Steroid metabolism in Actinomycetales and the relation to pathogenicity

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Abstract

Steroids are group of molecules with a specific 4-ring structure which are found in Eukaryotic organisms. Steroids have various functions; they can be hormonal compounds, but can also, for example, modulate cell wall fluidity/rigidity.

The order of Actinomycetales is known to contain species of bacteria which thrive in a broad range of environments. Due to this property the species within this order have developed the ability to degrade a variety of organic compounds, including m-toluate, polycyclic aromatic hydrocarbons and polychlorinated biphenyls and steroids.

Mycobacterium tuberculosis is the causative agent of tuberculosis worldwide and is known to be able to catabolize cholesterol, a steroid. M. tuberculosis infects an estimated third of the world population and causes three million deaths annually, making it a very successful human pathogen. The hallmark property of M. tuberculosis is its ability to cause a persistent lung infection involving multiplication inside phagosomes of alveolar macrophages following phagocytosis. Multiple studies have shown that various genes which are implicated in cholesterol catabolism are also essential to establishing persistent infection in murine models. These findings suggest that cholesterol catabolism is required for persistent infection. Although, it remains unknown how cholesterol catabolism plays a role in vivo infection models it is tempting to speculate that M. tuberculosis requires cholesterol as a carbon and energy source during survival and growth in the macrophage phagosome.

In this work various genes which are known to be implicated in cholesterol catabolism and pathogenicity in various actinomycetales are reviewed and discussed. From the conducted research it seems that there are multiple factors accounting for the pathogenicity of M. tuberculosis. The mycobacterial cell wall contains mannosylated lipoarabinomannan, which is likely the main factor responsible for inhibition of maturation of the phagosome. Suspectedly it prevents the fusion of the phagosome with lysosomes which would otherwise form a phagolysosome. Additionally, the findings include that the production of a diterpenoid seems to assist in prevention of acidification of the phagosome. Moreover, a deletion of genes implicated in cholesterol metabolism (e.g.: mce4, kshA, kshB, igr, kstD and hsaABCD) seems to abolish the ability of M. tuberculosis to develop a persistent infection. Nevertheless, this does not seem to be true for the deletion of hsd (cholesterol oxidase), as hsd does not seem to be required for growth in macrophages.

This leads to the conclusion that multiple virulence factors account for the pathogenicity of M. tuberculosis and related bacteria. Furthermore, the utilization of cholesterol in M. tuberculosis is still not fully understood and this merits further research which would be of interest to the biotechnological and pharmaceutical industries.
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1. Introduction
Steroids are a group of polycyclic hydrocarbons which are generally only found in plants, animals and fungi. Steroids have various functions, some are precursors for biologically important compounds, and some are hormones. (e.g., in humans, cholesterol - a steroid - can react to vitamin D - a hormone - under influence of UVB radiation.) Others modulate cell membrane fluidity.

1.1. Steroid structure and general biological functions
Steroids have a specific 4 ring structure consisting out of 17 carbon atoms or a structure derived therefrom by one or more bond scissions, ring expansions or ring contractions. The basic ring structure includes 3 linked 6-carbon rings and a linked 5-carbon ring. These rings are referred to as the A, B and C rings for the 6-carbon rings and D for the 5-carbon ring (figure 1A). In most cases a methyl group is present at C-10 and C-13 and an alkyl side chain might be present at C17 (figure 1B).

Sterols or steroid alcohol are steroids which have a hydroxyl group present at C-3 (e.g.: cholesterol, figure 1B), and steroidal hormones occasionally have a carbonyl group at C-3 (e.g.: progesterone).

Steroids have various functions in animals, mainly as hormones (e.g.: vitamin-D$_3$, testosterone, progesterone and cortisol). Others are usually precursors to various hormones and/or cover a different function, such as cholesterol.

Cholesterol inserts in mammalian cell membranes to decrease fluidity / increase rigidity of the membrane. This happens due to the polar interactions between the hydroxyl-group of the cholesterol with the phosphate group of the phospholipids. This interaction allows the steroid core to insert in the membrane. Additionally, non-polar interactions of the rigid steroid core of cholesterol with the membrane phospholipid fatty-acid chains increases membrane packing, and thus increase membrane rigidity.
2. The role of steroid metabolism in pathogen virulence

Rhodococci are a genus of aerobic, non-motile, Gram-positive, G+C rich bacteria within the order Actinomycetales which includes mycobacteria. The order of Actinomycetales is known to contain species of bacteria which thrive in a broad range of environments (1). Due to this property the species within this order have developed the ability to degrade a variety of organic compounds, including m-toluate, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and steroids, especially Rhodococci seem to be very versatile (2). This gives members of the Actinomycetales order the potential to play a role in bioremediation of contaminated soils, or in biotechnology with industrial processing of organic compounds (3).

Most species in the Actinomycetales order are not pathogenic. However, Rhodococcus equi (R. equi) is known to cause pneumonia in foals by a mechanism similar to the way the closely related Mycobacterium tuberculosis (M. tuberculosis) and Mycobacterium bovis (M. bovis) causes tuberculosis in humans and cattle (4) (5).

2.1. Cholesterol metabolism in Mycobacterium tuberculosis

Of particular interest to both the biotechnological and pharmaceutical industry are the genes responsible for the catabolism of steroids found in R. equi RHA1. Bioinformatic analyses revealed that 51 genes in R. equi RHA1 which are up regulated while cultured in presence of cholesterol are conserved within a gene cluster of 82 genes in M. tuberculosis H37Rv and M. bovis bacillus Calmette-Guérin (6). In this paragraph various genes implicated in cholesterol metabolism and virulence in M. tuberculosis will be discussed.

Recent research has shown that ABC-transporter mce4 (Mammalian Cell Entry) is responsible for cholesterol import in M. tuberculosis. Deletion of mce4 has shown that it resulted in a growth defect in vitro when cholesterol was used as the primary carbon source (7). Furthermore, this research has also shown that the ability of cholesterol uptake by mce4 and subsequent catabolism is essential for persistent respiratory murine infection (8). Orthologous mce4 loci have been identified in other species a well, including but not limited to: Mycobacterium bovis, Rhodococcus jostii and Nocardia farcinia (9) (10).

After import either the terminus of the substituent group on C-17 of the cholesterol steroid ring is oxidized by Cyp 125 (figure 2) (11) to a carboxylic acid group or the hydroxyl group on C-3 of the cholesterol steroid ring is oxidized to a carbonyl group by cholesterol oxidase (hsd) (figure 2) (11) (12). After these two oxidations (However, in M. tuberculosis degradation of the side chain and degradation of the steroid core may happen simultaneously.) which can occur in both orders, the substituent group on the C-17 of the cholesterol steroid ring is shortened in a fashion similar to β-oxidation in fatty acids. This process yields acetic acid and propionic acid which are both able to be processed in the carboxylic acid cycle, generating CO₂ and energy (13). The final product of this C-17 substituent group degradation is a C-17 keto-steroid.

Additionally, this research group has also identified two additional genes, kstD and kstD2 (3-KetoSTeroid Δ¹-Dehydrogenases) in Rhodococcus erythropolis. Activity of 3-ketosteroid Δ¹-dehydrogenase has been found in other bacteria as well, including Mycobacteria. This enzyme is essential in the opening of the steroid B-ring which enables further degradation (14) (15) (figure 2).
Research by Maurine D. Miner, et al. has shown that the iger (Intracellular GRowth) locus is essential to cholesterol metabolism (16). Their research has shown that in contrast to the WT strain, iger mutants of M. tuberculosis H37Rv are unable to grow using cholesterol as the sole carbon source. Moreover, additional experiments revealed that the iger mutants were unable to grow in presence of cholesterol, even with addition of a preferred carbon source, such as dextrose/glycerol. The additional deletion of mce4 yielded the iger Δmce4 mutant strain, which partially compensated for the growth defect in cholesterol containing media. These results are consistent with a model in which attenuation of the iger strain results from the inability to degrade cholesterol along with the subsequent build-up of toxic intermediates. However, the exact function of the iger locus remains unknown. Because of this, and the only partial compensation by deletion of mce4 the attenuation of the iger mutant is only partially attributable to toxicity.

R. van der Geize, et al. have shown that ksha and kshb (3-KetoSteroid 9α-Hydroxylase) are the components of a two-component oxygenase which is involved in cholesterol catabolism (17) (figure 2). The deletion of ksha or kshb yielded mutants which are unable to survive in in vivo murine infection models of BALBc mice and in γ-Interferon - activated macrophage growth models (18). Furthermore, the ksha and kshb mutants showed attenuated growth on cholesterol and some other sterols as a primary carbon source, but did not show decreased growth in presence of cholesterol, unlike the previously mentioned iger mutants (16). These results indicate that the growth attenuation of the ksha and kshb mutants on cholesterol as the primary carbon source is not due to toxicity caused by accumulation of toxic cholesterol metabolites. The kshb mutant, unlike the ksha mutant, also showed altered lipid metabolism due to changes in its cell wall, indicating that kshb is also implicated in lipid metabolism. The results of this study give further confirmation that cholesterol catabolism is a crucial factor for infection.

Figure 2: A schematic representation of the proposed cholesterol catabolic pathway in M. tuberculosis, M. bovis and R. equi based on identification of cholesterol degradation intermediates in the Gram-negative bacterium Sterolibacterium denitrificans by Mirjan Petrusma, et al. (11). Dashed arrows indicate multiple enzymatic steps. The depicted steroids are 5-cholestone-3β-ol (cholesterol), 25-cholestone-26-oic acid-3β-ol, 3β-cholestone-3-one, 4α-cholestone-26oic acid-3-one, 5-4-cholestone-24oic acid-3-one, 6-3-oxo-23,24-bisnorchole-4-ene-22-oic acid, 7-4-androstene-3,17-dione (AD), 8 1,4-androstadiene-3,17-dione (ADD), 9 9α-hydroxy-4-androstene-3,17-dione (9OHAD), 10 9α-hydroxy-1,4-androstadiene-3,17-dione (ADD), 11 3-hydroxy-9,10-secoandrost-1,3,5(10)-trien-9,17-dione (3-HSA), 12 3,4-dihydroxy-9,10-secoandrost-1,3,5(10)-trien-9,17-dione (3,4-DHSA), 13 4,5-9,10-diseco-3-hydroxy-5,9,17-triandrosta-1(10),23-diene-4-oic acid (4,9-DSHA), 14 9,17-dioxa-1,2,3,4,10,19-hexanorandrost-5-oic acid (DOHNA), 15 2-hydroxyhexa-2,4-diene-oic acid (HHD). The compound between brackets is chemically unstable. Hsd 3β-hydroxy steroid dehydrogenase, Cyp 125 cytochrome P450 CYP125, Ksd 3-ketosteroid dehydrogenase, KSH 3-ketosteroid 9α-hydroxylase, HsaAB 3-hydroxy-9,10-secoandrost-1,3,5(10)-trien-9,17-dione 4-hydroxylase, HsaC 3,4-dihydroxy-9,10-secoandrost-1,3,5(10)-trien-9,17-dione dioxygenase, HsaD 4,5-9,10-diseco-3-hydroxy-5,9,17-triandrosta-1(10),23-diene-4-oic acid hydrolase. The A, B, C and D rings are shown in compound 1. Mce4 mediated import is not shown. This image is adapted from M. Petrusma, et al. (11).
Carola Dresen, et al. have shown that the *HsaA* and *HsaB* genes form an oxygenase/reductase enzyme pair which is capable of effectively adding a hydroxyl group to the (now-aromatic) ring which used to be the A-ring of the steroid ring structure (figure 2) (19). This research group also reports that a Δ*HsaA* mutant is unable to grow on cholesterol and is unable to survive in macrophage phagosomes (19). They also mention that a Δ*HsaB* mutant is likely able to survive in macrophage phagosomes because the reduction can probably be catalysed by other enzymes.

The same research group has shown that *HsaC* which is an extradiol dioxygenase, is responsible for an iron-dependent cleaving of catechols (20). In other words it is responsible for catalysing the cleavage of the ring which used to be the A-ring of the steroid ring structure (figure 2). They also report that Δ*HsaC* mutants are unable to grow on cholesterol as a primary carbon source (20). Additionally immunocompromised SCID mice survived 50% longer when intravenously infected with 10⁵ CFU of a *M. tuberculosis* H37Rv Δ*HsaC* strain compared to the wild-type strain. Moreover, the Δ*HsaC* mutant disseminated more slowly to the spleens and persisted less successfully in the lungs in guinea pigs compared to the wild-type strain. These data corroborates the hypothesis that while cholesterol metabolism seems most important during the chronic stage of infection, the importance begins much earlier and may contribute to pathogen dissemination within the host (20).

Similarly, Jyothi Rengarajan, et al. have shown that *HsaD* is critical for intracellular survival of *M. tuberculosis*. This was discovered by transposon site hybridisation (TraSH) analysis, a microarray-based technique which identifies genes from a large pool of transposon mutants that are essential for growth of *M. tuberculosis* under different conditions (21). Research by Nathan A. Lack, et al. has shown that *HsaD* is responsible for cleaving off the residue, generated by opening of the A and B ring (figure 2) (22). The cleaved off residue effectively contains the carbon atoms which used to make up ring A of the original steroid core ring structure.

In contrast to the previously described genes, *hsd* (3β-hydroxysteroid dehydrogenase) which is responsible for oxidation of the C-3 hydroxyl group to a carbonyl group is not required for growth in macrophage-like cells or in guinea pig lungs. However, *hsd* is required for growth on cholesterol as the sole carbon source (23). This data suggests that cholesterol is not the sole carbon source utilized by *M. tuberculosis* during infection.
2.2. Pathogenicity of *Mycobacterium tuberculosis*

When in human lungs, *M. tuberculosis* is phagocytized by alveolar macrophages. While in the phagosome of the macrophage, *M. tuberculosis* prevents the maturation of the phagosome and the fusion of the phagosome with lysosomes to prevent the formation of a phagolysosome. However, this does not prevent the fusion of phagosomes containing *M. tuberculosis* with nutrient-containing endosomes (24). Although various other studies hypothesize that *M. tuberculosis* utilizes sterols, such as cholesterol, which is present in the phagosome cell wall as a carbon source during this stage of growth (8) (25) (26).

Apparently, this ability in combination with the thick hydrophobic mycolic acid cell wall coating enables the bacterium to survive, and even multiply inside the alveolar macrophage, protected from the immune system. Eventually the macrophage harboring the *M. tuberculosis* cells suffers from necrotic cell death, releasing the bacterial cells. This cycle ultimately results in a persistent tuberculosis infection and cavitation of the pulmonary tissue, which creates a more protective microenvironment.

The prevention of maturation and therefore acidification of the phagosome is possibly an effect of the production of the diterpinoid edaxadiene. A study by K. Pethe, et al. has shown that *M. tuberculosis* with deletions of the genes Rv3377c and Rv3378c are deficient in arresting the acidification of the phagosome (27). They also showed that these genes are responsible for synthesis of a diterpinoid termed edaxadiene. An independent study by F.M. Mann, et al. has shown corroborating results, showing that the pH in phagosomes which absorbed beads coated with edaxadiene declined to pH 5.3, while the phagosomes containing negative control beads declined to pH 4.8. A pH difference of 0.5 units, the same value found in the gene deletion study by K. Pethe, et al. (28).

The prevention of the phagosome fusing with lysosomes suspectedly happens due to the presence of mannosylated lipoarabinomannan (LAM), a complex cell wall component, characteristic of mycobacteria. Mannosylated LAM inhibits activity of hVPS34, a phosphatidylinositol 3 (OH) kinase, which presumably synthesizes phosphatidylinositol 3-phosphate (PI3P) (29). PI3P is a crucial component of the cell / endosomal membrane involved in protein trafficking and recruitment. Therefore, a lack of PI3P would lead to decreased recruitment of early endosomal antigen 1 (EEA1), a tethering molecule playing a role in endosome-endosome fusion. This would inhibit the fusion of the phagosome with other endosomes, including lysosomes.

Another branch of *M. tuberculosis* research has found several genes which are likely responsible for reactive nitrogen intermediate (RNI) and or reactive oxygen intermediate (ROI) resistance. These genes have been termed *noxR1*, *noxR3* and *ahpC*. These genes are responsible for the resistance to RNI and ROI generation in activated macrophages and facilitate survival in the macrophage (30) (31) (32). These genes are unlikely to be related to cholesterol catabolism, but they likely play an important role in persistence in combination with cholesterol catabolism.
3. Discussion

From the research above it seems that cholesterol catabolism is an essential factor for persistent infection of *M. tuberculosis* and likely also for related bacteria such as *M. bovis* and *R. equi*. Whether cholesterol is solely utilized as an energy and carbon source or is used to make compounds which assist in persistent infection remains a topic of interest and for future investigation. My personal opinion is that most merit lies in research which investigates the environment for *M. tuberculosis* in the macrophage phagosome and the response of the bacterium to this environment. The response of the bacterium to a change in the environment is measureable. Not only biochemically (The ability to metabolize cholesterol) but the underlying genetic changes can also be measured by transcriptional analyses. Owing to the fact that transcriptional changes is the underlying cause of the biochemical changes.

A different perspective is obtained when the study by Xinxin Yang, et al. is taken into account (23). These results suggest cholesterol catabolism is not required for infection. More specifically that cholesterol is not required as a carbon source during infection. The observation that at least some cholesterol catabolism intermediates are toxic could play a role in the hypothesis that cholesterol catabolism is required for infection. However, the data from Xinxin Yang, et al. shows that cholesterol accumulation itself is not toxic, at least, not on the levels which were observed (23). The fact that some catabolic intermediates are less toxic than others could account for the fact that some cholesterol catabolism deletion mutants are still able to grow in presence of cholesterol.

This means that in all cases cholesterol is imported into the bacterial cell (except in ∆mce4). In the ∆hsa study cholesterol is not degraded but accumulated, not leading to toxicity and leading to growth (23). In other mutation studies the bacteria partially degrade cholesterol, leading to toxicity and cell death (or, no growth)(16-20). In the WT strain, cholesterol is degraded, not accumulated. This does not lead to toxicity, but leads to growth. The ∆mce4 strain does not import and therefore does not accumulate or degrade and does not grow (8). These observations leave at least three possibilities; either cholesterol metabolism is not required for growth in macrophages at all, which means that the cholesterol catabolism enzymes have multiple functions which are directly or in directly essential for growth in macrophages. Or only cholesterol import is of importance, depriving the macrophage of cholesterol is essential for growth in macrophages. The third possibility would be that cholesterol is alternatively degraded. The ∆hsa study only shows the absence of the formation of the hsa catalysed reaction product. This study did not take alternative, pathways into account. For example: the usage of cholesterol to make a molecule essential to virulence. Or maybe even an alternative catabolic pathway. The latter could be disproved by measuring if a culture grown on 14C-4 cholesterol emits 14CO2.
This second hypothesis might be able to be tested by co-infecting macrophages with *M. tuberculosis* H37rv Δhsa and a *M. tuberculosis* H37rv Δmce4 GFP+ strain. The *M. tuberculosis* H37rv Δhsa strain will import cholesterol allowing a *M. tuberculosis* H37rv Δmce4 GFP+ strain to grow which is not able to import cholesterol, and not able to infect a macrophage on its own. Fluorescence of GFP in the macrophages would mean the *M. tuberculosis* H37rv Δmce4 GFP+ strain is able to survive because the macrophage is cholesterol deprived by the Δhsa strain. Admittedly it might prove very difficult or maybe even impossible to get 2 different strains of bacteria in the same phagosome.

Figure 4: Schematic overview of the expected effect of having 2 strains of bacteria in a single phagosome. The Δhsa mutant would import cholesterol which would result in non-toxic accumulation. The Δmce4 GFP+ mutant strain would not have the need to import cholesterol, would not have accumulation and therefore would grow and cause GFP fluorescence.

Another interesting experiment would be to construct double mutants of Δhsa and kshA and see if this Δhsa ΔkshA mutant is still able to grow in macrophages like the Δhsa strain. As in the generally supported model that cholesterol catabolism is required for growth in macrophages there should be no growth differences between the Δhsa mutant strain and the Δhsa ΔkshA double mutant strain. Because the deletion of hsa would mean that the substrate for kshA would not be available, unless kshA plays an as of yet unknown role.

Another interesting double mutant would be Δhsa Δigr to see if the deletion of hsa compensates for the attenuated growth in presence of cholesterol as the additional deletion of mce4 does.
4. Conclusion

The pathogenicity of some Actinomycetes and in particular \textit{M. tuberculosis} is very likely to be attributed to multiple virulence factors.

The main virulence factor is probably the ability to survive and develop inside the phagosome in macrophages. This ability is presumably mainly caused by the prevention of phagosome maturation in the macrophage by the mycobacterial cell wall and by uptake and utilization of cholesterol and lipids as a carbon and energy source during infection.

Secondary effectors are probably the production of edaxadiene to prevent or slow acidification of the phagosome and possibly similar, but as of yet unknown compounds. Additionally, the production of reductase enzymes that gives the bacterium resistance to RNI and ROI species also plays a role in virulence.

The utilization of cholesterol in \textit{M. tuberculosis} is still not fully understood and this merits further research which would be of interest to the biotechnological and pharmaceutical industries.
5. Future outlook

I expect that in the future more transcriptional and bioinformatical analyses will be conducted to find the remaining pieces of the cholesterol catabolism puzzle. Additionally, I think it would be worth looking in to if there are genes of unknown function in *M. tuberculosis* which are down-regulated when cholesterol catabolism is disrupted (Or up-regulated in presence of cholesterol or in *in vitro* macrophage cultures) to potentially discover genes which are responsible for production of virulence-associated molecules. (i.e.: enzymes which use cholesterol metabolites to produce molecules which assist in persistent infection.) With this approach, metabolites which manipulate the human defense mechanism or maybe even additional catabolic pathways could be found.

Misschien eventueel microscopie met bepaalde moleculaire labels? Misschien niet te doen *i.v.m* langzame groeitijd. Maar misschien wel met *R. equi*?

Hier overwoog ik wat te schrijven over Vit-D induced antimicrobial activity vs *M. tuberculosis*.

Maar dit schijnt alleen in vitro te werken. In een in vivo clinical trial (eigenlijk te kleinschalig) hadden ze negatieve resultaten. Dus achteraar lijkt vitamine-D niet zo intressant. Ik geloof wel dat ik gelezen heb dat de antimicrobiële activiteit van inductie van een molecuul “cathelicidin” afhangt. En dat wanneer het gen hiervoor gesilenced is vitamine-D in vitro zelfs averechts werkt. (dus *M. tuberculosis* kan denk ik zelfs Vit-D afbreken.

Wat een andere studie met pakistanen gevonden heeft is dat er een verband is met lage Vit D waarden en de ontwikkeling naar actieve TB.

Maar voor zover ik weet zijn er geen positieve onderzoeken dat Vit-D behandelingen iets verbeteren.

Dus ik denk dat dit relatief veel afwijkt van het onderwerp: catabolisme van steroiden en ook dat er eigenlijk niet veel te vertellen valt.
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## List of abbreviations

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<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>UVB</td>
<td>Ultra Violet – B radiation</td>
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<tr>
<td>PAH</td>
<td>Poly-Aromatic Hydrocarbons</td>
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<tr>
<td>PCB</td>
<td>Poly-Chlorinated Biphenyls</td>
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<tr>
<td>ABC-transporter</td>
<td>ATP-binding cassette transporter</td>
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<td>AD</td>
<td>4-androstene-3,17-dione</td>
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