilutions.
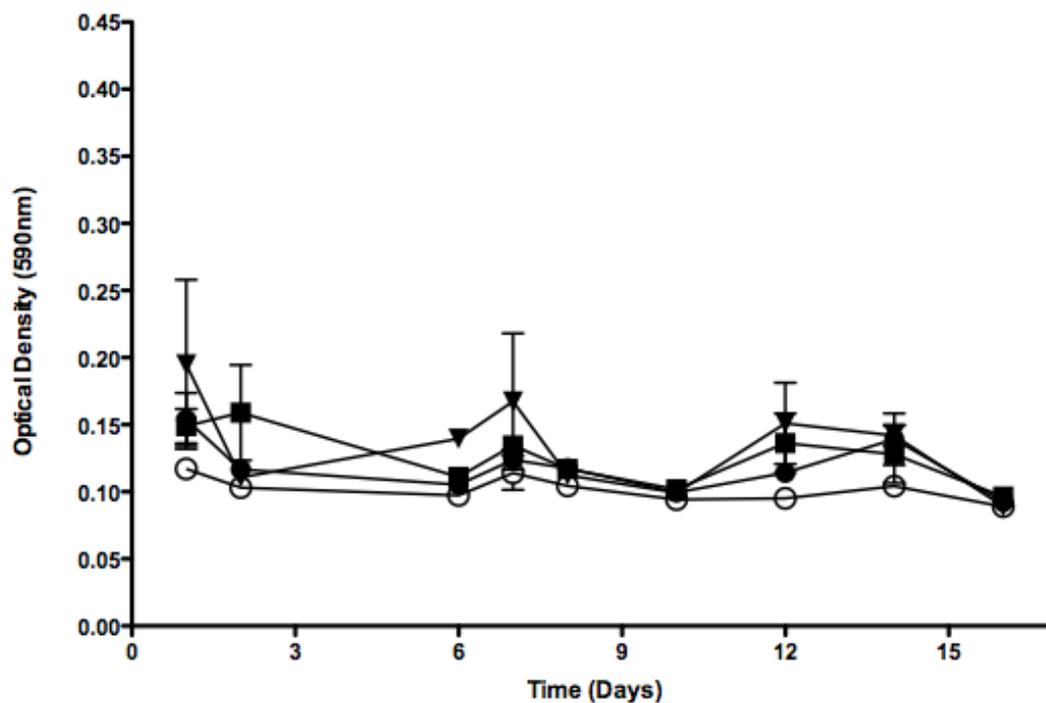
A3. Effect of fraction 30 on the recovery of anaerobically dormant Mpt: The

supernatant, which molecular weight was greater than 10,000 Da, was filtered through

30,000 Da filtered to obtain fraction 30. Two fold serial dilutions of fraction 30 in

Middlebrook 7H9 broth were added (100 μl) to the wells of microtiter plate, then 100 μl

of anaerobically Mpt dormant cells were added to all the wells. Plate was incubated for

16 days and optical density monitored every 48 hours at 590 nm. The control well

contained Middlebrook 7H9 broth and Mpt dormant cells only. Open circle- Control,

Square- 1:2 dilutions, Triangle- 1:4 dilutions, and Solid circle- 1:8 dilutions.
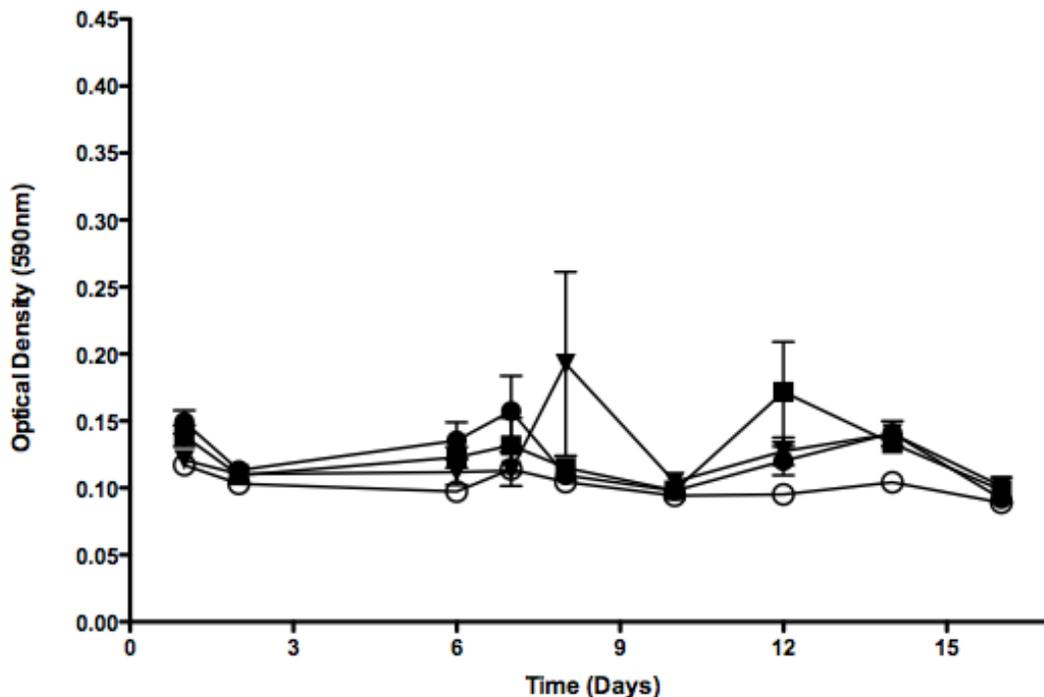
A4. Effect of fraction 50 on the recovery of anaerobically dormant Mpt: The supernatant, which molecular weight was greater than 30,000 Da, was filtered through 50,000 Da filtered to obtain fraction 50. Two fold serial dilutions of fraction 50 in Middlebrook 7H9 broth were added (100 μl) to the wells of microtiter plate, then 100 μl of anaerobically Mpt dormant cells were added to all the wells. Plate was incubated for 16 days and optical density monitored every 48 hours at 590 nm. The control well contained Middlebrook 7H9 broth and Mpt dormant cells only. Open circle- Control, Square- 1:2 dilutions, Triangle- 1:4 dilutions, and Solid circle- 1:8 dilutions.
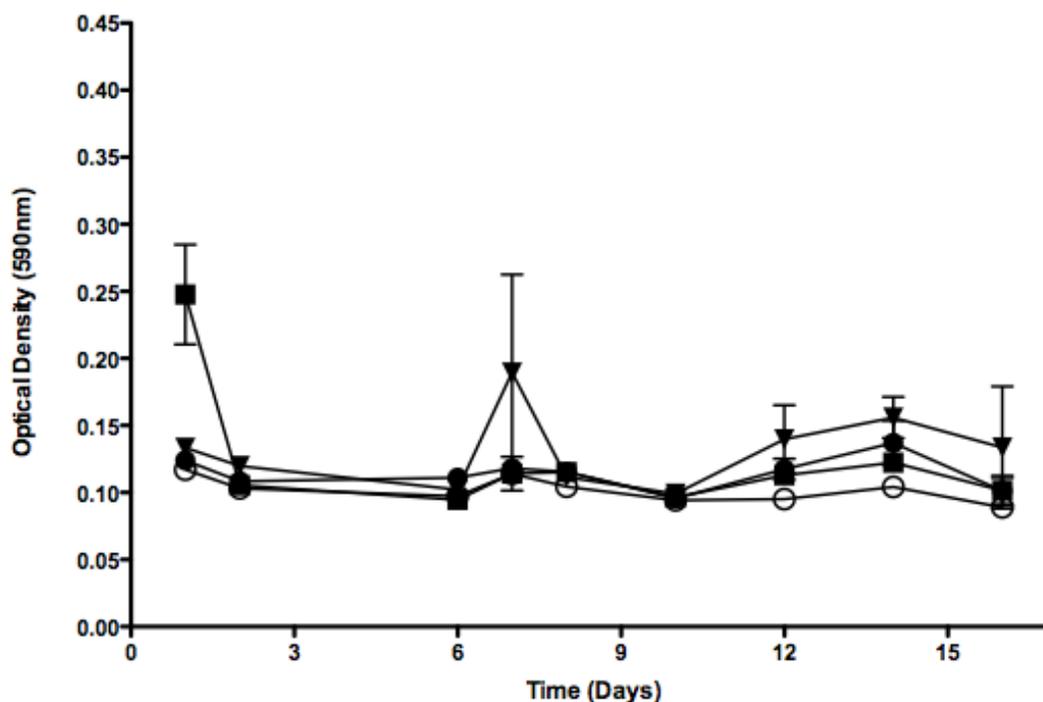
A5. Effect of fraction 100L on the recovery of anaerobically dormant Mpt: The supernatant, which molecular weight was greater than 50,000 Da, was filtered through 100,000 Da filtered to obtain fraction 100L. Two fold serial dilutions of fraction 100L in Middlebrook 7H9 broth were added (100 μl) to the wells of microtiter plate, then 100 μl of anaerobically Mpt dormant cells were added to all the wells. Plate was incubated for 16 days and optical density monitored every 48 hours at 590 nm. The control well contained Middlebrook 7H9 broth and Mpt dormant cells only. . Open circle- Control, Square- 1:2 dilutions, Triangle- 1:4 dilutions, and Solid circle- 1:8 dilutions.

A6. Effect of fraction 100U on the recovery of anaerobically dormant Mpt: The supernatant, which molecular weight was greater than 100,000 Da, was used as fraction 100U. Two fold serial dilutions of fraction 100U in Middlebrook 7H9 broth were added (100 μl) to the wells of microtiter plate, then 100 μl of anaerobically Mpt dormant cells were added to all the wells. Plate was incubated for 16 days and optical density monitored every 48 hours at 590 nm. The control well contained Middlebrook 7H9 broth and Mpt dormant cells only. Open circle- Control, Square- 1:2 dilutions, Triangle- 1:4 dilutions, Solid circle- 1:8 dilutions.

Appendix 2: HPLC for Peptide Report

A7. HPLC for Peptide Report: Media Blank
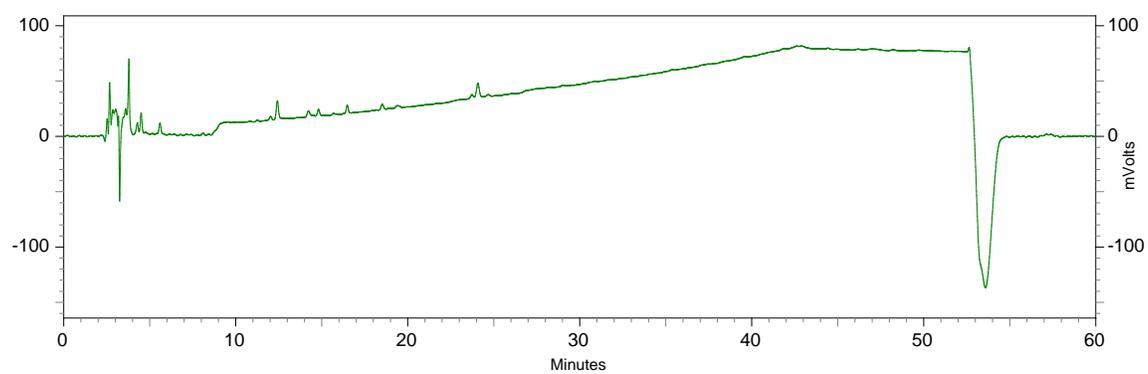
Channel 1 254 nm
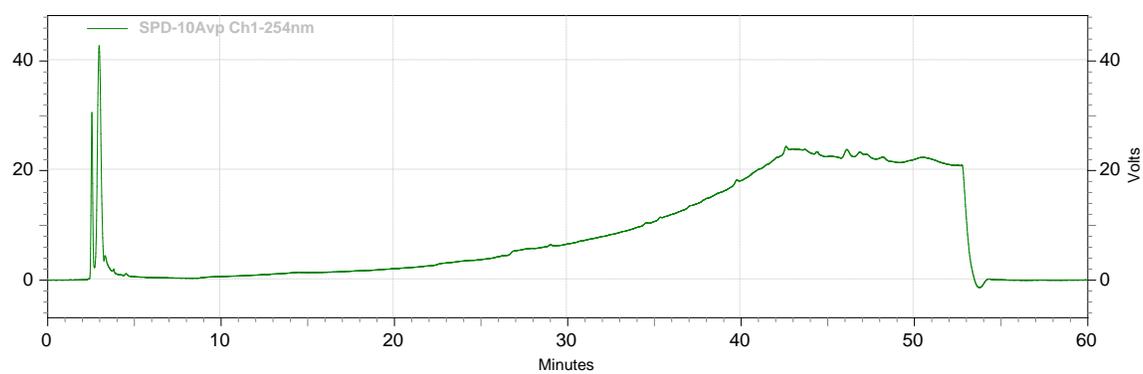
Channel 2 220 nm
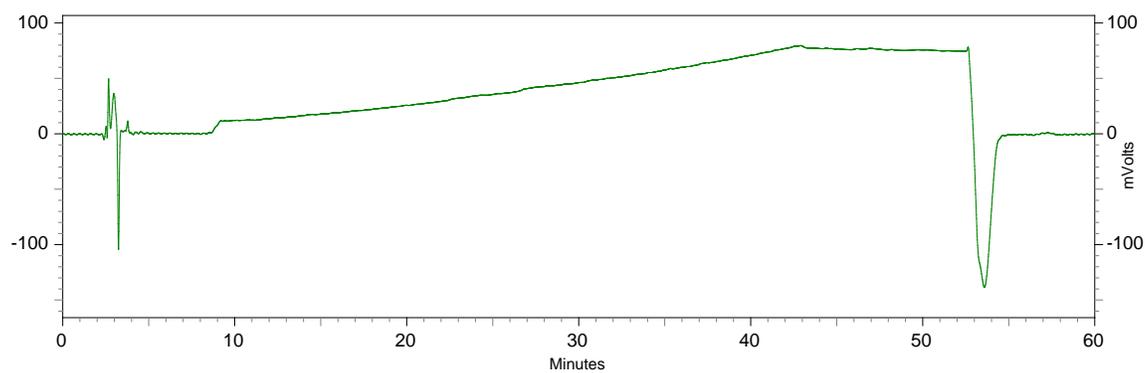
A8. HPLC for Peptide Report: F5 from 8-month-old culture
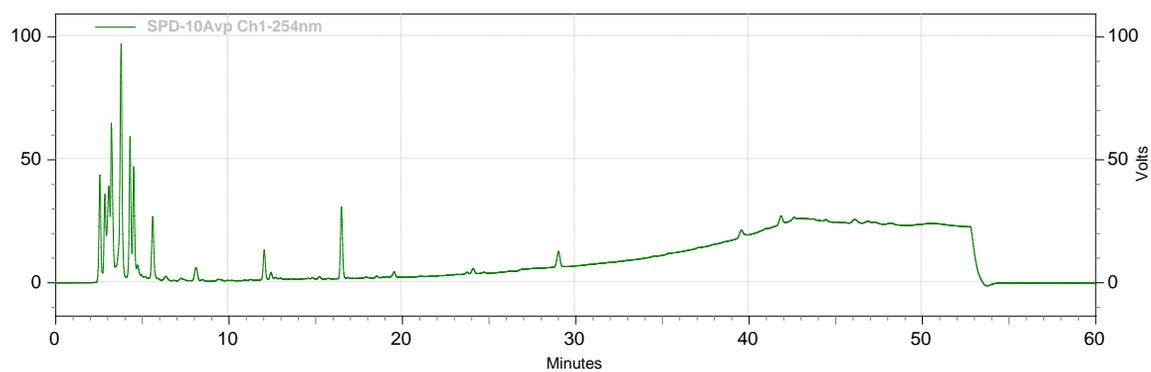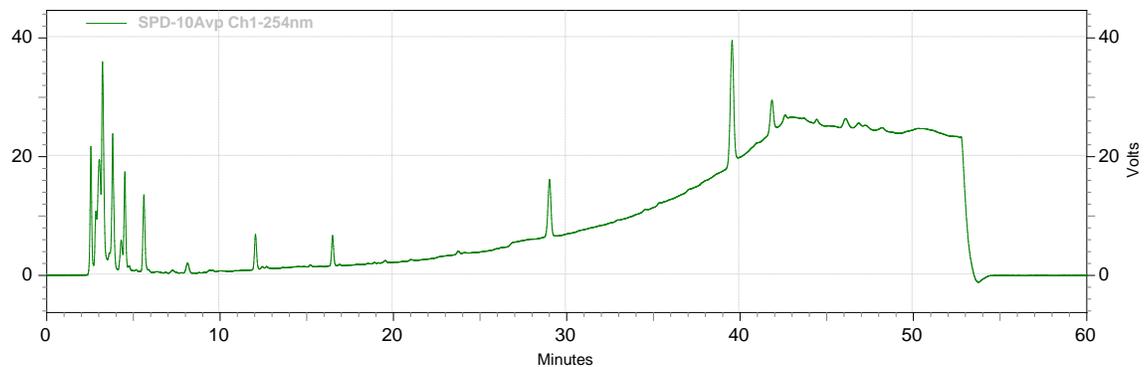
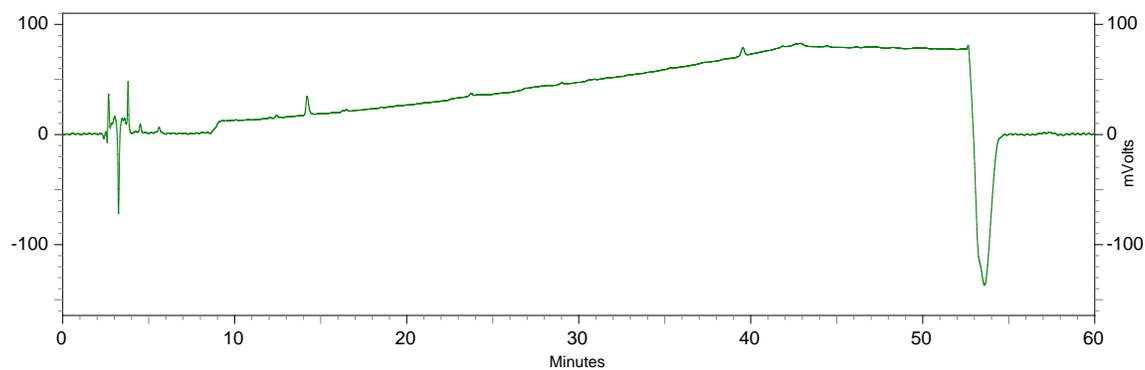Channel 1 254 nm

Channel 2 220 nm

A9. HPLC for Peptide Report: F5 from 6-month-old culture
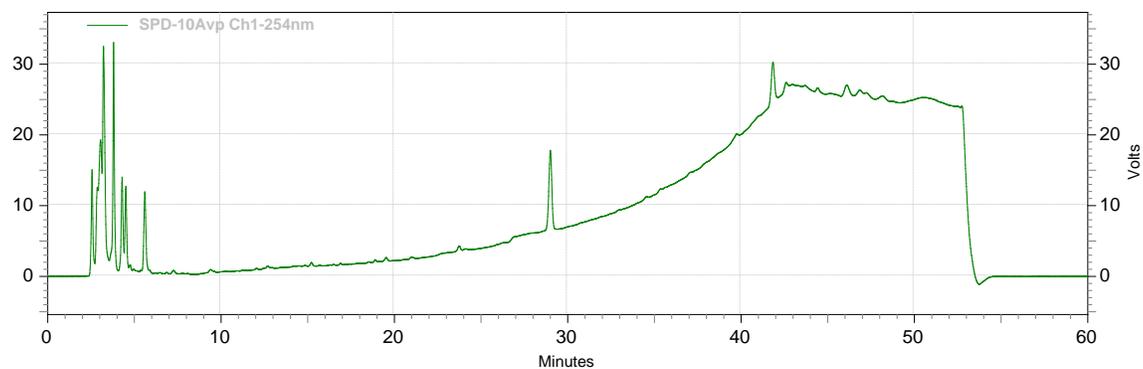
Channel 1 254 nm

Channel 2 220 nm

A10. HPLC for Peptide Report: F5 from 5-month-old culture

Channel 1 254 nm

Channel 2 220 nm