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1. Introduction and Background information

1.1. Overview of the *Mycobacterium* genus

The *Mycobacterium* genus is a member within the family *Mycobacteriaceae*, order Actinomycetales, and the phylum Actinobacteria (Stackebrandt et al., 1997). The genus comprises more than 130 species that are excessive in G+C content, aerobic, and non-motile. Though being classified as Gram positive, *Mycobacterium* spp. cannot be stained by the Gram staining method, owing to their acid-fast and lipid-rich cell wall that contains mycolic acids. Within the genus, most mycobacteria are non-pathogenic saprophytes, while others are remarkable human and animal pathogens. Majority of the non-pathogenic group is observed to have a faster grow rate compared to the latter, which usually takes longer than 7 d to form visible colonies (Grange, 1988).

1.2. Mycobacterial cell envelope

Mycobacterium spp. belong to the *Corynebacterium-Mycobacerium-Nocardia* branch whose members are characterised by a particular acid-fast cell wall, consisting a multiplex structure of peptidoglycan, arabinogalactan, and mycolic acids (Brennan and Nikaido, 1995, Brennan and Goren, 1979). Despite of being classified as Gram-positive, such a distinctive chemical constitution of their cell envelope distinguishes mycobacteria from both groups of Gram-stained bacteria. Additionally, their cell wall contains as much as 40% of lipid in contrast to less than 5% in Gram positive species and 10% in Gram negative ones (Fig. 1) (Palmer, 2012).

Excessive amount of lipid content in their cell wall enables mycobacteria to resist decolourisation with acid alcohol, attributing to their acid-fastness feature (Barksdale and Kim, 1977). Owing to

its heavy molecular weight, at room temperature, the lipid coat becomes waxy and prevents liquid-based Gram’s staining solutions from permeating to the peptidoglycan layer (Brennan and Goren, 1979). As a result, acid-fast stain methods, such as the Ziehl-Neelsen staining method is to identify acid-fast microorganisms (Fig. 2) (Swift et al., 2013). Generally, the method consists of concentrated carbol fuchsin as a primary stain, strong sulphuric acid (usually 20%) as a decolouriser, and Loeffler's methylene blue as a counterstain for the background. If present mycobacteria will appear in either red curved/rod singles or small clumps against a blue background (Costilow et al., 1981).

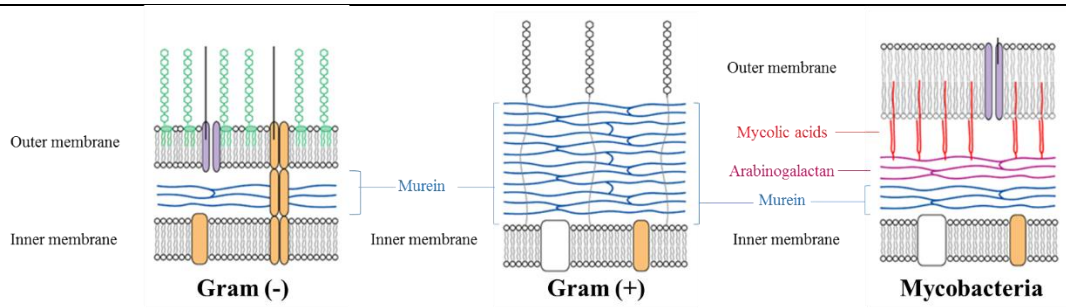


Figure 1: Mycobacteria cell wall as compared with Gram positive and Gram negative cell walls – Adapted from Palmer (2012)

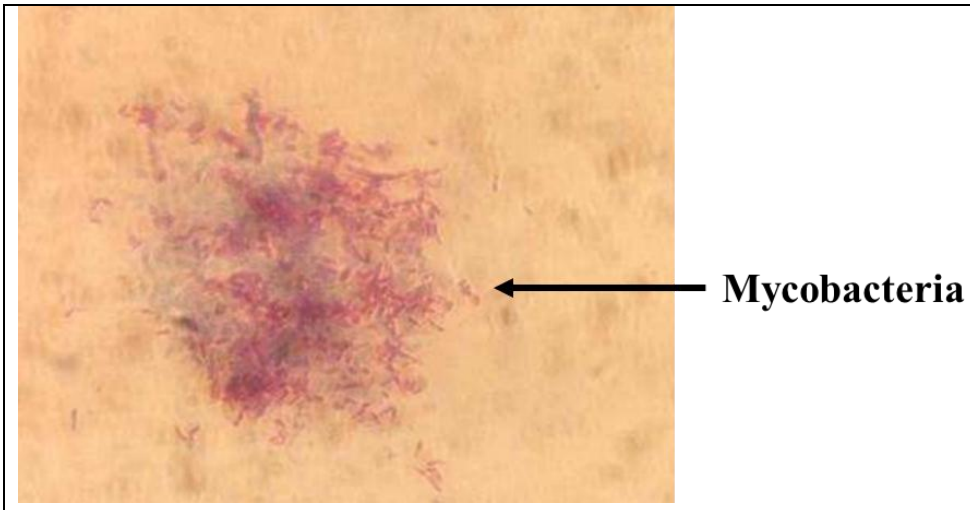


Figure 2: Mycobacteria under Ziehl-Neelsen stain appeared purple – Adapted from Swift et al. (2013)

1.3. Species of the genus

A large majority of mycobacteria are free-living saprophytes which can often be found in soil and water (Yates et al., 1984). However, some mycobacteria are dependent of a living host, mostly inside their diseased tissues. Humans and many animals such as birds, and ruminants, may be the ideal hosts for both obligate and opportunistic parasites (Wayne, 1974) . Species within the genus can be divided into fast growers or slower growers. Within the second group, the strains can be further classifies based on their pigmentation: photochromogens (pigment production under light), scotochromogens (pigment production in the dark), and non-chromogens (no pigment production) (Runyon, 1959). Pathogenic species are mostly slow growers, except for *Mycobacterium abscessus*, which is an emerging human pathogen causing cystic fibrosis (Bryant et al., 2013, Pawlik et al., 2013, Macheras et al., 2009). Among them, there are well-known human pathogens, such as *Mycobacterium tuberculosis, Mycobacterium leprae*, and *Mycobacterium ulcerans*, and also animal pathogens such as *Mycobacterium bovis*, *Mycobacterium avium* spp. *paratuberculosis* (MAP), and *Mycobacterium marinum* (Springer et al., 1996). Different mycobacteria species will have different sites of infection; serious infections can be in lungs or bowel areas, while others less invasive can elicit skin surface lesions (Yates et al., 1984, Magee et al., 1997). Below are highlights of some mycobacteria species that cause major diseases in both humans and animals.

1.3.1. The Mycobacterium tuberculosis complex (MTBC)

The *Mycobacterium tuberculosis* Complex (MTBC) currently comprises seven species and subspecies, which contains *M. tuberculosis, M. canettii, M. africanum, M. bovis, M. microti, M.*

pinnipedii, and *M. caprae*. However, this list is not exhaustive as genetic variations between current members are still under study (Ernst et al., 2007).

Species in MTBC are 99.9% similar at the nucleotide level and share the same 16S rRNA sequences (Böddinghaus et al., 1990, Telenti et al., 1997). However, they vary greatly in terms of host preferences, phenotypes, and pathogenicity; many infect humans, namely *M. tuberculosis, M. africanum* – both are the main agents of human tuberculosis, *M. canettii*, or plague animals like *M. microti* (Frota et al., 2004), or *M. caprae* (Niemann et al., 2002).

1.3.2. The Mycobacterium avium complex (MAC)

Species in *Mycobacterium avium* complex (MAC) are slow growing mycobacteria. They are environmental mycobacteria, including *M. avium* subspecies *avium*, *paratuberculosis*, and s*ilvaticum*, and *M. intracellulare* (Johnson and Odell, 2014). Before the postgenomic era, the species *M. avium* subsp*. avium* was identified as only *M. avium*. However, new evidence from genomic studies has divided *M. avium* into three subsets: *M. avium* subsp. *avium, M. avium* subsp*. paratuberculosis* (MAP), and *M. avium* subsp. *silvaticum* (Turenne et al., 2007)

MAC pathogens have often been found in soils and tap water (Falkinham et al., 2001), and transmission of MAC diseases is prevalently from tap water to host, not among the infected individuals themselves (Cosma et al., 2003). They also manage to survive under both chemical disinfectants and ultra violet (UV) irradiation, which makes water sources the constant reason for MAC infection (Von Reyn et al., 1994).

Strains that can infect humans are *M. avium* subsp. *avium* and *M. intracellulare*. MAP is the causative agent of Johne's disease in ruminants, and many studies have suggested a link between the pathogen and Crohn's disease in humans, a severe inflammation of the intestines (McNees et al., 2015).

In clinical setting of nontuberculous mycobacteria (NTM) cases worldwide, MAC isolates are often attributed to trains of *M. avium* subsp. *avium* and *M. intracellulare* (Griffith et al., 2007). Patients with MAC infection are immune-compromised, suffering from AIDS, leukaemia, or undergo immunosuppressive chemotherapy. Diseases associated with MAC infection in non-AID patients can be pulmonary disease, sub-acute lymphadenitis, and disseminated infection. In North America, MAC pathogens are known as the major NTM responsible for cystic fibrosis infections (Olivier et al., 2003). Furthermore, AIDS patients may show infections in the respiratory or gastrointestinal tract (focal disease), and disseminated infection in the lymph nodes, the central nervous system, the liver, the spleen, and the bone marrow (Inderlied et al., 1993).

Treatments of MAC diseases often employ antibiotic therapy of at least 12 months. However, though therapy can succeed, MAC pathogens may regrow (Johnson and Odell, 2014). For instance, 34 individuals were treated for MAC infection in 3 years (18 months) with combination drug therapy, 11 of them (32%) tested positive for MAC in sputum after one year off therapy (Hasegawa et al., 2009).

1.3.3. *M. avium* subsp. *paratuberculosis* (MAP)

MAP is an obligate pathogen causing critical hypertrophic enteritis – or Johne's disease (Kim and Keasling) – in mostly cattle, sheep, goats as well as other ruminants; this host preference distinguishes

the species from other strains of mycobacteria (Whipple et al., 1997). Taxonomically, MAP is a member of MAC and a subspecies of *M. avium*. Within the subspecies, MAP is different from the other two based on both phenotypic and genomic characteristics, its requirement for purified mycobactin in growth medium and the insertion sequence IS*900*, *f*57, HspX respectively (Turenne et al., 2006, Thorel et al., 1990).

Cultivation of MAP *in vitro* is time demanding and laborious, usually taking from 8 to 16 weeks. Due to such a long incubation period, contamination is often an issue which requires treatment of samples with selective agents to reduce numbers of non-mycobacterial organisms (Donaghy et al., 2003, Dundee et al., 2001, Grant and Rowe, 2004).). In some laboratories, before plating onto solid medium, samples might go through an enrichment liquid medium, such as Dubos broth. After successful cultivation, the bacteria collected will be identified based on following criteria (Cerf et al., 2007):

i. acid-fast positive
ii. slow growth with typical colony morphology
iii. IS*900* PCR-positive
iv. requirement of mycobactin J for growth

Within the MAP species, there are various type and sub-type strains. From the first isolates of MAP to be identified, two main types were chatecterised: type S and type C, according to their host preference, sheep and cattle, respectively (Collins et al., 1990). By using modern molecular techniques such as pulse-field gel electrophoresis, classification of MAP has brought forth new discoveries. There are three sub-types under Type S, Type I and Type III, and a newly sequenced camelid strain (Stevenson et al., 2002, Castellanos et al.,

2007b, De Juan et al., 2005, Castellanos et al., 2007a). Type II is the same as Type C, which has a sub-group called Type B, including USA Bison Type and Indian Bison Type (Fig. 3) (Stevenson et al., 2002, Whittington et al., 2001, Sohal et al., 2010, Thibault et al., 2012, Stevenson, 2015).

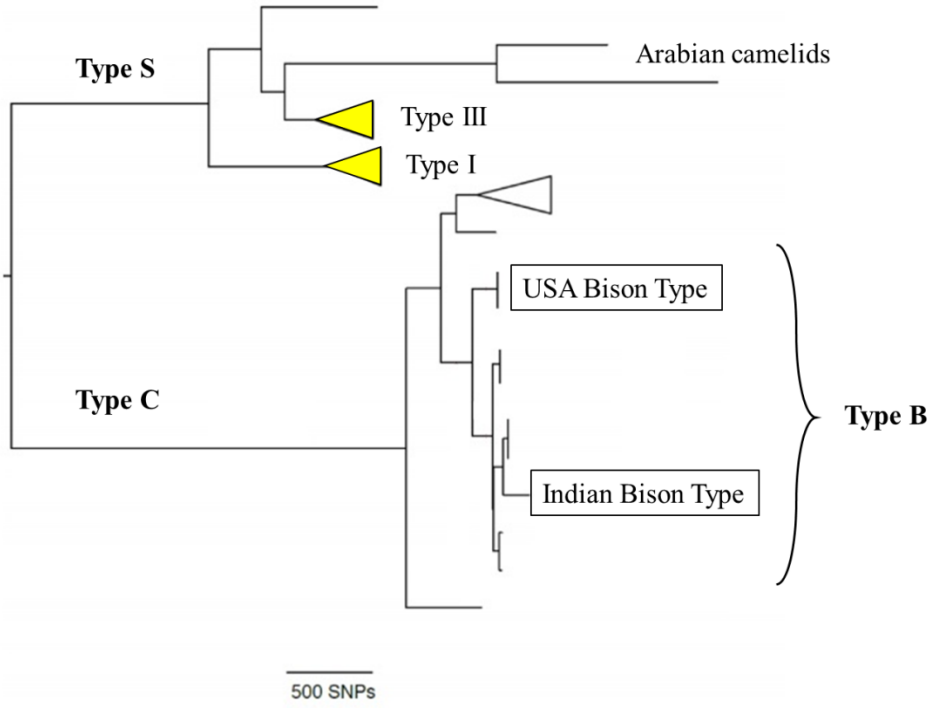


Figure 3: Evolutionary tree of MAP showing different subtypes – Adapted from Stevenson (2015)

1.3.4. MAP as a zoonotic pathogen

The question as to whether or not MAP is a zoonotic pathogen is a controversial issue. In 1913, Dalziel reported a strong similarity in appearance between the disease in his patients with chronic intestinal inflammation and JD (Corman, 1989). In addition, recent reviews and studies have provided evidence to support zoonotic capacities of MAP (Chiodini et al., 2012, Greenstein, 2003, Rosenfeld and Bressler, 2010), especially the genomic evidence by Wynne et al. (2011) has shown that there was MAP transmission from domestic animals to humans. Although MAP has already been proven to have a strong association with Crohn's disease, whether MAP is the primary causation for the stated disease or just an opportunistic pathogen is still debatable. As a consequence, more studies are needed to validate the presence of MAP in patients with Crohn's disease.

1.4. MAP and associated diseases

1.4.1. Johne's disease

MAP causes paratuberculosis – Johne's disease or JD – in ruminants, a chronic enteritis, resulting in malnutrition, diarrhoea, and eventually death (Ayele et al., 2001). Although the pathogen is known to infect mainly cattle, sheep, and goats, there are strong epidemiological evidence showing that domestic ruminants can transmit paratuberculosis to wild animals (deer, wild boars, and rabbits), and vice versa, meaning wild animals can also be the sources of JD in domestic ruminants (Raizman et al., 2005, Reyes-García et al., 2008).

Economically, JD possesses a real problem to the dairy industry worldwide. A report in 2004 estimated that, the average cost of paratuberculosis per animal per year was £26.00 for dairy cattle and £17.00 for beef cattle (Hasonova and Pavlik, 2006). Annually, the US cattle industry loses approximately $1.5 billion owing to JD's impact (Liu et al., 2001). Despite all problems, there is currently no known effective treatment for paratuberculosis infected cows (Sweeney et al., 2012).

MAP survives well under extreme environments. The species can resist high and low temperatures, low humidity, low pH, and UV radiation (Donaghy et al., 2009, Emery and Whittington, 2004). Consequently, high-temperature and short time (HTST) pasteurisation appears to incompletely inactivate viable MAP in raw milk (Dundee et al., 2001, Grant et al., 2002). A number of studies have detected paratuberculosis pathogens in raw milk, owing to ineffective pasteurization (Grant et al., 2002, Corti and Stephan, 2002). Greenstein and Collins (2004) reported other possible transmission routes can also be undercooked beef, water and contacts with infected animals. As a result, presence of MAP in food raises a concern in food security, as the bacterium can be transmitted to humans via infected milk.

1.4.2. Crohn's disease (CD)

Crohn's disease (CD) is an inflammatory bowel disease, which results in swelling of the lining along gastrointestinal tract, and patients will experience abdominal pain, chronic diarrhoea, fever, weight loss and blood and mucus in faeces (Chacon et al., 2004, Chiodini et al., 1986).). Is is also a significant economic burden, with CD costing the United States an estimated $10.9–15.5 billion

annually, and around €2.1–16.7 billion in Europe (Peng Yu et al., 2008).

Factors leading to CD continue to be unknown. However, a considerable number of theories have been hypothesised (Chiodini et al., 1986):

i. a continuous infection which probably involves mycobacteria (especially MAP), *Listeria* spp., or *E. coli*;
ii. a defective mucosal barrier which leads to invasion of bacteria;
iii. host immune response dysfunction;
iv. genetic susceptibility factors; or
v. a combination of some or all of the theories

The disease is similar to JD in terms of gross pathology and clinical symptoms (Fig. 4)(Greenstein, 2003). Furthermore, Hermon-Taylor (2009) has put a link between MAP and CD based on the correlation between the increased cases of CD with the incline cases of JD. All in all, it is clear that MAP has an association with CD, yet current evidence is not enough to establish a direct relationship of MAP as the cause of CD. As a consequence, further genomic analyses with regard to MAP identification and characterisation in both diseases may provide us with better evidence to validate such a view.

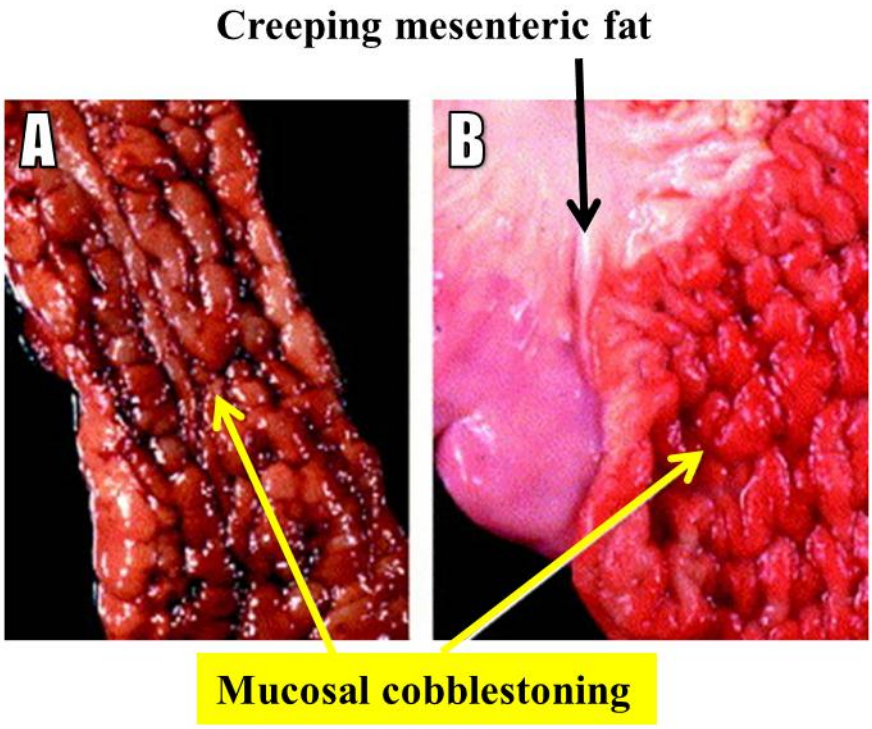


Figure 4: Gross pathology between infected intestinal samples in humans with CD (A) and cows with JD (B). Similar cobblestones on the intestinal lining in both samples were noted. Creeping mesenteric fat, a characteristic of CD, was also spotted on B – Adapted from

1.5. Pigmentation overview

Pigment can be used to classified non-tuberculosis (NTB) mycobacteria. Pigment production in most cases results in yellow, orange, red or rarely pink – either in the dark (scotochromogens), or on exposure to light (photochromogens) (Runyon, 1959, Runyon, 1970). These mycobacterial pigments have been indicated as carotenoids, a class of polyterpene lipids that occur widely throughout nature, and usually act as free radical scavengers to protect the cells against photodynamic injuries (Vershinin, 1999).

Nonetheless, there are studies that show carotenoid functions in bacteria are also involved in protection against environmental stress, and in some cases, bacterial pathogenesis and survival (Hümpel et al., 2010, Liu et al., 2005). Some bacteria are natural pigment producers, like *Staphylococcus aureus* produces a golden yellow pigment to aid the species in terms of virulence and neutrophil killing (Liu et al., 2005). Meanwhile, other species generate pigments due to environmental stress. *Synechococci,* for example, build up carotenoids when iron level is low (Ivanov et al., 2000), or exposure to methanol can trigger pigment production in *Acinetobacter wofii* (Ghosh et al., 2007). Likewise, a number of mycobacteria appear to produce pigments when there is oxidative stress applied (Provvedi et al., 2008, Saviola and Felton, 2011).

1.5.1. Functions of carotenoids

Carotenoids are pigment molecules that give colours of red, yellow and orange in plants, algae and bacteria. In plants, they support light absorption during photosynthesis. Apart from that, they are antioxidants which scavenge free radicals and thus protect the cells. To date, there are approximately 750 natural carotenoids that have

been found, which are believed to play a significant role in safeguarding bacteria from external stresses like UV light, anaerobic stress, activated oxygen damages, and cellular membrane regulation (Table 1) (Vershinin, 1999).

The basic structure of carotenoids is a conjugated system of single and double bonds. At their core, usually there are 40 core carbon atoms, and different number of hydrogen atoms. The determining structural property of carotenoids is their conjugated double bonds, which is responsible for their pigment characteristics and rigidity (Fig. 5)(Rao and Rao, 2007). The number of double bonds affects absorption of different light waves which yields to the variety of pigmentation, such as red, orange or yellow. Individual carotenoids are characterised by modifying either one or two tail-ends cyclization, hydrogenation level, or adding functional oxygen group. Subsequently, there are primarily two classes of carotenoids molecules: carotenes – made of entirely hydrocarbons, and xanthophylls – with modified oxygen functional groups (Gruszecki and Strzałka, 2005).

Biological functions	**Other functions**
Vitamin A precursor	Hydrophobic
Protection against oxidative damage	Antioxidative agent
Preventing cancer	Colouration (red, orange, and yellow)
Treatment of erythropoietic protoporphyria	
Imparting colouration in plants	

Light and spectral filteration screening	
Protection of egg protein against protease	
Retinal visual pigment	
Environmental bioindicators	
Antimicrobial agent	
Food and nutrition	

Table 1: List of natural carotenoids properties – Adapted from Vershinin (1999)

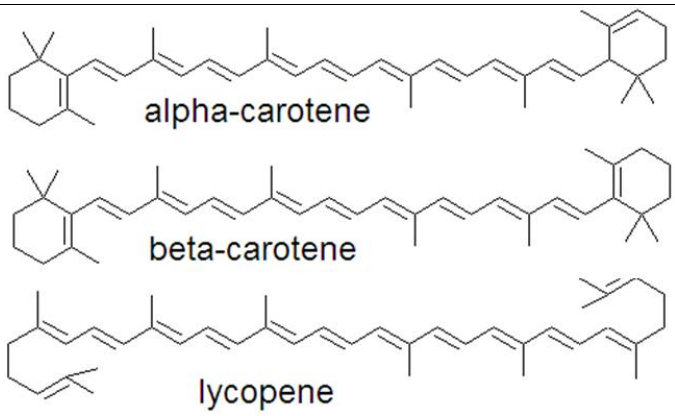


Figure 5: Structure of some carotenoids molecules (alpha-carotene, beta-carotene, and lycopene), showing conjugated double bonds – Adapted from Rao and Rao (2007)

Carotenoids have ability to localise and position themselves to fit in and interact with surrounding molecules, according to their individual characteristics and functional groups. Because of their rigid conjugation double bonds system, carotenoid molecules can significantly support the stabilization in lipid cellular membranes and proteins (Britton, 1995). In brief, by localising themselves at an integral part of cellular membrane complex and in combination with interactions with other proteins, they bring about the increase rigidity and mechanical support to membrane fluidity (Armstrong, 1997). Different carotenoids have different locations, β-carotene and lycopene, for example, are positioned at membrane hydrophobic core for mobility support (Fong et al., 2001).

Carotenoids synthesis pathway in bacteria is believed to begin with the transformation of nonmevolanate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Next, condensation reaction of both IPP and DMAPP results to geranylgeranyl

pyrophosphate (GGPP). From two molecules of GGPP, with regulation of different *crt* genes coding for different enzymes along the pathway, eventually beta-carotene would be obtained (Fig. 6) (Kim and Keasling, 2001),(Brennan et al., 2003)

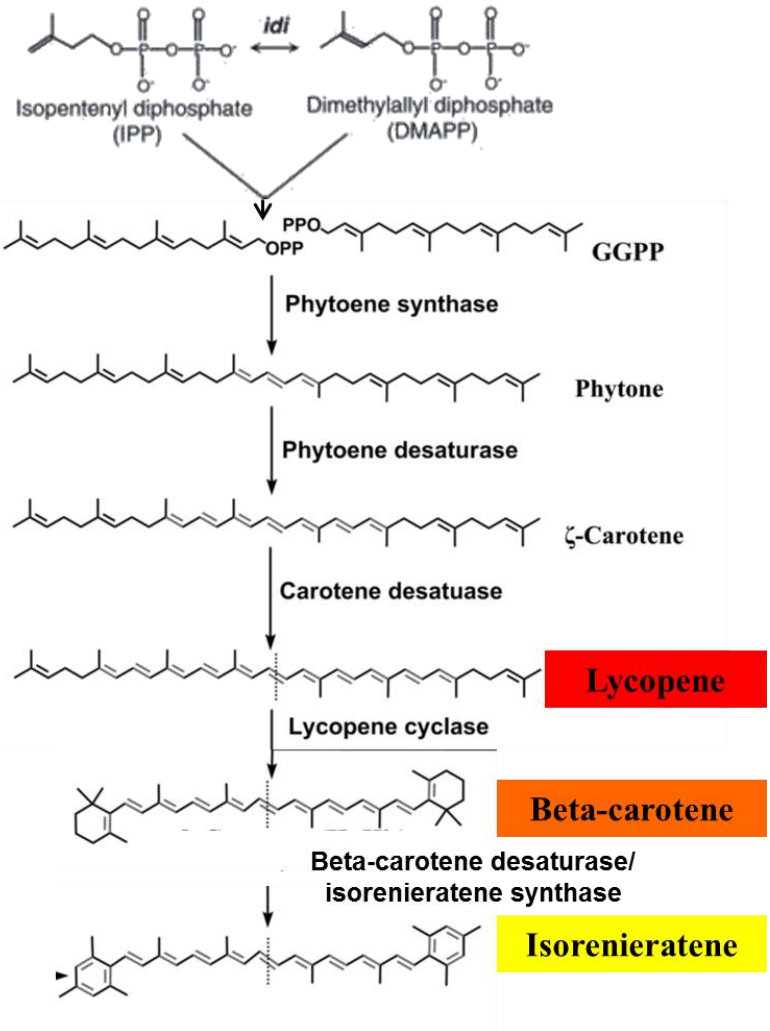


Figure 6: Carotenoids biosynthesis pathway in bacteria. Different pathway end products will yield different colours correspondingly. Lycopene: red, beta-carotene: orange, and isorenieratene: yellow – Adapted with additional information from (Brennan et al., 2003)

1.5.2. Pigmentation is important to bacteria's pathogenesis and survival

Pigment functions in mycobacteria may be unclear, but other extensive studies on consecutive pigmented bacteria can offer a brief understanding of pigmentation benefits in mycobacteria. Pigment plays a significant role in bacterial pathogenesis. It renders the effectiveness of host immunology to kill foreign pathogens, thus strengthening bacterial survival and virulence. It can also generate inflammatory reactions within the host to further damage cells and tissues (Liu and Nizet, 2009).

Studies conducted on *S. aureus*, a yellow pigmented human pathogen, have revealed that carotenoids are important to the species pathogenesis. Mutants without pigment biosynthesis gene (*crtM*) turned colourless and more susceptible to be killed by host phagocytosis. Furthermore, on mouse models for murine skin abscess or systemic infection, virulence of colourless *S. aureus* appeared to decrease significantly. Survival of *S. aureus* non-pigmented mutants were shown to be weak in environments challenged with hydrogen peroxide, superoxide radical, hydroxyl radical, hypochloride and singlet oxygen, as the conjugated double bonds system in carotenoids absorbs free radicals (Liu et al., 2005, Liu et al., 2008). On the other hand, on skin infected mouse models, when pigment genes, *crtM* and *crtN*, were inserted to group A *Streptococcus*, pigmentation and larger skin lesions were noted (Singer and Talan, 2014).

Melanin is a pigment molecule that often found in fungal pathogens like *Cryptococcus neoformans* and *Aspergillus fumigatus* (Gómez and Nosanchuk, 2003). Studies in *C. neoformans in vitro* shown that melanin plays a key role in bacterial pathogenesis. Melanin does not only prevent macrophage phagocytosis but also without melanin production, the fungal pathogen are less invasive and with low survival rate in infected models (Kwon-Chung et al., 1982, Salas et al., 1996, Wang et al., 1995). In addition, melanin enhances *C. neoformans*; because of the molecule's charge, it can successfully neutralise and hinder effectiveness of antimicrobial peptides and pharmacologic antifungal agents (Doering et al., 1999).

In general, the lack of pigment in consecutive pigment producers often results in reduced virulence, and lower rate of survival both in environment and in host cells. This can as well be applied to mycobacteria, and thus studies into pigmentation may reveal more about mycobacterial pathogenesis pathways and treatments.

1.6. Pigmentation in Mycobacteria

Pigment production in mycobacteria is thought to be a stable genetic property, or specifically a response to light (Barksdale and Kim, 1977, Rilling, 1962). Runyon (1970) proposed a classification system for non-TB mycobacteria, which bases on growth rate and pigment production of different mycobacterial strains. There are 4 groups: Groups I and II consist of slow-growing and pigment production species (*M. kansasii, M. marinum, M. gordonae,* and *M. scrofulaceum*); Group III lists slow growers with no pigment production (*M. avium, M. intracellulare*); whereas Group IV include rapid growers (≤7 days).

1.6.1. Diseases by pigmented Mycobacteria

Pigmented mycobacteria have been reported to cause significant infections in both humans and animals.

M. marinum is a pigmented slow-grower that is usually associated with disease in fish and skin disease in humans (Aronson, 1926, Norden and Linell, 1951). Dodiuk-Gad et al. (2007) found out that *M. marinum* is responsible for 64% of skin disease cases in their study. Moreover, Ho et al. (2006) in Hong Kong reported the result of their 10-year retrospective NTM infections study, that *M. marinum* was responsible for 50% of recorded cases. Serious lesions caused by the bacteria may not heal, and there are possibilities of re-infected (Bhambri et al., 2009).

Another pigmented slow-grower is *M. kansasii* that usually infects lung tissues. It is prevalent both in hosts – cattle and swine, and the environment, such as tap water (Weitzul et al., 2000). The organism is usually associated with people suffering from chronic obstructive pulmonary disease and cystic fibrosis (Groves, 1995).

M. scrofulaceum is a pigmented slow-grower that turns light yellow or orange in both light and dark conditions. The organism has been found in food – raw milk, dairy, oysters, and the environment – soil, water (Jang et al., 2005). *M. scrofulaceum* is often isolated from the submandibular or submaxillary region, mostly in immunodeficient patients (Jang et al., 2005, Weitzul et al., 2000). Until 1970s, the organism was responsible for most cases of lymphadenitis in children. To date, 10% to 20% of unilateral cervical lymphadenitis infections were reported to be caused by *M. scrofulaceum* (Bartralot et al., 2005, Falkinham, 2003).

In general, pigmented mycobacteria are of clinical interest, to differentiate from tuberculosis and for disease prevention. Studies in

pigmented mycobacteria therefore broaden our understanding on *Mycobacteria* spp. diversity and diseases.

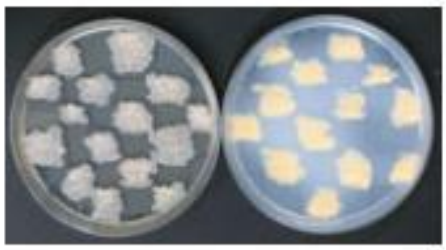
1.6.2. Pigmentation in non-chromogenic Mycobacteria

Although being classified as nonchromogens, rapid growers like *M. smegmatis* and *M. goodii* formed pigment after prolonged cultivation period from 7 to 10 d (Brown-Elliott and Wallace, 2002, Brown et al., 1999).

Moreover, recent studies have discovered that non-TB mycobacterial species do produce pigments in response to other environment stress factors. Acidic stress condition *in viro* by lowering acidic level of culture media can successfully induce pigment generation in *Mycobacteria* spp. At pH 5.0 – 6.0, *M. smegmatis* pigmented after 7 - 10 d of incubation; pigmentation results were also obtained with rapid growers like *M. fortuitum* and *M. chelonae* (Fig. 7) (Saviola and Felton, 2011, Stormer and Falkinham, 1989).

Similarly, Rynyon Group III species including slow-growers like *Mycobacterium avium intracellulare* and *Mycobacterium avium* were observed to generate pigments at pH 5.5 – 6.0 under acidic stress (Stormer and Falkinham, 1989)

Pigmentation process in the above cases may happen to protect mycobacterial cells from damage owing to acidic stress. Another explanation can due to carotenoids acting as free radicals to resist the concurrent oxidative stress *in vivo* after pH reduction. Overall, further studies are needed to understand better the role of carotenoids play in mycobacterial survival in case of environment stress, and acidic stress in particular.



M. smegmatis 7.0 M. smegmatis 6.0



Figure 7: Rapid-growing mycobacterial nonchromonogens produced pigments in response to acidic stress – Adapted from Saviola and Felton (2011)

1.7. Pigmentation in MAP

As previously mentioned, there are two main types of MAP, Sheep Type (Type S) and Cattle Type (Type C). Methods to differentiate the two types vary from growth characteristics, disease presentation, molecular typing, to colony pigmentation (Taylor, 1945, Taylor, 1951, Stevenson et al., 2002). MAP sheep strain colonies are often observed to be smooth, and pigmented (Fig. 8) (Behr and Collins, 2010). Besides, MAP cattle strains are not classified as consecutively pigmented in Runyon's classification system.

Clinical cases of pigmented MAP are not common, neither are they regionally connected. In the UK, there are past findings of pigmented MAP sheep strains by Taylor (1945) and Stamp and Watt (1954). More recently in Morocco, Africa, Benazzi et al. (1996) investigated three sheep flocks, and reported three strains of yellow colonies. Molecular typing in infected sheep reported that there is mostly pigmented sheep Type I (Behr and Collins, 2010). Nonetheless, Pulsed-field gel electrophoresis profiling (PFGE) in sheep and goats in Spain identified type III pigmented MAP strains (Sevilla et al., 2007). Furthermore, recently molecular typing in infected sheep has singled out a case of pigmented isolates from non-pigmented sheep Type I (Stevenson, unpublished results).

Studies have shown that pigmentation in MAP sheep type, which appears to be either yellow or orange, seems to be an inherent characteristic, existing in all growth stages, and is not affected by the host's passage (Stevenson et al., 2002). Nonetheless, the reasons for such pigmentation are still of unknown (Stevenson, 2015). In general, from the literature, clinical pigmented MAP isolates are often seen in sheep type, not cattle type.

However, despite the fact that pigmentation is mainly observed in MAP sheep type, an experimental infection using pigmented MAP strains can cause inflammation in cattle (Taylor, 1953). Stamp and Watt (1954) reported a natural infection with pigmented strains in bovine. These rare reports do put a question to our current understanding, as in whether or not pigmentation is an exclusive characteristic of MAP sheep type strains. More recently, in Portugal, pigmented MAP isolates were identified in goat faecal samples (Célia Leão, 2016), the first evidence for pigmented MAP cattle strains. This finding prompted us to believe that pigmentation is not only exclusive in MAP sheep strains. As a result, MAP cattle strains are believed to be able to produce pigment. However, specific conditions and triggers for such phenomenon have not yet been well-studied.

In our laboratory, observations *in vitro* had been made to show that cattle strains of MAP cultured on acidic solid agar became pigmented whereas those cultured on neutral pH agar did not (Swift, unpublished material). This finding confirmed that MAP cattle type strains are able to form pigment under specific conditions. Further investigation of pigmentation in MAP in this case will provide us with a deeper understanding of MAP diversity, as well as better knowledge of the pathogen's behaviour in hosts. In this study, the aim was to induce pigmentation in MAP cattle type strains under acidic conditions, and to determine the genetic mechanism that enables the organism to produce pigment.

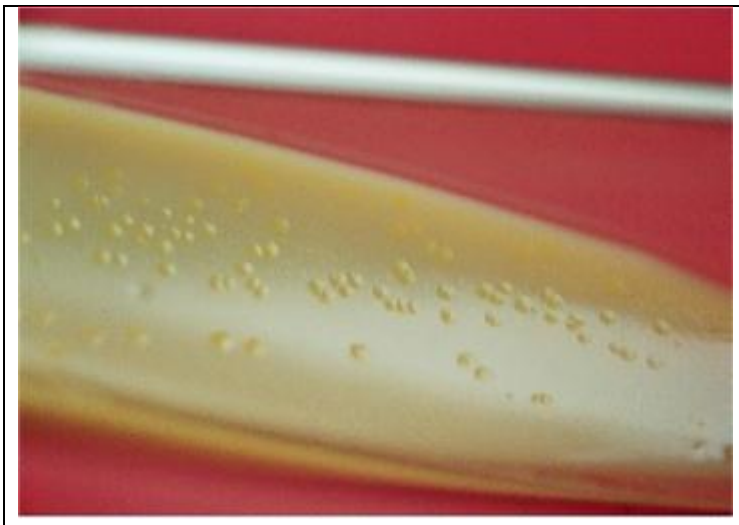


Figure 8: Pigmented MAP ovine isolates on on a Lowënstein-Jensen slant, from the Basque Country (Spain) – Adapted from Sevilla et al. (2007)

1.8. The study of gene expression

1.8.1. Mycobacterial carotenoid biosynthesis pathway

In species with orange pigment, namely *Mycobacterium aurum*, *M. phlei*, *M. avium* and *M. intracellulare*, isorenieratene and β-carotene are common carotenoids (LEVY-FREBAULT and DAVID, 1979, Tarnok and Tárnok, 1970, Zureck, 1985), while pigments in *M. kansasii* and *M. marinum* are characterised solely by β-carotene (Tarnok and Tárnok, 1970, Zureck, 1985). The formation of isorenieratene in *M. aurum* A+ and other organisms have been well studied and analysed, which can form a model carotenogenesis pathway applied for research in other mycobacterial species (Armstrong, 1997, Tarnok and Tárnok, 1970, Zureck, 1985).

Genes that code for the enzymes involve in carotenoid biosynthesis can be located in the *crt* cluster; in *M. aurum*, there are 5 enzymes take part in the manufacturing of isorenieratene, namely CrtE, CrtB, CrtI, CrtY, and CrtU (Viveiros et al., 2000). As illustrated in Fig. 9 and Fig. 10 (Brennan et al., 2003, Viveiros et al., 2000), from Farnesyl pyrophosphate, GGPP synthase (CrtE) directs the production of Geranylgeranyl pyrophosphate (GGPP), which then is transformed into phytoene by the enzyme phytoene synthase (CrtB). Desaturation of phytoene to lycopene is completed via lycopene desaturase (CrtI). Lycopene next is converted to β-carotene by lycopene cyclase (CrtY). And in the end, desaturation of β-carotene yields isorenieratene, by β-carotene desaturase (CrtU). In most species and in *M. smegmatis*, there is one CrtY domain for all lycopene cyclase genes. Nonetheless, in *M. aurum* and *Brevibacterium linen*, the existence of such a domain has not yet been determined (Viveiros et al., 2000), but the combination of two

different gene segments *crtYc* and *crtYd* is proven to be able to form functional lycopene (Sandmann, 2002).

Homologous gene sequences with the *crt* cluster in *M. aurum* have also been detected in non-pigmented species in *M. avium* complex (Viveiros et al., 2000) such as *M. avium* and *M. intracellulare*. Likewise, pigmented MAP, as a member of MAC, is believed to possess similar characteristics.

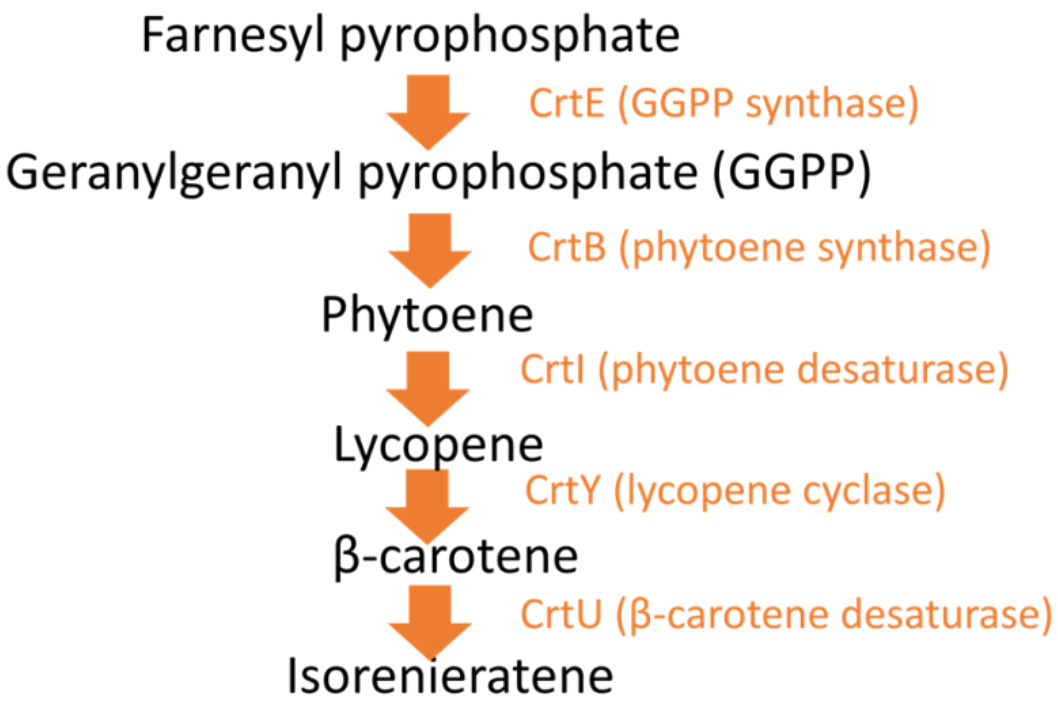


Figure 9: Presumptive metabolic pathway leading to the production of isorenieratene in *M. aurum* and *M. smegmatis* – Adapted from Viveiros et al. (2000)

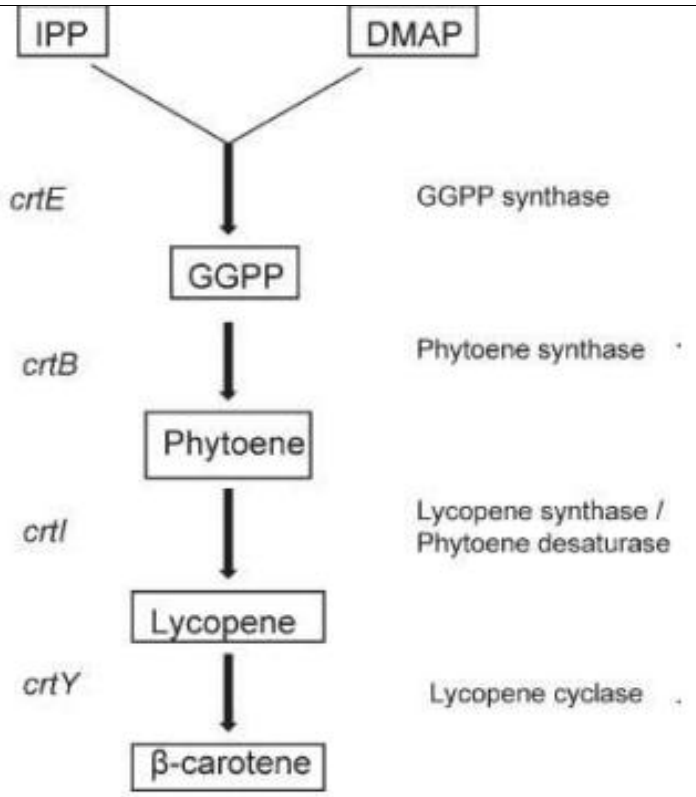


Figure 10: Presumptive metabolic pathway leading to the production of beta-carotene in *M. tuberculosis* – Adapted from Brennan et al. (2003)

1.8.2. Regulation of bacterial gene expression

As more evidence supports the importance of pigmentation's role, more studies are required to explore deeper into the regulatory mechanism behind it. Generally, the answer to the question as to whether or not pigment genes are expressed in mycobacteria can be similar to the principal prokaryotic regulation mechanism of gene expression, which is transcriptional control.

Gene expression in bacteria is regulated primarily at transcription initiation, which is controlled by the RNA polymerase (RNAP) holoenzyme, an enzyme consists of a five-subunit core RNAP and a dissociable subunit, sigma factor (σ). In bacteria, the binding of different sigma factors with core RNAP subunit generates specific cellular responses via redirection of transcription initiation at various promoter regions. There are two main types of sigma factors, one for housekeeping genes, and another one comprises alternative sigma factors which regulate gene expression in response to changes in environment (Gross et al., 1998, Gruber and Gross, 2003).

Sigma factors in mycobacteria, in particular, shows the most variation among species, for example, *M. tuberculosis* has 13 sigma factors, and *M. smegmatis* contains 28 sigma factors while *M. leprae* has only four functional sigma factors (Rodrigue et al., 2006, Waagmeester et al., 2005). Recently, a study in *M. marinum* which quantifies and compares mRNA levels of sigma factors under stress conditions is able to support the important role of sigma factors in bacterial survival and growth (Pettersson et al., 2015).

Overall, any study attempts to understand bacterial gene regulation should focus on sigma factors functions and regulations.

1.8.3. General methods in studying gene expression

Popular approaches for studying gene expression can either be genetic or biochemical ones.

Genetic methods usually employ gene fusions to study expression of one or many genes of interest. In this way, the gene of interest is fused to a reporter gene to create a chimeric DNA fragment. If the gene is only controlled at transcriptional level, the fusion can be called transcriptional or operon fusion. If the gene of interest results in a hybrid protein, means genetic materials has gone through both transcription and translation processes, the fusion is a translational or a gene fusion.

The second alternative for genetic approach is the adoption of luciferases as gene reporters. However, fusion of the genes can damage the stability and activity of transcriptional and translational products. As a result, biochemical methods are more feasible because they are faster, easier to visualise under gels, and the products can be quantified completely. Protein analysis is mostly used for immunodiagnosis and vaccines improvement, by employing techniques like western blot or SDS-PAGE.

Many studies have opted for RNA analysis to understand gene expression, utilising techniques such as northern blot, primer extension, RNase protection assay, and the modern one, reverse transcription quantitative PCR (RT-qPCR). This latest technique offers many advantages over traditional ones: better sensitivity, target a wide range of genes, and minimise the possibilities of contamination.

In RT-qPCR, the quality of RNA plays a crucial role in the validity the assay. In addition, close attention should be place on numerous RT-

qPCR inhibitors generating from RNA template. For example, a majority of inhibitors can be found in blood and tissue, also with environmental samples (Al-Soud et al., 2000, Al-Soud and Rådström, 2001, Panaccio and Lew, 1991). Likewise, RT-qPCR results can also be compromised by inhibitors from culture media, or reagents used in nucleic acids purification (Rossen et al., 1992). Overall, owing to its robust effectiveness, RT-qPCR has been a reliable tool for studying gene expression.

1.8.4. Initial RNA preparation step is the limiting factor for useful transcriptomic studies

Generally, the study of gene expression usually involves different types of designs to quantify mRNA levels, so figuring out how different genes expressed at different conditions. In fact, many researches have shown a clear influence of total RNA on gene expression profiling using RNA quantifying methods. Those approaches can employ various technologies namely real time PCR, microarrays, or RNA sequencing (Becker et al., 2010, Spellman et al., 2002).

Because RNA affects validity and reliability of downstream data collected, the quality of RNA input is the limiting factor for relevant gene expression evaluation (Copois et al., 2007, Imbeaud et al., 2005). Therefore, careful measures should be taken, especially as RNAs are sensitive molecules, which makes them susceptible to degradation by nucleases. There is also a risk of impurities by post-processes, and improbable sample handling or inadequate storage conditions (Pérez-Novo et al., 2005).

Bustin et al. (2009) and Fleige and Pfaffl (2006) have established criteria for the extraction and purification procedure of total RNA:

i. Free of protein
ii. Pure, free of genomic dna
iii. Intact, meaning not degraded
iv. Free of enzymatic inhibitors for downstream applications such as micro arrays or RT-qPCR
v. Free of any substances which complex essential reaction co-factors
vi. Free of nucleases for extended storage

As a result, there are aspects in term of ensuring RNA quality that are of great concern namely RNA extracting techniques and RNA purification (Copois et al., 2007). Therefore, it is important to develop a reliable method for total RNA extraction of high quality and purity yield in any transcriptomic studies.

1.8.5. Common approaches to RNA isolation

The most critical step in RNA isolation process is the rapid bacterial cell lysis, following by suspension of cellular content – meaning the RNA – in buffers containing RNase inhibitors (Dietrich et al., 2000). However, also this step is the challenge in mycobacterial RNA extraction, as *Mycobacteria* spp. are highly resistant to fast and efficient cellular lysis methods, considering their impermeable and waxy cell walls (Brennan and Nikaido, 1995).

To date, various methods are selected to break mycobacterial cell walls, such as enzymatic lysis, nitrogen decompression, sonication, shearing, or bead beating (Bashyam et al., 1996, Cheung et al., 1994, Mangan et al., 2002, Rajagopalan et al., 1995, Rezwan et al., 2007). All have the pros and cons, for example, although a nitrogen decompression chamber is sophisticated, it is rather expensive. While enzymatic lysis is more affordable, it is a lagging process which may results in high possibility of RNA degradation (Dietrich et al., 2000). Besides, methods ultilising toxic chemicals often yield irregular quality of RNA (Jahn et al., 2008).

In addition, as Bustin et al. (2005) and Fleige and Pfaffl (2006) have highlighted, other factors with respect to RNA sensitivity should be considered as well, such as RNA degradation and traces of contamination – genomic DNA or proteins fragments. A number of studies which include an RNA extraction step usually employ Trizol-based method or the Hot-phenol based method, and RNA isolation kits are also a valid option (Mangan et al., 1997, Rustad et al., 2009, Arnvig et al., 2011, Killick et al., 2011). However, the quality of RNA collected from these tests is not consistent, and more trials should be taken to evaluate and to verify the most suitable RNA

isolation approach for mycobacterial species on the whole, and MAP strains in particular.

1.9. Aims and objectives

Owing to the prevalence of MAP, its potential epidemiology and its correlation to Crohn's disease in humans, there is an urgent need to understand better about the species genetics and diversity. Pigmentation in MAP is well documented for sheep strains while there are currently only a few reports both *in vitro* and *in vivo* of pigmented cattle strains. Nonetheless, there is enough evidence to support the strong influence of pigmentation on mycobacteria virulence and especially the species' survival inside the host body. Therefore, in order to understand pigmentation pathway in MAP, there is a need to investigate further into cattle strains MAP pigment gene expression regulation.

This study was designed to repeat the experience by Swift (unpublished material) to induce pigmentation in MAP cattle strains. Subsequently, there would be RNA extraction for further transcriptional studies. Nevertheless, owing to the mycobacterial rigid integrity and bilayer of mycolic acids, the quality of RNA isolated is not always sufficient enough for a complete gene expression profile in MAP. Current studies use different RNA isolation techniques; however the results are non-consistent and non-standardised. Thus, in this study we try to evaluate different purification methods and then find a holistic approach for RNA extraction methods in MAP.

Another aim of this study is to use isolated RNA from pigmented MAP to measure the expression of pigmented genes. To make it clearer, this study hypothesises that by placing MAP in an acidic environment, carotenoid synthesis pathway in MAP was triggered and responsible genes were regulated at transcriptional level. In order to validate such hypothesis, quantitative real time PCR would

be used to understand regulation pattern of pigmented genes in MAP.

Finally, an extensive study of mycobacterial carotenoid operon in general would be conducted. It is aimed to inspect the connection of the carotenoid operon inter-species, and to get a grasp of its origin in MAP C strains.

2. Materials, methods and standard procedures

2.1. Strains and media

2.1.1. Bacteria strains

Bacteria	Strain	Reference
Mycobacterium avium subsp. *paratuberculosis*	K10	Type strain
Mycobacterium smegmatis	FPTB strain	Lab 21

Table 2: List of bacteria strains used in this study

2.1.2. Mycobacteria culture

All mycobacteria were cultured and maintained on Middlebrook 7H10 agar slopes (Becton Dickenson, France) by dissolving 9 g of media in 1 L of RO water and autoclaved at 121 ^{o}C for 15 min. Ten percent of Oleic Albumin Dextrose Catalase (OADC, Becton Dickenson, France) was supplemented into the culture media before preparing the slopes. When culturing MAP, the media was supplemented with 2 mg ml^{-1} of Mycobactin J (Synbiotic Corporation, France). All cultures were incubated statically at 37 ^{o}C.

2.1.3. Different pHs cultures

For preparing acidic cultures, Middlebrook 7H10 media was adjusting to different pH at 6.6, 6.0, 5.5, and 5.0 with HCl and autoclaved at 121 ^{o}C for 15 min.

2.2. Identification of MAP cattle strains

2.2.1. MAP-specific *f57* PCR

This PCR reaction was described by Coetsier et al. (2000), ultilising the primers *f57*f and *f57*r (Table 3), which amplified a DNA specific sequence on MAP IS*1311*. DNA (10 µl) extracted from cells was used in each of the PCR reactions. The 25 µl reaction mixture consisted of: 12.5 µl of Qiagen HotStarTaq Master Mix plus (Qiagen, UK), 250 ng of each primer; *f57*f and *f57*r and sterile molecular grade water to top up to the final 25 µl reaction volume.

The mixture was briefly pulse centrifuged to bring all the contents to the bottom of the 0.2 µl PCR tubes and placed in a thermo-cycler (Techne TC-3000). The parameters of the thermo cycler were set as followed: initial denaturing step of 94 ^{o}C for 5 min. This was followed by 37 cycles of; 94 ^{o}C for 30 s, 58 ^{o}C for 30 s, and 72 ^{o}C for 60 s. Final extension at 72 ^{o}C for 7 min.

The PCR products were visualised on a 1.5 % agarose gel containing 5 % (v/v) ethidium bromide. Two micro-litres of 5 x loading dye (25 mg bromophenol blue, 4 g of sucrose, and H2O to 10 ml) was added to each PCR product and 15 µl of each dyed product was placed in separate wells on gel. The products were separated for 1 h using 70 V. The gels were visualised under ultra-violet (UV) light. The expected product size was a single 329 bp band.

2.2.2. Restriction Endonuclease PCR (REA-PCR)

The protocol for REA-PCR analysis was adopted from the method of Marsh et al. (1999). In brief, each reaction needed 12 µl of Taq PCR Master Mix (Qiagen, UK), 1 mM MgCl2, 250 ng each of the primers M56 and M94 (Table…), 5 µl of sample DNA, and finally sterile distilled water was added up to final volume of 50 µl.

The mixture was briefly pulse centrifuged to bring all the contents to the bottom the PCR tubes and placed in a thermo-cycler (Techne

TC-3000). Reaction was set as followed: 1 cycle of denaturation at 94 ^{o}C for 5 min, 37 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 62 ^{o}C for 30 s, and extension at 72 ^{o}C for 1 min. Final extension at 72 ^{o}C for 4 min.

When the PCR amplification process was finished, 8 µl of the PCR product were cut using 2 U of the enzyme *Hinf*1 (Fisher Scientific), 1.6 µl of 10 x reaction buffer supplied with the enzyme, and sterile distilled water to a final volume of 16 µl. Enzyme reactions were incubated at 37 °C for 1 h.

Results was visualised on gel as described in 7.2.1. Two bands at 285 bp and 281 bp were expected positive result for MAP cattle strains.

2.3. Preparing samples for RNA extractions

Cells were to be collected in sterile condition, using sterile loops of 10 µl (VWR, UK). Collected cells were suspended in 1 X PBS made of RNase free H2O (Invitrogen, UK). Cultures were grown to stationary phase and the samples were adjusted to O.D. of 2.0 – 3.0, approximately 1.0 x 10^{9} CFU ml^{-1}. The samples were then homogenized using 1 ml 0.4 mm silica beads (VWR, UK) followed by high-speed vortex for 1 min. Next, 0.5 ml homogenised sample was to be transferred to a clean and RNase-free 1.5 ml container with approximately 0.5 ml acid-washed glass beads 400-600 µm (Sigma-Aldrich, UK) for subsequent RNA extraction process.

Primer name	Primer sequence (5’ to 3’)	Target DNA	Reference
*f57*f	GGTCGCGTCATTCAGAATC	MAP *f57* gene	Coetsier *et al.* (2000)
*f57*r	TCTCAGACAGTGGCAGGTG	MAP *f57* gene	Coetsier *et al.* (2000)
M56	GCGTGAGGCTCTGTGGT GAA	Specific for the 5’ region of the IS*1311* gene	Marsh *et al*.,(1998)
M94	CAGCGATCGTCGACAGT GTG	Specific for the 3’ region of the IS*1311* gene	Marsh *et al*.,(1998)
16sF	GCAAGCCTGATGCAGCGACG	16S ribosomal RNA	This study
16sR	CGGACAACGCTCGCACCC	16S ribosomal RNA	This study
GGPPF	GACCTCGGGGTGGCGTTT	*crtE*	This study
GGPPR	TCAGCTGTGCGCCGATGG	*crtE*	This study
PDF	CAGGCCGGACGCATCTCC	*crtB*	This study
PDR	AAGATGGTGTGGTGCGCGGT	*crtB*	This study

PSF	CACGGCCGCACCTTCTTCC	*crtI*	This study
PSR	GTAGCGGGCGAAGCCGTAC	*crtI*	This study
LC1F	TCTACCGGAGGTGGCGACG	*crtYc*	This study
LC1R	GCCACGGCGATCTCATCCCA	*crtYc*	This study
LC2F	ATGACCGGCCTGGGCTAC	*crtYd*	This study
LC2R	CTGCGTTTGGTCAGCCAGC	*crtYd*	This study
BCDF	ACCGACCTCGCGATGAACC	*crtU*	This study
BCDR	CCGCGTCGATGAGCGGGTA	*crtU*	This study
sigFF	ATGATGGTGGGCACCGCGA	sigma factor F	This study
sigFR	TCAACGCGGTGGTCCGGTT	sigma factor F	This study

Table 3: Primer sequences used in this study

2.4. RNA extraction methods

2.4.1. Modified Phenol:Chloroform:Isoamyl Alcohol (PCA) (25:24:1, v/v) method

The protocol was adapted from (Manganelli Dubnau Tyagi (1999) Differential expression). 1ml of PCA (Sigma-Aldrich, UK) was added to the sample, followed by high-speed vortex for a total of 3 min with periodic ice-chilling of 30 s in between. After that, samples were centrifuged at 10,000 x *g* at room temperature for 5 min, and supernatant was collected to remove cell debris. Another 1 ml of PCA was added to the sample and first step was repeated. Subsequently, samples were centrifuged at 16,000 x *g* at 4 ^{o}C for 15 min. Aqueous phase was collected for RNA precipitation in isopropanol.

2.4.2. Modified TRIzol method

The protocol was modified from (An Effective Method of RNA Extraction from Mycobacterium tuberculosis Tae Sang Oh). 0.5 ml of TRIzol reagent (Invitrogen, UK) was added to the sample, followed by high-speed vortex for a total of 3 min with periodic ice-chilling of 30 s in between. After 3 to 5 min incubation at room temperature, samples were centrifuged at 10,000 x *g* at room temperature for 5 min, and supernatant was collected to remove cell debris. Next, 0.2 ml of Chloroform added to allow better phase separation. After incubation at room temperature for 2 min, samples were centrifuged at 16,000 x *g* at 4 ^{o}C for 15 min. Aqueous phase was collected for RNA precipitation in isopropanol.

2.4.3. Modified cell lysis methods using commercial kits

In brief, when using RNeasy Mini Kit (Qiagen, UK), 0.7 ml of RNA Lysis Buffer with 10% (v/v) of Beta-Mercaptoethanol was added to

the sample, followed by high-speed vortex for 3 min then chilled on ice. Samples were then centrifuged at 10,000 x *g* at room temperature for 5 min, and supernatant was collected to remove cell debris. 0.5 ml of isopropanol was added to lysate; approximately 10% of total volume was used for RNA precipitation and visualization on gel. The rest was proceeded to wash and elute steps as per manufacturer's instructions.

With SV Total RNA Isolation System (Promega, UK), 0.3 ml of RNA Lysis Buffer with 10% (v/v) of Beta-Mercaptoethanol was added to the sample, followed by high-speed vortex for a total of 3 min with periodic ice-chilling of 30 s in between. Subsequently, 0.35 ml of blue RNA Dilution Buffer was added to lysate, and samples were incubated at 70°C for 3 min. Samples were then centrifuged at 10,000 x *g* at room temperature for 5 min, and supernatant was collected. 0.5 ml of isopropanol was added to lysate; approximately 10% of total volume was used for RNA precipitation and visualization on gel. The rest was proceeded to wash and elute steps as per manufacturer's instructions.

2.4.4. RNA precipitation

After collection of aqueous phase or RNA, 0.5 ml of isopropanol was added to the sample. Tubes were inverted gently to allow complete mixing. Samples were then to be chilled at -20 ^{o}C from 1 hr up to 24 hr, followed by high-speed centrifugation at 16,000 x *g*, 4 ^{o}C for 10 min. Supernatant was to be discarded, and pellet was to be washed with 1 ml of RNase-free 75% ethanol. Subsequently, samples were air-dried for 20 min to remove traces of alcohol. In order to obtain completely soluble RNA extracted, RNA pellets are advised not to air-dry for more than 2 hr, and to bincubated at 55-60^{o}C for approximately 5 to 10 min in RNase-free water.

2.4.5. Visualisation of RNA products

Before RNA clean-up, the RNA products were visualised on a 2.0 % agarose gel containing 5 % (v/v) ethidium bromide to check for cell lysis effectiveness. Two micro-litres of 6 x loading dye (25 mg bromophenol blue, 4 g of sucrose, and H2O to 10 ml) was added to each extracted sample and 5 µl of each dyed product was placed in separate wells on gel. The products were separated for 1 h using 60 V. The gels were visualised under ultra-violet (UV) light. The expected product size was two separate bands, 23s and 16s.

2.4.6. RNA clean-up

RNA clean-up was achieved using RNase-Free DNase Set (Qiagen, UK) to digest contaminated DNA. 1 ml of RNase-free 75% ethanol was added to lysate and mixed by inverting. 0.7 ml of mixture was then transferred to spin-column for high speed centrifugation at 12,000 x *g*, room temperature for 15 sec. If there was more lysate, the step was repeated. Subsequent wash and elute steps were followed manufacturer's instruction.

2.4.7. Quantification of RNA

RNA and cDNA quantifications were performed using a NanoDrop ND 1000 spectophotometer (Thermo scientific, USA). 2 ul of RNase free H2O (Invitrogen, UK) was loaded to the optic measurement area as blank. Subsequently, 2 ul of samples was loaded to measure absorbance ratios at A260/A280 and A260/A230.

2.4.8. RNA Quality Control

RNA quality control parameters were achieved using 2200 TapeStation Instrument (Agilent, UK). 1 ul of samples was mixed with 5 ul of RNA Sample Buffer (Agilent, UK). Next, samples were heated to 72 °C for 3 min, then chilled on ice for 2 min before

measuring using RNA ScreenTape (5067- 5576) and equipment’s internal RNA ladder. Yield and RIN of each sample was recorded.

2.5. Gene expression study

2.5.1. Primers designs

Primers for target gene sequences in this study (Table 3) were designed to be 50 to 200 nucleotides long, melting temperature approximately 60 – 65 ^{o}C, and GC content around 70%. Design template used at www.ncbi.nlm.nih.gov/tools/primer-blast/, and manufactured by Eurofins Genomics, UK.

2.5.2. cDNA synthesis

Up to 1 µg of total RNA (2 µl) was reverse transcribed by SuperScript® III Reverse Transcriptase (Introvigen, UK) master mix according to the manufacturer's instructions. Prior to use in subsequent quantitative real time PCR, cDNA was diluted to 1:20 with nuclease free water included in the kit, stored at -20 ^{o}C.

2.5.3. Quantitative real time PCR (RT-qPCR)

After building up the MAP cDNA library, gene expression study was carried on by RT-qPCR using GoTaq RT-qPCR Systems (Promega, UK). Identical supplied master mix was used during the PCR reaction to ensure consistency between samples, with GoTaq Hot Start Polymerase, MgCl2, dNTPs, BRYT green dye, and a reaction buffer. Total reaction volume is 50 µl, with 25 µl master mix, 10 µl template cDNA, 2 µl primer each, 1 µl DMSO, 1 µl CXR reference dye, and nuclease free H2O up to required volume. For negative control, nuclease free H2O was used as templates. Each sample was triplicated in 48 wells plate, well-shaken with a plate vortex, and spin down to make sure target samples were at the bottom of the plate.

Setting up details were quantitative amplification with SYBR green, and cDNA template. Amplification process was set as followed:

Initial denaturation at 95 °, 10 min; then 40 cycles of denaturing at 95 °C for 15 s, and annealing at 60 °C for 60 s.

For validation of reference and target genes, 10-fold dilution of cDNA from Control MAP K10 was used as per normal RT-qPCR set up. Standard curve was constructed by plotting Ct values against dilution factors.

All reaction were conducted on ice, samples well-kept from light, and using Step One Real-time PCR instrument (Applied Biosystems, UK).

2.5.4. Quantitative analysis of the data

Ct values obtained from RT-qPCR reactions were collected and processed with Office Excel 2010 (Microsoft). One-tailed paired t-test was conducted between reference and target genes with *p* value cut-off at 0.05. Fold changes were calculated based on the formula below (Table 4)

$\text{Fold difference} = 2^{-\Delta\Delta C_t}$ $\Delta C_{t\ \text{sample}} - \Delta C_{t\ \text{calibrator}} = \Delta\Delta C_t$ $C_{t\ GOI}{}^{s} - C_{t\ norm}{}^{s} = \Delta C_{t\ \text{sample}}$ $C_{t\ GOI}{}^{c} - C_{t\ norm}{}^{c} = \Delta C_{t\ \text{calibrator}}$
Table 4: Calculation of fold difference from Ct values using ΔΔCt method. Sample = pigmented MAP. Norm = control MAP. GOI = gene of interest / target gene. Calibrator = reference gene

2.6. Comparative genomic analysis of the carotenoid locus

Below is the list of bacteria from NCBI Database used for comparative study in this project.

Bacteria strain	Pigment characteristic	Accession number
MAP K10	Unknown	AE016958.1
MAP S397	Unknown	AFIF00000000
Mycobacterium kansasii 824	Beta-carotene	NZ_CP009483
Mycobacterium marinum M	Beta-carotene	CP000854.1
Mycobacterium aurum A+	Beta-carotene and eschscholtzxanthin-like substance	AJ133724.1
Mycobacterium smegmatis	Induced in acidic condition	CP009494.1

Table 5: List of bacteria strains used for comparative study

Comparative analysis was conducted using Genomic BLAST, Protein BLAST, and Constraint-based Multiple Alignment Tool (COBALT) which are available on www.blast.ncbi.nlm.nih.gov

3. Induction of pigment in MAP K10

3.1. Introduction

In the literature, non-chromogenic mycobacteria were reported to form pigment upon acidic stress (Brown-Elliott and Wallace, 2002, Brown et al., 1999).

Previous observations made in our laboratory had shown that cattle strains of MAP cultured on solid agar for an extended time in plastic Universal vessels became orange-pigmented whereas those cultured in glass Universal vessels did not (Fig. 11) (Swift, unpublished material).

Colonies from the slope were sub-cultured to eliminate the possibility that other contaminated bacteria had caused the change in colour. To determine the identity of pigmented colonies, they were also subjected to a MAP-specific *f*57 gene PCR. The results show that each pigmented colony was positive for the PCR test, and no contamination was noted.

At further investigation, REA-PCR analysis of the amplified IS1311 gene had shown that the pigmented colonies observed in our cultures belonged to cattle type MAP classification, and not the well-known pigmented sheep strains of MAP (Fig. 12).



Figure 11: Image shows the observed pigmented presumptive MAP colonies cultured on Middlebrook 7h10 (Becton Dickenson) for over 2 years.

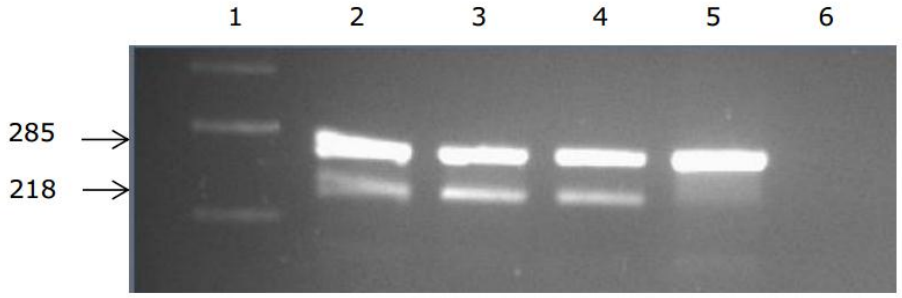


Figure 12: REA-PCR Analysis of MAP isolates from two pigmented cattle MAP strains (Lane2: presumptive K10, and Lane 3:ATCC 19851), after treatment with the restriction enzyme HinfI. . Lane 4: positive control with MAP DNA. Lane 5: genomic DNA extracted from a pigmented sheep strain DNA (Kindly donated by K. Stevenson). Lane 6: negative control (sterile distilled H2O). Bands at 285 bp show the presence of the IS*1311* element in all MAP strains. Bands at 218 bp show the point mutation in the insertion element found only in cattle strains of MAP.

The initial observations of pigmented MAP could have been because of the extended length of culture. Old cultures of mycobacteria have been reported to become pigmented over time (Dworkin and Foster, 1958). In addition, there are some differences between glass and plastic vessels in term of air permeability, and light absorbance. We suspected that in plastic vessels, there could have been more air and thus more oxygen compared to the glass one. In this condition MAP cells would be less likely to go completely into a dormant state (Swift…). As a result, long culturing period in the presence of excess oxygen might result in a drop in pH (REF?) and creating an acidic condition which could probably triggered pigmentation in MAP.

To evaluate all possible cases, *M. smegmatis* was used as a fast-growing model organisms to determine whether they have the ability to become pigmented. It has already been described by Saviola and Felton (2011) that *M. smegmatis* can produce pigments when grown on acidic agar. In this study the author attempted to repeat the experiments by Saviola and Felton (2011). *M. smegmatis* and MAP were cultured on 7h10 agar slopes, at pH 6.5, pH 5.5 and pH 5.

3.2. Results

The results showed *M. smegmatis* did not produce pigments at pH 7, but did at pH 6 (Fig. 13). No growth was seen by either of the cells at pH 5 (data not shown).

At pH 6.6, MAP colonies were observed to be of sporadic form, creamy colour, rough surface, and raised but uneven elevation. At pH 6.0, colonies with yellow pigments were spotted and they formed brighter colour at pH 5.5. At further acidic condition, i.e. pH 5.0,

scattered small colonies of MAP were seen which suggested that little or no growth of MAP was present.

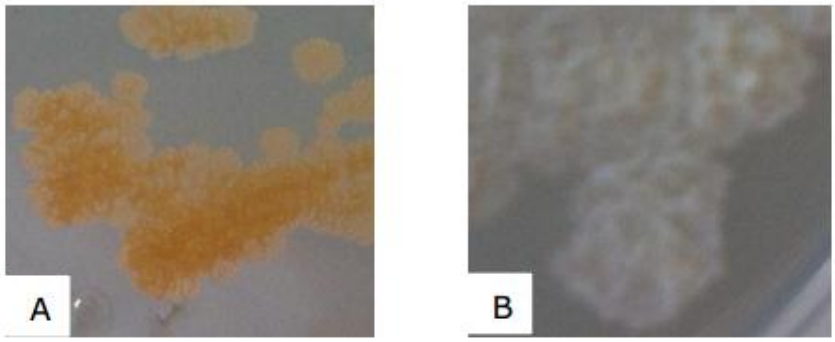


Figure 13: Figure shows *M. smegmatis* (FPTB strain; panel A and B) grown on 7H10 agar adjusted to pH 6.5 (A) or pH 5.5 (B). Orange colonies can clearly be seen after 4 d incubation at 37^{o}C for *M. smegmatis*

pH 6.6	pH 6.0	pH 5.5	pH 5.0
			

Figure 14: MAP strains K10 formed colonies with yellow pigments after 3 months of incubation when exposed to acidic culture media

3.3. Discussion

This observation supported our hypothesis and agreed with previous studies which found that pigmentation can be induced in atypical non-pigmented strains of mycobacteria patched on acidic agar (Saviola, B. and Felton, J., 2011.).

Carotenoid biosynthesis can be responsible for pigmentation in MAP which results in carotene and lycopence, both are free radical scavengers protecting the cells against photodynamic injuries (Vershinin, 1999), and strengthening bacterial cell envelope (Kubler Baumeister 1978). Suitably, it is reasonable to deduce that MAP produced carotenoids as a mean to withstand changes in the environment. Acidity is a challenge that MAP in particular and mycobacteria in general has to deal with, both in the environment and inside the hosts. In the former scenario, natural acidic environments like in water bodies or the soil possess a threat to MAP's survival. In mammalian bodies, as macrophages engulfing bacteria, phagocytosis process can bring the pH down to 4.5-5.5 (Rohde, K.H., Abramovitch, R.B. and Russell, D.G., 2007). All in all, stressors are abundant in both the environment and inside host cells, thus in MAP there should be a counter-stress mechanism which would help the organism overcome the challenge and propagate.

One of many ways mycobacteria protect themselves from stressors may be their special cell wall. Mycobacteria overall have developed an extensive cell wall, composed of mycolic acids and other lipids, as an intrinsic mean to protect themselves from environmental shifts (Hoffman et al; 2008, Neiderweis et al; 2010). Apart from it, mycobacteria also regulate necessary genes that can help them

persist acidic stress (Saviola, B. 2012). Wu (2007) took a close look at the stressome of MAP and discovered that in acidic condition, up to 195 genes are upregulated.

In our experiment, as the drop in pH can damage cell membrane, MAP would possibly activate carotenoids biosynthesis pathway to resist the disturbance, which would explain the presence of pigment. However, the specific function for pigment in this case is still a question as to whether it plays a direct role in counteracting acidic damage or an indirect role in response to other circumstantial stimuli, such as oxidative stress.

In summary, pigmentation in mycobacteria is of clinical interest for identification and diagnostic purposes as well as understanding infectious pathway. We have shown in our laboratory that there is a confirmed case of pigmented cattle strains of MAP, which brought about by acidic condition. acidity changes possibly triggered carotenoid production mechanism in mycobacteria. Nonetheless, there has not yet been a clear perception on the exact roles carotenoids play in mycobacterial acidity response. Consequently, a further investigation into the regulation of carotenoid biosynthesis pathway would reveal more understandings regarding this ambiguity.

4. Carotenoid biosynthesis operon

4.1. Introduction

From the literature, pigment synthesis genes found in *M. aurum* and *M. smegmatis* were used to find homologues in the MAP K10 genome using BLAST (NCBI Database). The results identified an intact operon for carotenoid pigment production (Fig 15) and therefore MAP K10 strains do have the genetic potential to produce carotenoids.

However having the genes does not necessarily mean that MAP K10 is able to use them, which requires more investigations to figure out the exact functions this carotenoid operon plays in MAP K10. This study attempted to compare homologous pigment gene sequences and related proteins in the MAP carotenoid operon with other mycobacteria, so that we may have better understandings regarding the MAP carotenoid operon.

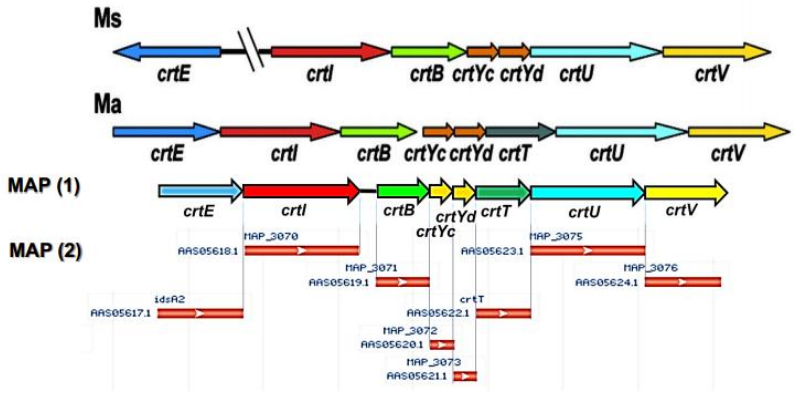


Figure 15: Comparative genomic analysis of the carotenoid locus in *M. smegmatis* (Guthertz et al.), *M. aurum* (Ma) and MAP(1) which deduced from the BLAST result MAP(2) of carotenoid locus gene in M. aurum in MAP K10 strain (Accession number: AE016958.1)

4.2. Results

4.2.1. Carotenoid biosynthesis proteins alignment

Proteins that are essential in carotenoid biosynthesis pathway found in MAP K10 were BLAST against similar proteins found in chromogenic mycobacteria, and mycobacteria that are known for producing carotenoids (Fig 16).



```
>MAP_K10                  1    MPSgreRWD---TMGALSF-----SPTTTAAAPAWTRidDFGG   WRAHVRAMVLDEVADFVGMRRADELD--RAGIGT  67
>Mycobacterium_kansasii   1    MCAsfsRQPptaELPTVSVaagVDSPPTTAGYRSPTV--DSAS   WRAMFRRAVLATVGEFVVTRSASQLD--EAASAA  73
>Mycobacterium_marinum    1    MVS-------trQRPTLSLataVDSPDS--GRMASTG--DAVS[5]WRSRLRTAALAQIADFVSDRSASALG--GSGWEV  69
>Mycobacterium_aurum      1    MAS---RSQ-------------VATDPFVGAGPTATR------   WLAPLRRAVLGAVTEFLDTHCREALY--GAHVDI  53
>Mycobacterium_smegmatis  1    MDT---------------------------AAPSAVE------   ----LAGAVTEQLREYLRERRSDCAYigFVYAEL  40

>MAP_K10                  68   AGDILVQFVSGGKCLRSTFVYLGWLCGAPRDRAA   LRAAASFELLHAFALLQDDVMDGAQERRGRPAAHLQFGRWHQD  144
>Mycobacterium_kansasii   74   AGEILLTFVSRGKCLRSTFMYLGWLCGAAPSRAA   LSAAASLELLHAFALLQDDVMDRSSQRRGSPAVHVQLAEWYRL  150
>Mycobacterium_marinum    70   AGDILLRFVSEGKCLRSTFMYLGWLCGAGPNNAA   LSAAASLELLHAFALLQDDVMDQSPQRRGSPAVHVQLARCHQR  146
>Mycobacterium_aurum      54   TELVLRGMLAEGKCVRSTYLYLGWQCGAPADEAA   LRAAASLELLHAFALLQDDVMDESPMRRGRPSAHRAFERWHRE  130
>Mycobacterium_smegmatis  41   TAGVEEFVLRGGKRVRPAFAYWGWRSVIEPADAV[5]LRLFSALELLQACALIHDDVIDDSATRRGLPTVHRLFTEQHHE  122

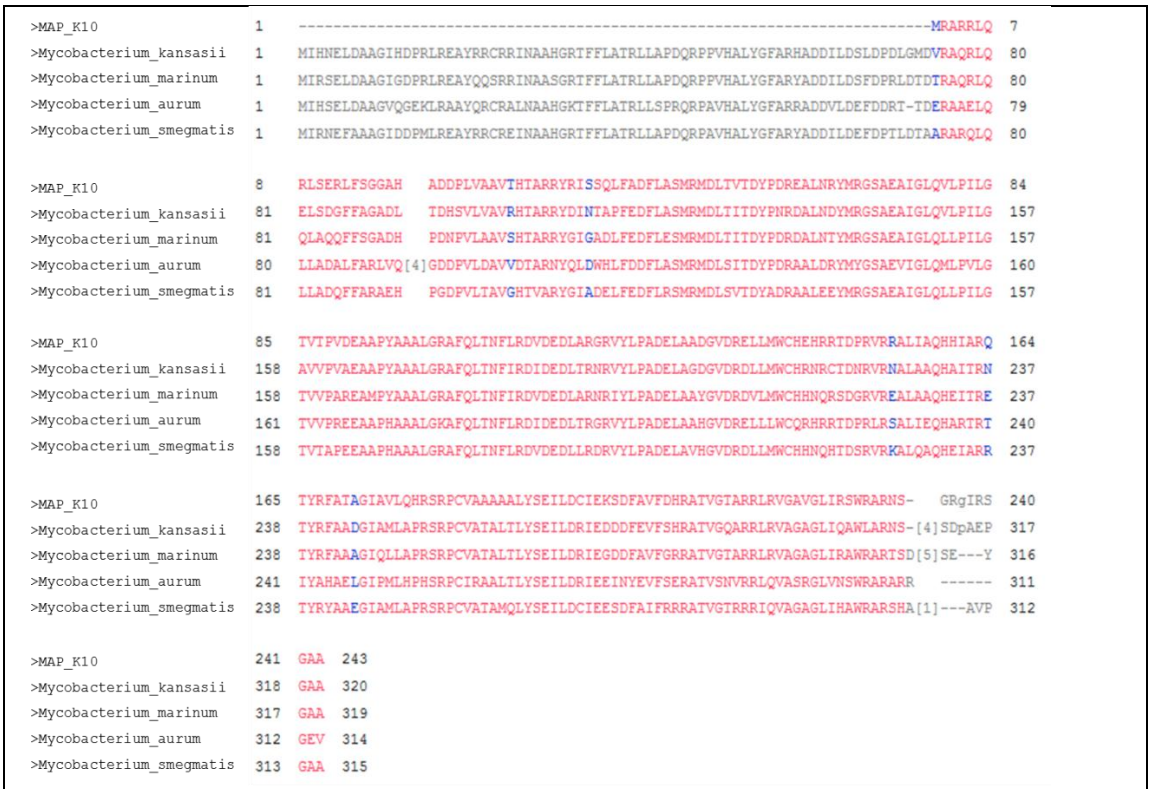
>MAP_K10                  145  RGLSGSPRRFGESAAILLGDLCLIWAEQMLRDSGLEARRLQRAWPRYDAMRVELAVGQFADLTNDIRQSPGLDAVLEVAR  224
>Mycobacterium_kansasii   151  RGLSGPAGRFGESAAILLGDVCLIWAEQMLRESGVEARRLQQLWPRYDAMRIELAVGQFADLANDIRDRPSLRTVLDIAR  230
>Mycobacterium_marinum    147  RGLSGAAARFGESAAVLLGDICLVWAEQMLRESGVEDRRLRQVWPRYDAMRIELAVGQLCDLSNDIRNLPTLDAVLDVAR  226
>Mycobacterium_aurum      131  RGLPGSSARFGESAAVLLGDLCLVWAEQMLRESGVDEAALTRAWPRYDAMRTELAVGQFADLVNDTAGFPQLDEVLDVAR  210
>Mycobacterium_smegmatis  123  RGWLGSERQFGLSAAILAGDLALSWADDIVAGADLFVDAHRRVQTVWRHIRTEVLGGQYLDIVIESSGDESVASALNVNT  202

>MAP_K10                  225  RKSGNYTVRRPLEIGATMAGCDDRTVARLSRYGAAVGEAFQLRDDVLGVFGSPAVTGKPCAADLFERKATAVVVSAHQLA  304
>Mycobacterium_kansasii   231  RKSGNYTVRRPLEMGAAMADCDDRTLTQLGLYGADVGEAFQLRDDLLGVFGPPATTGKPNGGDLLDRKATSVVVAAHEMA  310
>Mycobacterium_marinum    227  RKSGNYTVRRPLEMGAAMADCDERTLEQLARYGDAVGEAFQLRDDMLGVFGTPAITGKPSGDDLRERKATSVVVAAYEMA  306
>Mycobacterium_aurum      211  RKSGNYTVRRPLEIGAALAGCDAEVITVLGRYGEAIGEAFQLRDDLLGVFGAPQTTGKPTGTDLVARKATTVVVAGYELA  290
>Mycobacterium_smegmatis  203  FKTASYTVARPLQLGAAAAADRPDVQELFHRIGNDLGVAFQLRDDVLGVFGDPAVTGKPSGDDLRSGKRTVLLAEALELA  282

>MAP_K10                  305  DP--PVRRELIELATRDVLDAAAIERWKALIVETGAVRLIERMISDR-VASAREQ--LCGLPVDESVRAALAEMAAVCTD  379
>Mycobacterium_kansasii   311  DQ--SARRELAELVRADHLDDAALDRFRAVIIATGAAQRIEGMIRRR-VDRARNV--LDHIVIDCAARTALATLAEACVE  385
>Mycobacterium_marinum    307  DP--PTRRQLHELMHRAELDDAAVDHCKRLITATGAVQRIEDMIDRR-VDRVRTL--LADMTIGQQTRAALGDLAAACVQ  381
>Mycobacterium_aurum      291  TG--AQQRQFKEFMTAPELSSGDVTRWQALISEVGTVQRIEDMIHRRfAVAVQALdsLDATRLDADARSALVAMASACTE  368
>Mycobacterium_smegmatis  283  DAsdPAAAALLRRSVGTELSDIQVRELCDAIESVGALAAVEQRIETL---TRDALdvLAAANVHPQAKAGLSELARSAAN  359

>MAP_K10                  380  RAE  382
>Mycobacterium_kansasii   386  RTA  388
>Mycobacterium_marinum    382  RST  384
>Mycobacterium_aurum      369  RIA  371
>Mycobacterium_smegmatis  360  RTA  362
```

CrtE (GGPP Synthase) protein homology



```
>MAP_K10                    1   ------------------------------------------------------------MRARRLQ  7
>Mycobacterium_kansasii     1   MIHNELDAAGIHDPRLREAYRRCRRINAAHGRTFFLATRLLAPDQRPPVHALYGFARHADDILDSLDPDLGMDVRAQRLQ  80
>Mycobacterium_marinum      1   MIRSELDAAGIGDPRLREAYQQSRRINAASGRTFFLATRLLAPDQRPPVHALYGFARYADDILDSFDPRLDTDTRAQRLQ  80
>Mycobacterium_aurum        1   MIHSELDAAGVQGEKLRAAYQRCRALNAAHGKTFFLATRLLSPRQRPAVHALYGFARRADDVLDEFDDRT-TDERAAELQ  79
>Mycobacterium_smegmatis    1   MIRNEFAAAGIDDPMLREAYRRCREINAAHGRTFFLATRLLAPDQRPAVHALYGFARYADDILDEFDPTLDTAARARQLQ  80

>MAP_K10                    8   RLSERLFSGGAH   ADDPLVAAVTHTARRYRISSQLFADFLASMRMDLTVTDYPDREALNRYMRGSAEAIGLQVLPILG  84
>Mycobacterium_kansasii     81  ELSDGFFAGADL   TDHSVLVAVRHTARRYDINTAPFEDFLASMRMDLTITDYPNRDALNDYMRGSAEAIGLQVLPILG  157
>Mycobacterium_marinum      81  QLAQQFFSGADH   PDNPVLAAVSHTARRYGIGADLFEDFLESMRMDLTITDYPDRDALNTYMRGSAEAIGLQLLPILG  157
>Mycobacterium_aurum        80  LLADALFARLVQ[4]GDDPVLDAVVDTARNYQLDWHLFDDFLASMRMDLSITDYPDRAALDRYMYGSAEVIGLQMLPVLG  160
>Mycobacterium_smegmatis    81  LLADQFFARAEH   PGDPVLTAVGHTVARYGIADELFEDFLRSMRMDLSVTDYADRAALEEYMRGSAEAIGLQLLPILG  157

>MAP_K10                    85  TVTPVDEAAPYAAALGRAFQLTNFLRDVDEDLARGRVYLPADELAADGVDRELLMWCHEHRRTDPRVRRALIAQHHIARQ  164
>Mycobacterium_kansasii     158 AVVPVAEAAPYAAALGRAFQLTNFIRDIDEDLTRNRVYLPADELAGDGVDRDLLMWCHRNRCTDNRVRNALAAQHAITRN  237
>Mycobacterium_marinum      158 TVVPAREAMPYAAALGRAFQLTNFIRDVDEDLARNRIYLPADELAAYGVDRDVLMWCHHNQRSDGRVREALAAQHEITRE  237
>Mycobacterium_aurum        161 TVVPREEAAPHAAALGKAFQLTNFLRDIDEDLTRGRVYLPADELAAHGVDRELLLWCQRHRRTDPRLRSALIEQHARTRT  240
>Mycobacterium_smegmatis    158 TVTAPEEAAPHAAALGRAFQLTNFLRDVDEDLLRDRVYLPADELAVHGVDRDLLMWCHHNQHTDSRVRKALQAQHEIARR  237

>MAP_K10                    165 TYRFATAGIAVLQHRSRPCVAAAAALYSEILDCIEKSDFAVFDHRATVGTARRLRVGAVGLIRSWRARNS-   GRgIRS  240
>Mycobacterium_kansasii     238 TYRFAADGIAMLAPRSRPCVATALTLYSEILDRIEDDDFEVFSHRATVGQARRLRVAGAGLIQAWLARNS-[4]SDpAEP  317
>Mycobacterium_marinum      238 TYRFAAAGIQLLAPRSRPCVATALTLYSEILDRIEGDDFAVFGRRATVGTARRLRVAGAGLIRAWRARTSD[5]SE---Y  316
>Mycobacterium_aurum        241 IYAHAELGIPMLHPHSRPCIRAALTLYSEILDRIEEINYEVFSERATVSNVRRLQVASRGLVNSWRARARR   ------  311
>Mycobacterium_smegmatis    238 TYRYAAEGIAMLAPRSRPCVATAMQLYSEILDCIEESDFAIFRRRATVGTRRRIQVAGAGLIHAWRARSHA[1]---AVP  312

>MAP_K10                    241 GAA  243
>Mycobacterium_kansasii     318 GAA  320
>Mycobacterium_marinum      317 GAA  319
>Mycobacterium_aurum        312 GEV  314
>Mycobacterium_smegmatis    313 GAA  315
```

CrtB (Phytoene Synthase) protein homology

```
>MAP_K10                  1    M-RAIEGSADHVVVIGAGLAGLSAALHLAGRGRAVTVVEREAWPGGRAGRRDIDGYRIDTGPTVLTMPDIIEDTFAAVGD  79
>Mycobacterium_kansasii   1    M-RTIQGRTDHVVVIGAGLAGLSTALHLAGRGRSVTVVEREPWPGGRAGRLDVGGYQIDTGPTVLTMPDIIDDVFAAVGE  79
>Mycobacterium_marinum    1    M-RTIEGTADHVVVIGAGLAGLATALHLAGRGRSVTVVEREAWPGGRAGRLDIDGYRIDTGPTVLTMPDLIDEVFAAVGE  79
>Mycobacterium_aurum      1    M-RTVGGRTDNVVVVGAGLSGLSAALHLAGRGRRVTVVERGSHPGGRVGRADLSGYRIDTGPTVLTMPDILDETFAAVGE  79
>Mycobacterium_smegmatis  1    MsRAIPGPTDHVVVVGAGLSGLSAALHLAGRGRRVTVVEREPWPGGRAGRIDTGGYRIDTGPTVLTMPDILDDVFAAVGE  80

>MAP_K10                  80   TLARRLELCTLDPAYRAVFADGSSLDIHRDPDRMADAVAEFAGREQATGYRRLRAWLTRLYRTEFDGFIAANFDSPLSLL  159
>Mycobacterium_kansasii   80   TSSGRLELLPVDPAYHAMFADGSSLDVHCDADRMASAIQTFAGSREAVGYRQLREWLTRLYRTEMDGFIAANFDSPLSLL  159
>Mycobacterium_marinum    80   TISDRLELLPVDPAYTAMFADGSSIDVHCDADRMVAAIENFAGPEQATGYLRLREWLTRLYRIEFAGFIGSNFDSPLSLL  159
>Mycobacterium_aurum      80   STADRLELIRVDPAYRASFADGSSLHVHSDAATMAAEIERFAGRAEADGYLRLRSWLARLYQLEFDGFIASNFDSPLSLL  159
>Mycobacterium_smegmatis  81   SVGDRLQLLPVDPAYRAQFADGSALEVHSDADRMAAAIADLAGPSEAAGYRRLRDWLTRLYRIEIDGFIGANFDSPLSLL  160

>MAP_K10                  160  TPRLAQLIAIGGFRNWDRMVKRYLSDPRLQRIFTFQSLYAGVAPRDALAVYAVIAYMDTVSGVVFPRGGVRALPDALAAA  239
>Mycobacterium_kansasii   160  TPQLARLAAIGGFRNWDAMVGRYLSDPRLRRIFTFQSLYAGVAPRDALAAYAVIAYMDTVSGVFFPRGGVRALPDALAAA  239
>Mycobacterium_marinum    160  TPQLARLAAAGGFRNWDSMVKRHITDPRLRRVFTFQSLYAGVAPQDALAAYAVIAYMDTISGVFFPRGGVRALPDALAAA  239
>Mycobacterium_aurum      160  TPQLAKLAAIGGFRRWEPMVRRFITDERLLRVFTFQALYAGVPPNRALAAYAVIAYMDTVSGVYFPRGGMRALPDAMAAA  239
>Mycobacterium_smegmatis  161  TPQLAQLAAIGGFGKWDHMVRRHLRDPRLQRVFTFQSLYAGVSPQRALAAYAVIAYMDTISGVFFPKGGVRAVPDALAAA  240

>MAP_K10                  240  ASDAGVQFRYGTTVTALEKTGGRVGAVLTEQAGRISCDAVVLTTELPQTYALLGRTPRRALRLRHSPSAVVAHVGCPAVA  319
>Mycobacterium_kansasii   240  GTGAGVQFHYRCSATELEHSGNRITAVHTDRGQRIPCDAVVLTTELPTTYRLLGRTPRRVRALRASPSAVVVHAGCRSVS  319
>Mycobacterium_marinum    240  AACAGVQFHYGSQVSELERDDARITAVRTDRGERIACDAVVLTTELPTTYRLLGRTPRRLRRLRAAPSAVVVHAGCRSAS  319
>Mycobacterium_aurum      240  AADAGVEFRYDATVTELERTGSRVTAVRTDTGERFPADAVVLTTELPDTYRMLGRTPRRPLRLRPAPSAVVAHIGCRAT-  318
>Mycobacterium_smegmatis  241  GADAGVRFHYGATVNCLERSADRVTAVHTDTGERIPCDAVVLTTELPRTYELLGRTPRRPRPWRASPSAVVVHVGCRRI-  319

>MAP_K10                  320  S-----QTAHHTILFGDAWDHTFADIIGAGQLMRDPSLLVTRPTASDPTLAPRGRDLLYVLAPAPNLESGGIDWDSVGRG  394
>Mycobacterium_kansasii   320  SaD--RPISHHTILFGAAWEQTFTDIIRDGLLMRDPSLLVTRPTASDPTLAPTGRDLFYLLAPAPNLAAGAVDWPTAGNA  397
>Mycobacterium_marinum    320  TaS--EPAAHHTILFGQAWEQTFTDIIRDGRLMRDPSLLVTRPTAGDPALAPPGRDLLYLLAPAPNLTAGRVDWGSAGGS  397
>Mycobacterium_aurum      319  --DpaADAAHHTILFGGEWDRTFEQIIDEGRTMSDPSLLVTRPTAGDPTLAPDGRDLLYVLAPAPNTAVSDKDWDADRDA  396
>Mycobacterium_smegmatis  320  --D--RPVAHHNILFGDAWHDTFTDIIDDGRLMRDPSLLVTRPTRGDPALAPAGRDLLYLLAPTPNLSSTAIDWETEGPA  395

>MAP_K10                  395  YVDDLIALVGQRLLPGLPGSATVLDVVTPADWARRGMAAGSPFALAHTFAQTGPFRPANMVRGIDNAVLAGSSTVPGVGV  474
>Mycobacterium_kansasii   398  YAESIIDTVGDRLLPELRDSADVLDVVTPLDWARQGMAAGTPFALAHTFGQTGPFRPANMVRGVDNAVLAGSSTIPGVGV  477
>Mycobacterium_marinum    398  YAQSMIDTVASRLLPDLRDSVELLDVVTPLDWARQGMAAGTPFALAHTFGQTGPFRPANTVRGIDNAVLAGSSTVPGVGV  477
>Mycobacterium_aurum      397  YTAQMLDTVTTR-LPHLGADAEVLHVVTPQDWARQGMLAGTPFALAHTFGQTGPFRPGNRVRGIDNAILAGSSTVPGVGI  475
>Mycobacterium_smegmatis  396  YAQRVVDTVAERLLPELRDDAQVLQVVTPADWARQGMVAGTPFALAHTFSQTGPFRPGNTVRGVANAVLAGSSTVPGVGV  475

>MAP_K10                  475  PTTLISGRLAADRITGVSKRTPARIDMK   ----AGSP  506
>Mycobacterium_kansasii   478  PTTLISGRLAADRITGGVRRQARNFDMR   VDVKAQMS  513
>Mycobacterium_marinum    478  PTTLISGRLAADRITGNTTRSIRH----   LDLKAQLS  509
>Mycobacterium_aurum      476  PTAIVSGRLAADRISSNSMMGART----   --------  499
>Mycobacterium_smegmatis  476  PTALLSGRLAADRVTGTVRRTRTHHDIN[6]ADRKARMP  517
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CrtI (Phytoene Dehydrogenase) protein homology

```
>MAP_K10                  1    MSDRWQYLVVLAACLVVTAPLEIAGAGVYRRWRRLAAAVLPVAAVFLLWDEIAVAARVWTYDHAYLCGLSIPVRVPIEEL  80
>Mycobacterium_kansasii   1    MNDHWQYLLVLAACLAITAPLEFFGNGVYRQPLRLLRAVLPVAAVFLIWDEVAVAAGIWTYDARYISGVAVPFRVPVEEV  80
>Mycobacterium_marinum    1    MSDRWQYLLVLLACLAITAPLEVFGAGVYRRPRRLLRSVLPVAAAFLVWDELAVAARVWTYDPRYISGLNVPFQVPIEEV  80
>Mycobacterium_aurum      1    ----------MVGCLAITAPLEVFGDGVYRQLRRAAAAILPVAAVFVLWDLLAILGDVWSYNPQYVTGFHIWV-FPIEEL  69
>Mycobacterium_smegmatis  1    MSENWHYLLVLAACVAITAPLECLGDGVYRQPRRVLQAVLPVAAVFLLWDEIAVAFDVWGYNPDYITGLTIPFDVPIEEV  80

>MAP_K10                  81   LFFLIIPVCAVLTYNAVSTILDKVRRR      107
>Mycobacterium_kansasii   81   LFFVVIPICALLTYNAVSTILDRLTRR      107
>Mycobacterium_marinum    81   LFFLVIPICALLTLNAVSTILDRLRRR      107
>Mycobacterium_aurum      70   LFFIVIPLCGLLTYSAVSTILAWVARR[11]  107
>Mycobacterium_smegmatis  81   LFFLVIPLCALLTYNAVSTILERVRRR      107
```

CrtYc (Lycopene Cyclase) protein homology

```
>MAP_K10                 1   MTGLGYTLPAALAVVIVCAAELTVLRTGLFRRPAYWLSMLIVLGFQVPVDGWLTKRSSPVVIYDDRQISGLRFPFDIPVE  80
>Mycobacterium_kansasii  1   MTGLGYTLPAVLAVVAVSALELKLLRIGMFRRPAYWVSMLIVLGFQIPVDGWLTKLNSPIVSYDERQTTGLRFPLDIPIE  80
>Mycobacterium_marinum   1   MTGLGYTLPAVLAVAGVGALELLVLRTGLFRRPAYWLSMVIVLGFQVPVDGWLTKLSAPIVSYNERQITGLRFPFDIPIE  80
>Mycobacterium_aurum     1   MTGLGYTLPAVIAVLVVIGWELRVLRTGLFRRAAYWLSMAIVLGFQVLVDGWLTKLSAPIVLYNEQHSTGIRFPWDIPIE  80
>Mycobacterium_smegmatis 1   MTGLGYTLPAVLAVIAVCGLELF-LRTGLFRRPAYWLSMLIVLGFQIPVDGWLTKLSAPIVIYDEQHTSGVRVFFDTPIE  79

>MAP_K10                 81  DFLFGFAMVTAVLLLWERRRARR-      103
>Mycobacterium_kansasii  81  DFLFGFAMVTAVLLLWERPRARR-      103
>Mycobacterium_marinum   81  DFLFGFAMVTAVLLLWERQREPGE[20]  124
>Mycobacterium_aurum     81  DFLFGWAMITAVLLLWERSAGRRN[ 2]  106
>Mycobacterium_smegmatis 80  DFLFGFAMITAVLLLWERRRVPTE[12]  115
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CrtYd (Lycopene Cyclase) protein homology

```
>MAP_K10                 1   M[4]TDRRRRILPAPTGLPDAGALPSRPRVAVVGGGIAGLIAATGLAERGVAVEVIEREHYLGGRVGGWTE-HHDGTDLA  8
>Mycobacterium_aurum     1   M   TDPRRVLHPATPGLPDAGHLPARPTVAVVGGGIAGLAAATALADRGVQVHLYEREPDLGGRVAGWTEtMPDGTPAA  7
>Mycobacterium_smegmatis 1   M   TDRLQRTHTAPRGLPHAGELPRRPRVVVVGAGIAGLAAATGMAERGVEVTVIERENYLGGRVGGWSE-QHDGAEYA  7

>MAP_K10                 81  MNRGFHAFFRQYYNLRALLVRLDPRLRMLVPVNDYPLIDAAGRRDSFRGLPRTPPLNAMAFAVRSPTFRLRDFARIDARA  1
>Mycobacterium_aurum     78  MNRGFHAFFRQYYNLRNMLRRIDSDLGMFAPLDDYPLVDGDGRRDTFRGLPRTPPFNAMAFALRSPTFRLRDMIHLDAKA  1
>Mycobacterium_smegmatis 77  MNRGFHAFFRQYYNLRALLARVDPQLRMLTPVEDYPLIDGEGRRESFRRLPHTPPWNALWFALRSPTFRLRDLMRLDAKA  1

>MAP_K10                 161 AAPLAAVSVPGTYRRLDHIDAATFLEDIRFPEAARHLAFEVFSRSFFADPAKLSAAELATMFHIYFLGSAEGLIFDVPSA  2
>Mycobacterium_aurum     158 AAPLAKVSVPQTYWQLDDQDAESFLQDINFPVAAQHLAFEVFSRSFFTRPEALSAAELATMFHIYFLGSSEGLVFDVATA  2
>Mycobacterium_smegmatis 157 AAPLAAVSVPGIYDRLDHIDAETFLKDINFPPAARHLAFEVFSRSFFADPSNLSAAELATMFHIYFLGSSEGLVFDVPTS  2

>MAP_K10                 241 NYDSALWQPLRSYLEQRHVRFRLGTSVGSID----AANRFAVHTDTDEELEVDAVVLAIDVAGLQRIVAASRGLANGEWR  3
>Mycobacterium_aurum     238 NFDVALWRPMEQYLRGKGVQVRTGIAVRGVTPVGL---GFLVTDDSGGTVRADGVVLATEVPALQRIVADSPDLGDSEWR  3
>Mycobacterium_smegmatis 237 NYDTALWEPLRRYLHEHGVRFHLGASVTRIDPVTTSPDAFAVHTDSGERLDADAVVLAADIAGLQRIVASSPGLGDDRWR  3

>MAP_K10                 317 DRIAGLRTAPPFAVHRFWLDRPVSPRRPAFLGTAGHKPLDNISVLDRYEREARAWAGAHHGSVVELHSYAL--DSAPSGA  3
>Mycobacterium_aurum     315 YRIGEMGSAAPFVVRRLWLDRPVQADRPAFLGTGGLPPLDNISVLNRYEVEATEWAQRTGGAVVELHAYSVaeDTPQLRE  3
>Mycobacterium_smegmatis 317 ARVQGLRTAPPFAVHRLWLDRPVTADRPAFMGTAGHKPLDNISVLERYERQSAQWARDNGGSAVELHAYAL--DTAESGP  3

>MAP_K10                 395 AALRELRRIYPETAAAQIVHETLLHRSDCPLFAPGGYPHRPTVVTPTPGLLLAGDAVRIDLPVALMERAATTGWCAANHL  4
>Mycobacterium_aurum     395 QLDARLHELYPETVGAGVVHEVTLCRADCPRLGPGDFARRPTVLTPQEGLVLAGDGIRIDLPVALMERAATTGMAAANAL  4
>Mycobacterium_smegmatis 395 GALAQLHRLYPETATAEPVFERVLVRDDCPLFAPGSHADRPGVVTGVPGLVLAGDGIRLDLPAALMERAATTGWAAANVL  4

>MAP_K10                 475 LKRWGLAGHPLVTVPTEGRSRLLRWLANREGA---TRS-  509
>Mycobacterium_aurum     475 LDRFGVRGHDLYTVPTQGRSAVLRTLAGRADRsdgRVSv  513
>Mycobacterium_smegmatis 475 LNRWGVAGHALRSVPTAGRFAPLRWLATREER---RAS-  509
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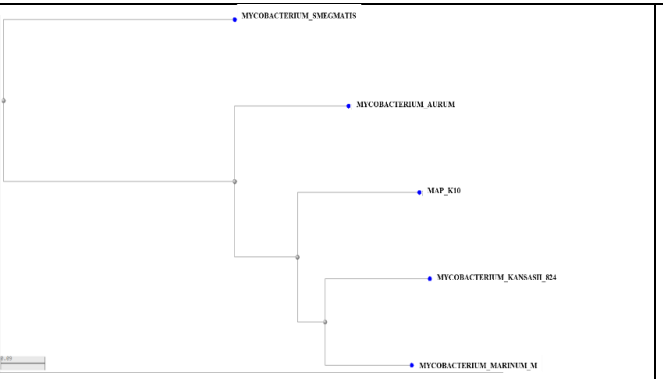
CrtU (Beta-Carotene Desaturase) protein homology

Figure 16: Comparison of carotenoid biosynthesis proteins in MAP K10 and other representative mycobacteria. All species in this study show a high level of sequence similarities. Pink means homologous

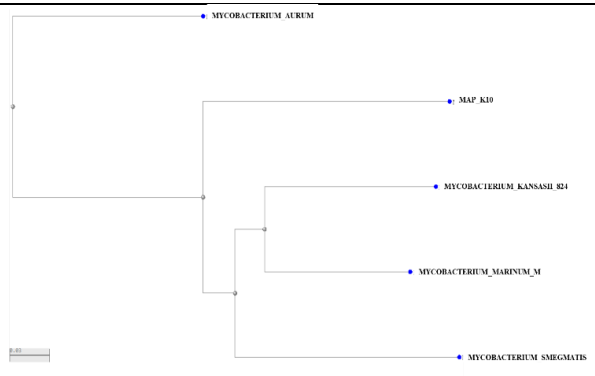
proteins. Blue means different proteins but similar function. Gray and dash means missing sequence.

4.2.2. A brief glance at evolutionary history of mycobacterial carotenoid operon

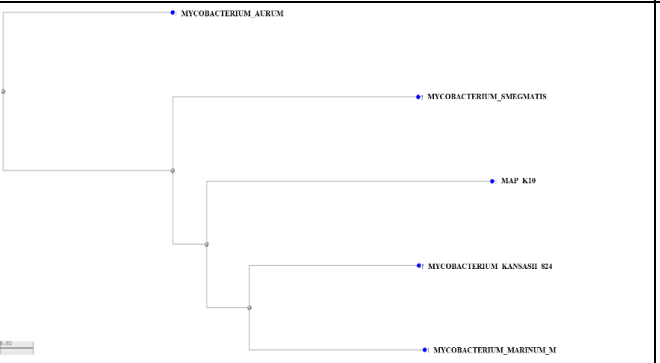
From Fig. 17 below, this study provided us with a brief understanding of mycobacterial carotenoid operon. It was predictable that *M. marinum* and *M. kansasii* share the same ancestors for most of the proteins in their pigment synthesis pathway. Likewise, *M. aurum* and *M. smegmatis* shared a close history of pigment genes evolution. Carotenoid proteins in MAP K10 appeared to share the same ancestor with, and more closely related to consecutive pigment producers, *M. marinum* and *M. kansasii*, than *M. smegmatis .*



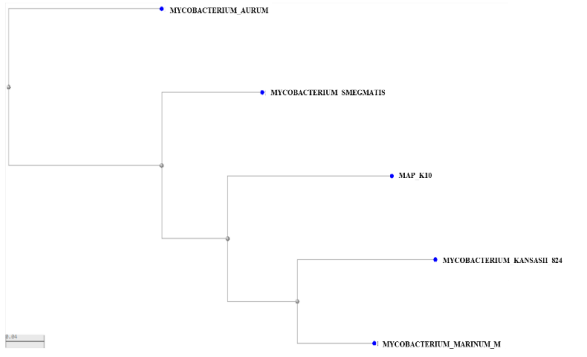
CrtE



CrtB

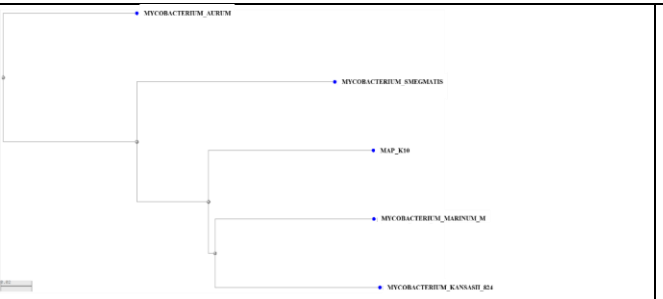
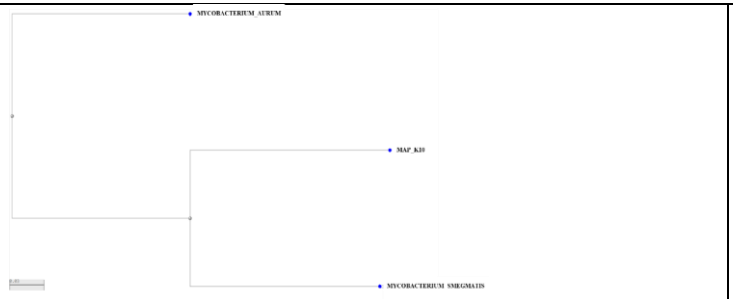


CrtI



CrtYc

Figure 17: Phylogenetic trees of proteins in representative mycobacterial carotenoid operons. The root represents the ancestor and the tips represent the descendants. When a speciation event happens, the ancestor splits into two or more branches. The longer the branch, the more changes occur (The bar provided for scale).

		
CrtYd	CrtU	

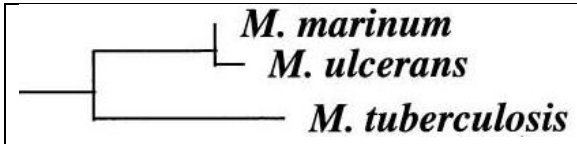


Figure 18: Adapted from (Tønjum T (1998)) which reveals a close relation between *M. marinum* and *M. ulcerans* and a shared ancestor between *M. tuberculosis* and the two mycobacterial species.

4.2.3. MAP K10 carotenoid operon

BLAST result of carotenoid operon from a well-studied species *M. aurum* against MAP K10 revealed 72% identities, which only covers the core of the locus (Fig. 19).

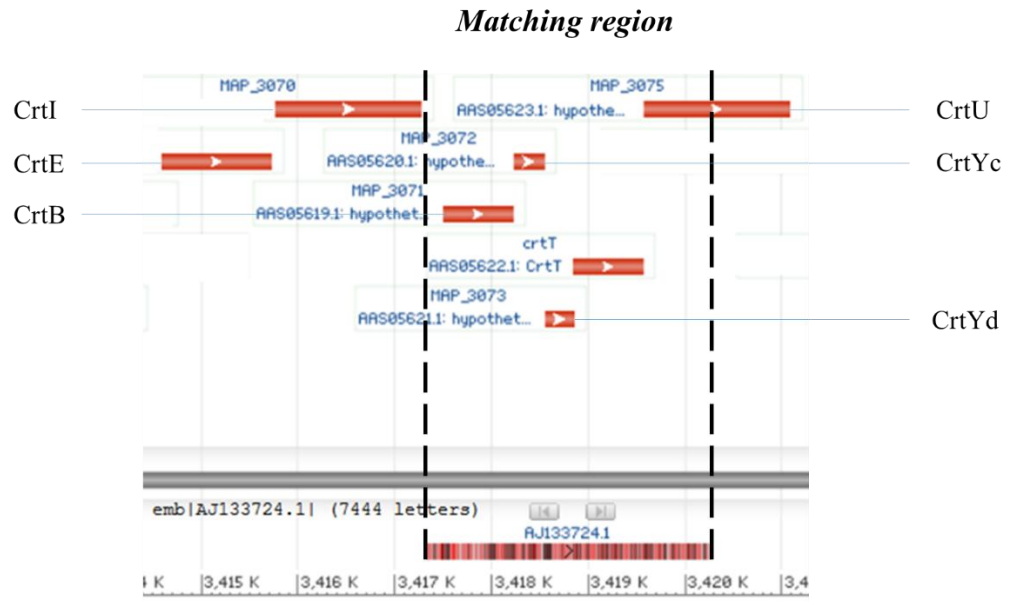


Figure 19: BLAST result of carotenoid operon search on MAP K10 and presumptive enzymatic pigment proteins

Which agreed with phylogenetic result in previous section showing a distant relationship between *M. aurum* and MAP K10. Therefore, to understand more about the functions of different proteins in MAP K10 locus, we conducted further investigate from available data on NCBI (Table 6).

Presumptive protein	Coded in MAP K10	Identities (%)	Bacteria strain	Aligned protein
CrtE	MAP3070	100	*Mycobacterium avium* subsp. *paratuberculosis* MAP4	Geranylgeranyl pyrophosphate synthetase
		99	*Mycobacterium avium*	Polyprenyl synthetase*
CrtI	MAP3072	100	*Mycobacterium avium*	Phytoene dehydrogenase
		99	*Mycobacterium avium* subsp. *paratuberculosis* 10-4404	Phytoene dehydrogenase
CrtB	MAP3071	99	*Mycobacterium avium* subsp. *paratuberculosis* S397	Phytoene/squalene synthetase
		99	*Mycobacterium avium*	Phytoene synthase
CrtYc	MAP3073	100	*Mycobacterium avium* subsp. *paratuberculosis* 10-4404	Lycopene cyclase
		99	*Mycobacterium avium* 11-0986	Lycopene cyclase

CrtYd	MAP3074	100	*Mycobacterium avium* complex (MAC)	Lycopene cyclase
		99	*Mycobacterium avium* subsp. *hominissuis* TH135	Lycopene cyclase
CrtU	MAP3075	100	*Mycobacterium avium*	Isorenieratene synthase
		99	*Mycobacterium avium* subsp. *paratuberculosis* S397	Isorenieratene synthase

Table 6: Carotenoid biosynthesis protein orthologs in MAP K10 against different mycobacteria

(*: Belongs to Geranylgeranyl pyrophosphate synthetase family, which catalyses a 1'4-condensation between 5 carbon isopreneunits)

The search result showed that hyphothetical proteins in the found locus in MAP K10 matched enzymes synthesising carotenoids in other mycobacteria. Closest alignment for MAP K10 carotenoid biosynthesis proteins came from *M. avium*.

4.3. Discussion

From 4.2.1. *M. kansasii* and *M. marinum* are photochromogenic bacteria which produce only beta-carotene, hence the gene sequence coding for beta-carotene desaturase or isoneriatene synthase was not found in their genome. The protein that responsible for synthesis of isorenieratene however is found in *M. aurum* and *M. smegmatis*, which may explain the presence of eschscholtzxanthin-like substance in *M. aurum*'s pigment and suggests a similar pigmentation product in *M. smegmatis. M. smegmatis*, surprisingly, has its *crtE* sequence not included in the locus but upstream 1.8 Mb (Roberta Provvedi et la 2008).

The high level of sequence similarities in our search (Fig. 16) suggested that proteins in the carotenoid operon are highly conserved in mycobacteria, be it chromogenic or not. This could mean that pigmentation ability is not resident solely in chromogenic mycobacteria but non-chromogens as well. In other words, non-chromogens are capable of produce carotenoids as long as the conditions allow. Nevertheless, we do not know yet of the specific circumstances that could bring about such transformation. In chromogens, the triggers can be light, which creates oxidative stress in the environment (REF). (REF) has shown that *M. smegmatis* was able to produce pigment in acidic condition, and this study had successfully induced pigment in MAP K10 in the same setting (Section 3). As a result, we prompted to believe that carotenoid pigments in this case play a protective function in induced species like what pigments do to chromogenic ones.

From 4.2.2., the fact that *M. marinum* and *M. kansasii* cluster tends to locate towards the right side of the map indicates that they have been through more evolutionary changes compared to the rest of mycobacteria in this particular search. From the results, this study was prompted to believe that carotenoid locus presents in a majority of mycobacteria, and only in recent evolutionary history, some special events happened and turned on permanently the trigger for consecutive pigment production in

chromogenic mycobacteria. Which might also explain why the gene *crtU* is found in *M. aurum* and *M. smegmatis* but not in *M. marinum* and *M. kansasii*, because over time, mycobacteria leave out redundant genes and only retain necessary ones to produce essential carotenoid products fitted to their needs. Similarly, a study by TP Stinear (2008) reported a shared 300 orthologs in *M. tuberculosis* and *M. marinum*, and *M. tuberculosis* seemed to evolve before and in a different way than *M. marinum*(Fig. 18) (Tønjum T (1998)), but the former mycobacteria is lacked carotenoids and has to utilise carotenoids from their hosts (Scherzinger D (2010)).

The fact that MAP share a high level of homology with *M. avium* carotenoid biosynthesis proteins might suggest that either the genes in MAP K10 are from *M. avium* or vice versa, or the two species share the same ancestor for the genes. Comparison to the list of possible lateral gene transfer in MAP by Marri (2006), genes coding for carotenoid operon were not found. Consequently, we speculated that the genes had been vertically transferred to MAP K10, which is consistent with the result reported in Section 4.2.1.. However, remarks were made that carotenoid biosynthesis is an ancient process, and the whole operon could have been laterally transferred between phyla to Actinobacteria, since there is a close relationship between Actinobacteria, Archaea and Bacteroidetes (JL Klassen 2010).

The fact that carotenoid operon is highly conserved in mycobacteria indicates a significant role to pigmentation, to both chromogens and non-chromogens. Another highlight is that the two consecutive pigment producers in this study, *M. marinum* and *M. kansasii*, appeared to share the same ancestor with MAP K10 for pigment genes. Further investigation into this may reveal cattle strain physiological diversity, and pathogenesis.

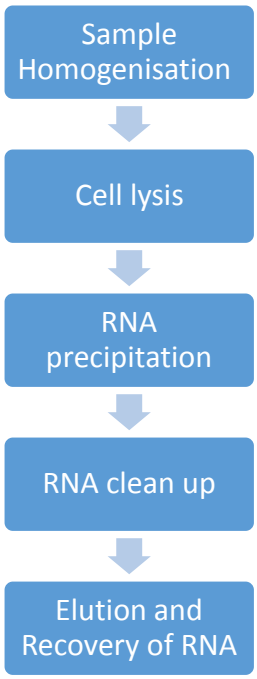
5. Evaluation of RNA extraction methods

5.1. Introduction

In this study, common and easy-to-use methods of total RNA extraction were chosen and the quality of result RNA would be used for evaluation. Up to date, methods using phenol as lysis reagent are widely applied such as TRIzol and Phenol:Chloroform:Isoamyl Alcohol reagent (REF). Commercial kits from Qiagen and Promega are also reported to be reliable in most RNA purification process (REF). These methods are believed to be time and cost efficient, and at the same time, able to yield high quality of RNA from culture cells.

Purification of pure and intact RNA requires four important steps: effective cells lysis, inactivation of nucleoproteins, denaturing of endogenous ribonuclease (RNase) and elimination of DNA and proteins. It is because the presence of RNases means RNA will be degraded along the process, thus RNA recovery rate and integrity will be lower respectively. Owing to the nature of mycobacterial cell wall, this study placed a strong focus on the step of cell lysis and denaturing process of RNases (Bustin, Benes, Nolan, and Pfaffl (2005) and (Fleige & Pfaffl, 2006)).

Flowchart of overall total RNA extraction process applied in this study


Sample Homogenisation
Cell lysis
RNA precipitation
RNA clean up
Elution and Recovery of RNA

5.1.1. Phenol based methods

Phenol is a weak acid, which dissociate negligible amount of hydrogen ion, and thus not able to solubilise completely in water. In consequence, by adding phenol into a liquid sample and using appropriate centrifugal force, there would be a formation of different phases – an aqueous water phase above, an interphase in between, and an organic phenol phase on the bottom, as phenol is denser than water.

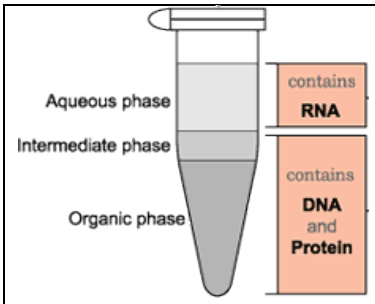


Figure 20: The homogenate is separated into 3 phases: an aqueous phase, an organic phase and an intermediate phase. In the aqueous phase, only RNA is present, and DNA and protein are present in the intermediate phase and the lower phase.

In nucleic acids extraction, owing to their negative phosphate backbone, DNA and RNA are more soluble in water than in phenol, which makes them remain in the aqueous phase. Next, by adjusting pH of phenol solution, either DNA or RNA can be obtained. At a more acidic pH, approximately 5 – 4.5, DNA – more negatively charged than RNA, being double stranded – is made soluble in phenol and thus retained in the interphase (Fig. 20).

In cytoplasmic environment where water is the dominant solvent, proteins are usually folded in a manner such that hydrophobic part forms the core inside most proteins while the hydrophilic part forms the outer part. When the solvent is switched to phenol, a less polar and more hydrophobic one, the folding of cellular proteins changes accordingly to be more soluble in

the new solvent. Therefore, majority of proteins is denatured, and proteins remain exclusively in the organic phase.

In addition, to further improve phenol extraction performance, chloroform is often added to phenol. Chloroform help to prevent the loss of poly(A)+ mRNA into the lower phase, lower the generation of insoluble RNA protein complexes in the upper phase, boost RNA yield, and maximise total RNA gained. (Kedzierzki W and Porter JC 1991, Palmiter RD 1974, Perry RP, La Torre J, Kelley DE, Greenberg JR 1972)

Many of studies today still employ this phenol approach owing to the fact that RNase would be dissolved in phenol and thus minimise the risk of RNA degradation. Below are two phenol based methods adapted to use for RNA extraction in this study.

Modified TRIzol method

Firstly introduced by (Chomczynski P and Sacchi N (1987)) TRIzol is a mono-phase solution of phenol and guanidinium isothiocyanate that stimulate both process of cell lysis and denaturing of RNase proteins. This method of RNA isolation is effective for small RNAs purification. (TRIMay2014)(Wu, LC (1997) Isolation and Long-Term Storage of Proteins from Tissues and Cells Using TRIzol Reagent) However, RNA pellets are reported to be difficult to solubilise in some cases (Rio, 2010). This study took note of such a drawback and provided some modification to the original TRIzol method to solve the problem.

Modified Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) method

Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) solution is a mixture of 1:1 ratio phenol:chloroform, at low pH, this ratio ensures a maximal retrieval of total RNA in the water phase but also some remains of DNA . While a 5:1 ratio can yield no DNA remains, but it is at the cost of losing some RNAs (Kedzierzki W and Porter JC 1991). Isoamyl alcohol is

included in a ratio of 24 : 1 of chloroform to isoamyl alcohol to avoid foaming. (REF?)

This method was developed to aim for total RNAs recovery and modified to effectively disrupt mycobacterial cell wall.

5.1.2. Commercial RNA purification kits

Other than the phenol based methods described above, there are other methods to purify RNA that do not use phase separation approach, but instead utilising silica-matrix-membrane column to bind and retain RNA (Esser K-H, Marx WH, Lisowsky T.). The recipes for buffers used are varied from kits to kits by different brands, nevertheless, the three main critical steps for a spin-column RNA extraction experiment are as follows: cell lysis, RNA binding to the matrix, and lastly, elution. (Kojima K, Ozawa S.)

There is a great diversity for lysis methods, but overall, it usually consists of a lysis buffer with chaotropic salt in high concentration. Proteins, and the main target is RNase, in view of chaotropes, are denatured, as the salt breaks chemical bonds and disrupt their tertiary structure. The kits used in this study are from QIAGEN and Promega, both make use of guanidine isothiocycanate as chaotropic salt, to aid in the binding of RNAs to the silica matrix membrane. However, even chaotropes cannot permanent disrupt all RNase and some proteins are able to renature, folding back to their functional shape. Therefore, ß-mercaptoethanol (BME) is applied to the buffer to effectively denature RNAses in samples. BME will deactivate disulfide bonds in RNases, thus consequently inactivate the proteins, to limit the process of RNA degradation (Chirgwin, J.M. et al . (1979).

The silica matrix membrane is often made to suitable for selective RNA binding. There are several types of silica materials: glass particles and hydrated matrix (Padhye VV, York C, Burkiewiez A. Nucleic acid

purification on silica gel and glass mixture, Woodard DL, Howard AJ, Down JA. Process for purifying DNA on hydrated silica.). Sodium in the matrix functions as a cation bridge to capture negatively charged nucleic acids (Esser K-H, Marx WH, Lisowsky T. MaxXbond: first regeneration system for DNA binding silica matrices.). As nucleic acids have been secured in the silica membrane, repeated and extensive washing would take place to wash away all contaminants and unwanted molecules such as proteins. Subsequent elution step using solutions with low ionic strength such as TE buffer or RNase free water will release RNA out of the membrane (Tan, S. C., & Yiap, B. C. 2009)

Column-based commercial kits provided a straightforward approach for RNA purification. However, they are costly, which possesses a real problem to less-funded laboratories. Furthermore, problems of exogenous RNase are still a main concern for this method (REF?), which call for extra cautions when handling samples during RNA extraction procedure.

5.1.3. Cell lysis for mycobacterial samples

Mycobacteria tend to clump which further complicates the quest of disrupting their cell wall to release cellular content. Therefore to ensure effective RNA purification experiment, bacteria in samples used for extraction must be dispersed to avoid clumping and allow effective cell lysis.

Owing to the fact that incomplete cell lysis in mycobacterial RNA isolation would result in low yield, from literature, after sample preparation, mycobacteria were often undergone an initial lysis treatment before proceeding to further treatment with selective reagents. In this study, we choose the method of bead beating as it is cost effective, and as the beads are newly sterile, little chance that they are contaminated with RNases, so to minimise RNA degradation. In comparison, sonicating method usually operates in a less controlled environment, and heat treatment to burst cells may made RNA degraded, as it is heat sensitive.

5.1.4. Removal of genomic DNA

High quality total RNA extracted should be free from proteins and genomic DNA (Bustin, Benes, Nolan, and Pfaffl (2005) and (Fleige & Pfaffl, 2006)), because such contaminations can affect downstream analysis and application.

As a result, careful approach should be taken to remove chromosomal DNA and assess the quality of RNA before ensuing the next step.

5.2. Results

Culture of MAP is difficult and time-consuming, so in this study we utilised a fast growing mycobacterial species, *M. smegmatis*, as a model before applying the result on MAP.

5.2.1. Cell lysis

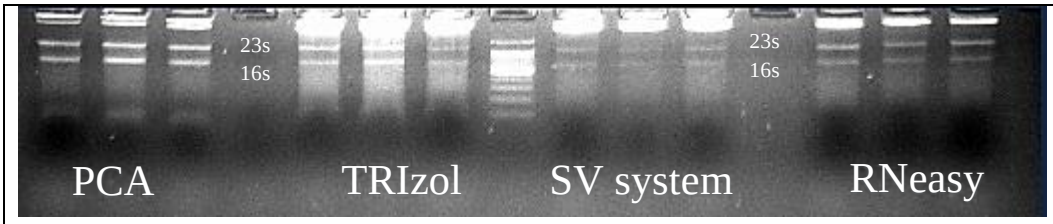


Figure 21: Gel electrophoresis visualization of M. smegmatis samples after complete cell lysis by different methods.

All 4 methods were able to give apparent and clear 23S and 16S bands. Nonetheless, without RNA clean up step, samples were contaminated with chromosomal DNA, which appeared to be found more in method using TRIzol reagent than other methods.

5.2.2. DNA digestion

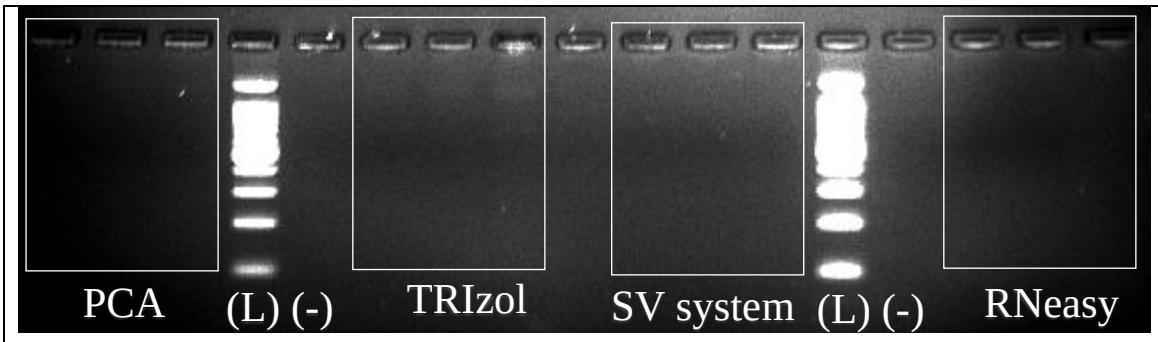
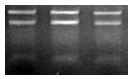
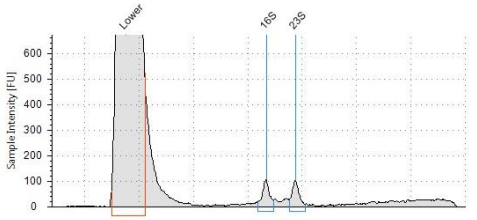
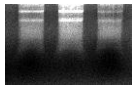
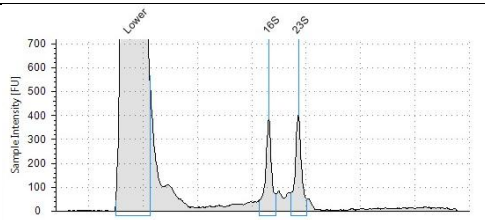
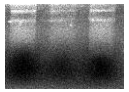
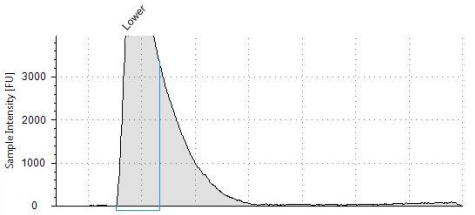


Figure 22: Gel electrophoresis visualization of M. smegmatis samples after RNA clean up

After RNA clean up treatment, residues of chromosomal DNA were not seen on gel. Although yield of RNA collected from different methods was too little to be visualised, there were dim traces of 23S and 16S bands from TRIzol treatment.

5.2.3. RNA quality assessment

Fig. 23 summarised result of RNA isolated using different methods. A260/A280 ratios were high and of acceptable range, but A260/A230 ratios were low and indicated that samples were contaminated with salts and phenols. Methods using PCA and RNeasy Mini Kit gave total RNAs of low concentration, while other two methods brought about total RNA amount of acceptable value, approximately 8-10 ng/ul. Among the four methods tested in this study, RIN from TRIzol method was the highest (8.4) while the lowest RIN (1.0) was from method employing SV system.

Method	Visualisation	A260/A280	A260/A230	Concentration (ng/uL)	RIN	Electropherogram
PCA		1.46	0.40	2.31	8.2	
TRIzol		1.57	0.63	8.14	8.4	
SV System		1.64	0.50	13.0	1.0	

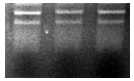
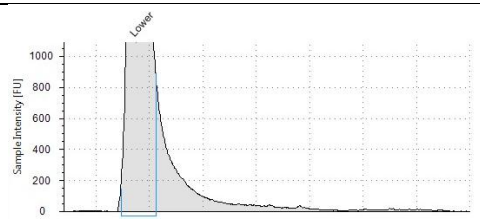
RNeasy		1.45	0.37	4.72	2.6	

Figure 23: Summary of RNA isolation assessment using different methods. Two distinct bands at 23S and 16S show that RNAs obtained was intact. The higher the RIN, the better the quality of RNA.

5.3. Discussion

In this unique study we have put together four different RNA extraction methods, based on two separate lysing and isolating techniques, to verify their efficiency in purifying total RNA from representative mycobacterial species, *M. smegmatis.*

While obtaining pure and intact RNA is of critical importance for any RNA based studies, such as gene expression, microarray experiments, or RNA sequencing (Fleige and Pfaffl 2006, Copois 2007), getting RNA out of mycobacterial cells is a difficult task due to their waxy cell wall. On one hand, the author had demonstrated that the four chosen methods were effective in breaking cell membrane and able to result in high integrity RNA, illustrated by two clear distinctive 23S and 16S bands shown in Fig 21. On the other hand, the reading for A260/A280 and A260/A230 ratios was not of ideal. Nevertheless, as stated by (Imbeaud Graudens Boulanger Barlet 2005), such ratios are informative in terms of RNA purity only, not reflecting the whole RNA quality. In addition, although RNA obtained from all methods was contaminated with peptides, phenols, aromatic compounds or carbohydrates owing to low A260/A230 ratio, and normally pure RNA of this ratio is expected to be close to 2.0, there has not yet been a clear understanding of what acceptable lower limit for A260/A230 ratio, and a low ratio does not equivalent to a limit to downstream applications.

Since interpreting from A260/A280 and A260/A230 ratios did not provide a reliable understanding of isolated RNA integrity and quality, quality control by using the RNA Integrity Number (RIN) has gradually become a popular method in the literature. RIN algorithm

classified total RNA from 1 to 10, the higher the more intact RNA is. In this light, comparison between samples can easily be made. Normally, total RNA with RIN from 7-8 and above should perform well in most downstream applications (Mueller, O., Lightfoot, S., & Schroeder, A. (2004)). In this particular study, liquid phase separation appeared to work better than their opposing methods, both PCA and TRIzol displayed a RIN above 8.0. Low RINs usually mean that extracted RNA are degraded, which can be possible as methods for solid phase separation of are susceptible to contamination of exogenous RNases (REF?).

In summary, by comparing the performance different RNA extraction methods and kits, this study have selected a preferred approach for isolating total RNA from mycobacteria, modified TRIzol method. Using TRIzol allowed a faster, more robust, and reliable total RNA for further RNA analysis.

6. Differential expression of proteins in MAP K10 carotenoid biosynthesis operon upon acidic stress

6.1. Introduction

As discussed previously in section 3, upon stimuli from environmental stressors, mycobacteria have both their cell wall and their transcriptional regulation activities as defence systems. Cossu (2012) reported the upregulation in MAP of several response genes under acid-nitrosative stress and macrophage conditions (Fig 27). Similarly, Wu (2007) also made a remark that there were 597 genes in MAP upregulated upon acidic stress. In the same study (Wu (2007)), the author also suggested that in time of stress, sigma factors play a significant role in counter-react the stimuli, and emphasised that *sigH* involved in MAP's oxidative and heat shock response like in *M. tuberculosis* (Raman S 2001). Other sigma factors have not been well-studied in MAP. Nonetheless, there are documents in *M. tuberculosis* regarding involvement of *sigD* and *sigG* in persistent infection response, and *sigI* in cold shock reaction (Manganelli R 199). In *M. smegmatis*, *sigF* was reported to play a significant role in stress response mechanism, involving in carotenoid biosynthesis, acidic stress, oxidative stress, and heat shock (Susanne Gebhard (2008), Provvedi et al., 2008, Anja Humpel (2010), Singh AK (2015)).

In this study, we hyposthesised that pigmentation observed in MAP K10 upon acidic stress was due to the transcriptional activities in the *crt* operon, with involvement of *sigF* which had seen in *M. smegmatis*. To testify our hyposthesis, quantitative measurement of *crt* and *sigF* gene expression under both neutral and acidic conditions was conducted.

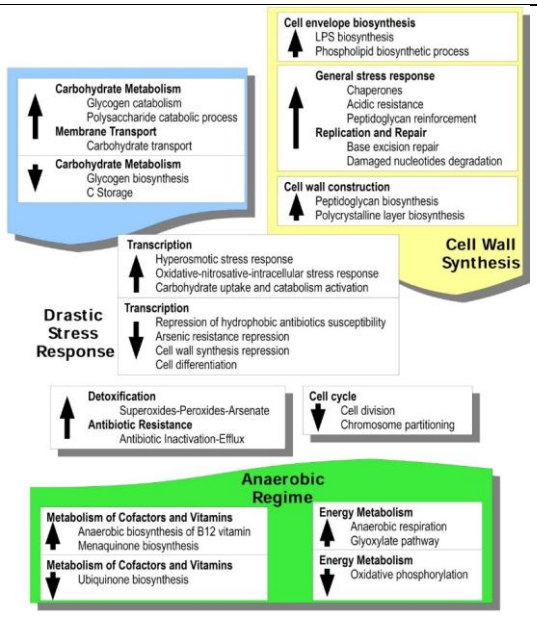


Figure 27: Schematic diagram of MAP transcriptional response during acid-nitrosative multistress. Differentially expressed genes during multi-stress were grouped based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) classification and sorted by function. Up arrows indicate an up-regulation of genes to the related metabolism whereas down arrows indicate a down-regulation.

6.2. Results

6.2.1. RNA quality control of Control and Pigment MAP K10

Sample	RIN	Concentration (ng μl^{-1})
Control MAP	RNA concentration outside recommended range for RINe	2.04
Pigment MAP	8.0	4.48

Table 7: MAP K10 extracted RNA integrity assessment by RIN

Sample	Concentration (ng μl^{-1})
Control MAP	945
Pigment MAP	1050

Table 8: Concentration of cDNA obtained from control and pigment MAP K10

Since the RNA collected from Control MAP samples was below detectable range for the software analysis, RIN could not be obtained (Table 7). Nevertheless, as there were no traces of DNA contamination, cDNA synthesis was still applied. Result was an increase in concentration (Table 8) and the cDNA samples displayed high integrity and free of contamination, with A260/A280 and A260/A230 ratios were approximately 2.00 (Data not shown).

6.2.2. Amplification efficiencies for target and reference genes

We used 16S rRNA as reference gene, and *crt* genes with *sigF* as target genes. Assessment for 16S primers displayed an amplification efficiency of 112% ($E=10^{(-1/slope)}$). (Fig. 28)

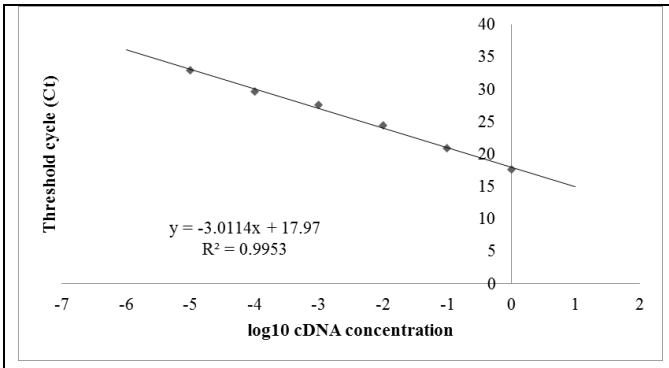


Figure 28: Validation of 16S as a reference gene using standard curve plotting Ct values against dilution log.

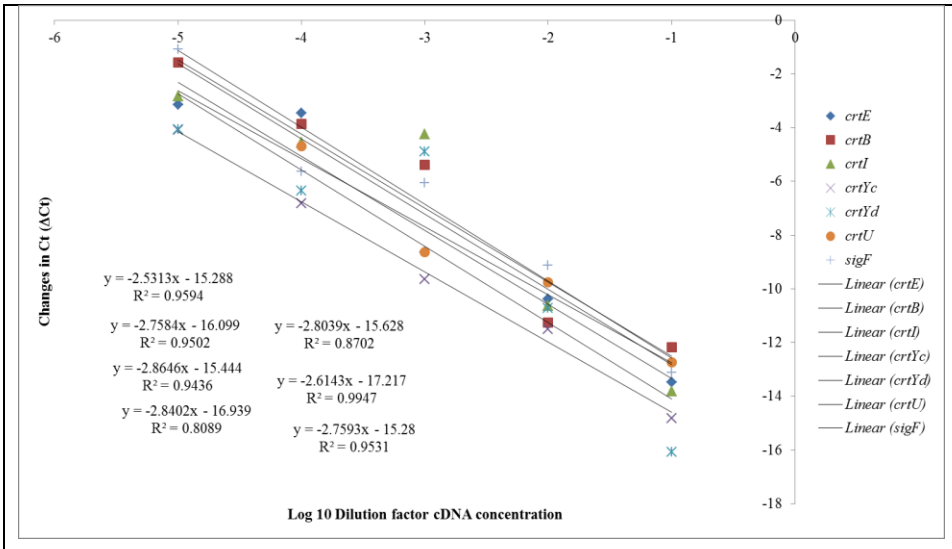


Figure 29: Relative efficiency plots of target *crt* genes against reference gene (16S), in which average ΔCt between them for each dilution were collected and a standard curve was generated.

We then validate the amplification efficiency between reference gene and target genes (Fig 29). A consistent decline was observed, which indicated variation between efficiencies for reference and target genes. However, a consistent efficiency between target genes was noted.

6.2.3. Differential expression of *crt* genes and *sigF* in MAP K10 carotenoid biosynthesis pathway

RT-qPCR readings showed a relative high up-regulation of *crt* genes with the exceptions of *crtI* and *crtU*, coding for phytoene desaturase and beta-carotene desaturase respectively, which displayed no significant fold differences. *sigF* revealed an increase in expression of 3 folds.

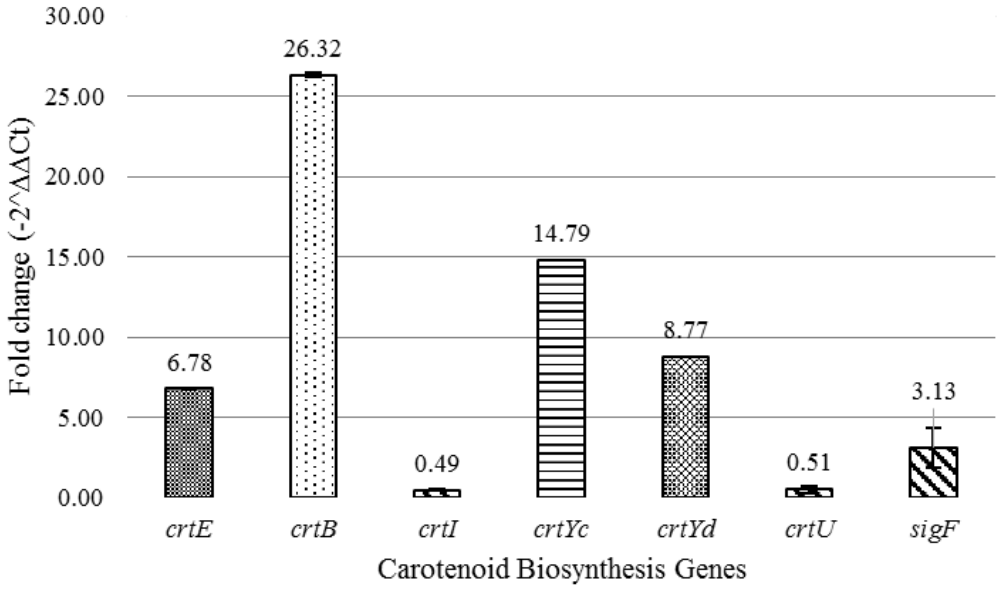


Figure 30: Differential expression of *crt* genes in MAP K10 upon acidic stress, Ct values between control and pigmented samples

were significantly different (p value < 0.05).

6.3. Discussion

MAP was shown to respond to external stress by regulating its genes. A relative up-regulating trend observed in *crt* genes suggested a role this carotenoid biosynthesis operon playing in the defense mechanism of MAP K10 in response to acidic stress. Together with *crt* genes, other genes such as acidic stress and heat shock response genes, the general stress response genes *htpX*, *clpX*, *relA,* and mycolic acid synthesis genes; they were all upregulated upon acidic stress (Wu 2007). This indicates a relationship between acidic stress and heat shock response mechanism. Furthermore, the importance of mycobacterial cell wall in defense mechanism was once again highlighted. However, in general, in acidic condition, the number of downregulated genes in MAP outnumbered upregulated ones (Wu 2007). As a result, those genes that were upregulated in MAP might play an important role in the organism's response to acidity challenge.

Sigma factor F is known to involve in stress responses in *Mycobacteria* spp. In *M. tuberculosis, sigF* expression was upregulated upon oxidative stress, low temperature and oxygen, and starvation (Manganelli et al., 1999; Betts et al., 2002). In *M.* smegmatis, apart from being responsible for stress response, *sigF* also involved in carotenoid biosynthesis (Provvedi R 2008). Consistent with these findings, we found that the expression of *sigF* in MAP was upregulated with acidity challenge. Increase in the amount of *sigF* was not as great as other genes analysed in this study, nonetheless, as sigma factors were known to be regulated by anti-sigma factors, such increase was predictable. The upregulation of *sigF* together with *crtE, crtB,* and *crtYcd* from our result suggested a link between ccarotenoid biosynthesis in MAP and *sigF*.

However, further investigations are needed to confirm the observation. Moreover, Riccardo Manganelli (2004) noted in their study about the different roles *sigF* involve in *M. bovis* and *M. tuberculosis,* although the two species are closely related. Therefore, functions of *sigF* in MAP response to acidic stress and pigmentation remain opened to discussion.

Another attempt to link *sigF* and *crt* genes was done by Srividya Kumar (2015). They reported an increase in *crtI* and *sigF* in *M. smegmatis* when growing the bacteria in glucose oriented media. It is suggested that glucose presence in the media might induce hypoxic challenge and stimulate the cells (Srividya Kumar 2015, Phong et al., 2013). Our result found an increase of 0. fold change in *crtI* which codes for Phytoene desaturase, compared to more than 2 fold increase in Srividya Kumar (2015)'s study. This comparison suggested that the increase of *crtI* upon acidic stress in this study was not accountable. Nonetheless, *crtI* anomalous did not change the fact that other *crt* genes were significantly upregulated, which implied that pigmentation in MAP might due to transcriptional regulation in response to external stress.

High integrity RNAs are crucial for RT-qPCR, but we should not underestimate the problems of PCR inhibitors and exogenous contamination (Wilson, 1997; Rossen et al., 1992). Besides, (Schoor et al., 2003) noted that partially degraded and low quality RNAs could still result in a successful RT-qPCR if carefully handled. Other factors which should also be taken into considerations for an efficient RT-qPCR reaction are length of the amplified sequence (approximately 50 – 200 bp), secondary structure and primer efficiency. As a result, amplification efficiency validation stands a must for any RT-qPCR reactions (MIQE guideline). In this study,

although the reference gene displayed a high amplification efficiency (E=112%), the unequal between that and target genes presenting in section 6.3.2. can jeopardise the results for gene expression. One possible reason is that the relative abundance of target genes in tested samples was low which resulted in high Ct reading, further illustrating the inconsistent variation between the reference and target genes ΔCt. Due to time constraint, this study did not manage to completely optimise reaction and primers efficiency.

7. Conclusion

This study demonstrated that pigmentation is not an exclusive characteristic of MAP sheep type, but upon stressed conditions such as acidity challenge, MAP cattle strains were as well observed to produce pigment. This finding together with the newly found pigmented MAP cattle strain clinical isolates in Spain by C Leao (2016) further illustrated the dynamic of MAP diversity.

By evaluating four popular RNA isolation methods, we reemphasized the need for finding and standardizing a method for effective cell lysis, especially on challenging organism like mycobacteria. From our comprehensive results, the modified method based on TRIzol reagent appeared to meet the criteria set by Flaff (year), and was of recommendation for any other studies employing RNA extraction step.

Both *crt* and *sigF* genes were found to be highly conserved among *Mycobacteria* spp. This finding suggested that pigmentation ability was available in a majority if not all mycobacteria. Therefore, this could serve as a call for more investigation into the regulation and expression of carotenoid in MAP in particular and other mycobacteria in general. There is also a need to confirm and establish a link between carotenoid biosynthesis and *sigF* regulation in MAP. The connection between acidity challenge and the expression of *crt* operon was not made clear in this study. However, our finding could serve as a foundation towards better understanding of the *crt* gene expression in MAP.

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