Potassium uptake systems of *Mycobacterium tuberculosis*: genomic and protein organisation and potential roles in microbial pathogenesis and chemotherapy

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Mycobacterium tuberculosis *(MTB) is a formidable microbial pathogen which uses multiple mechanisms to subvert host immune defences. These include the effective, protective barrier presented by the outer waxy coat, intracellular concealment from host defences, and the ability to enter a prolonged, dormant phase in the infected host. Priority strategies to combat the scourge of TB include the identification of novel and selective targets on/in MTB which are amenable to pharmacological or immune-mediated control. Because they are structurally different from their counterparts in eukaryotic cells and are likely to be essential for survival and growth, the major K^+ transporters of MTB represent alternative and novel targets for drug and vaccine design. These K^+-uptake systems of MTB are the primary focus of this review, with particular emphasis on their genomic and protein structures, properties and functions, and potential roles in intracellular survival.*

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Introduction

Mycobacterium tuberculosis (MTB) is a human pathogen that infects pulmonary macrophages and replicates within these cells. The MTB organisms are concealed in phagosomes that fail to fuse with lysosomes,[1] in which they are able to multiply and infect neighbouring immature monocytes. Several weeks after infection, the bacilli stop growing and caseous necrosis occurs, leading to tissue damage. At this stage, the majority of the tubercle bacilli are killed, while others survive extracellularly, but are unable to multiply because of the anoxic conditions, reduced pH and presence of numerous antimicrobial enzymes released from dead and dying cells. It is likely that viable extracellular bacilli persist in these caseous lesions without being metabolically active, or having limited metabolic activity, resulting in the latent, persistent stage of infection.[2]

During these changing environments, which occur at various stages of infection, the organisms express different sets of genes which are crucial to their adaptation and survival. Among the environmental factors that may influence expression of bacterial genes are the concentrations of metal ions within the phagosomes. Wagner *et al*[1] have reported differences in the concentrations of ions such as chlorine, calcium, potassium (K^+), manganese, copper and zinc between vacuoles containing pathogenic or non-pathogenic mycobacteria, underscoring the importance of the ionic environment in the adaptation of the organisms to intracellular survival.[3]

Of all the monovalent cations, K^+ is the most concentrated in both bacterial and eukaryotic cells, attaining concentrations ranging from 0.1 to 1 M in bacteria[4] and around 140 mM in eukaryotic cells relative to an extracellular concentration of 5 mM.[5] In bacteria, these high levels of intracellular K^+ are essential for a number of processes such as maintenance of turgor pressure, activation of enzymes, regulation of cytoplasmic pH, stress responses, and gene expression.[6-11] Most intracellular bacteria also require metal cations for the synthesis and function of the anti-oxidative enzymes, superoxide dismutase and catalase, which play an important role in microbial defence against macrophage-killing mechanisms.[3]

Although their roles in microbial pathogenesis and persistence remain to be fully characterised, the major structural differences between the K^+ transporters of MTB and those of eukaryotic cells, clearly underscore the potential of these microbial cation transporters as novel targets on which to base drug and vaccine design. Our aim, in this paper, is to highlight the genomic and protein structures and functional properties of the K^+ transporters of MTB, as well as their possible involvement in microbial pathogenesis.

The genomic organisation and protein structures of MTB potassium transporters

Most bacteria utilise several different K^+-uptake systems to maintain high intracellular K^+ concentrations, emphasising the importance of this cation for bacterial growth. These vary significantly between bacterial species. For instance, *Escherichia coli* utilises three K^+-uptake systems, viz the Trk, Kup and Kdp transporters,[12,13] while streptococcal species utilise the KtrI and KtrII systems.[14]

In MTB, two K^+-uptake systems, the Trk and Kdp, have been identified. The Trk system is a constitutively operative, moderate-to-low affinity system, comprised of two TrkA proteins, CeoB (24 kDa) and CeoC (23 kDa), which are encoded by the *ceoB* (684 bp) and *ceoC* (663 bp) genes. The *ceo* genes are so named because of their ability to complement the *E. coli* OxyR-knockout mutant.[15] The two genes,

which share 49% homology to each other, are tandemly arranged as an operon at position 3009.34 on the chromosome, with the *ceoC* gene overlapping the *ceoB* by one nucleotide.[16]

The genomic arrangements, together with the sizes of these genes of MTB and other mycobacterial species, some of which are clinically relevant in humans or animals, while others are non-pathogenic, are illustrated in Figure 1. Most of the mycobacterial species encode at least two *trk (ceoBC*) genes, which vary in size and orientation between species. Only *ceoB* and *ceoC* pseudogenes have been sequenced in *Mycobacterium leprae.* A high degree of sequence similarity exists between the *trk* genes of MTB and *Mycobacterium bovis/BCG,* in which the genes are tandemly arranged, are of equal size, and are similarly transcribed. Although the same orientation is observed in *Mycobacterium avium, Mycobacterium abscessus* and *Mycobacterium ulcerans,* the genes are tandemly arranged only in *M. abscessus* and are separated by several base pairs in other species. DNA sequence alignment of these genes in the other mycobacterial species in relation to those of MTB showed 99% similarity between MTB and *M. bovis*/BCG, >50% with the other mycobacteria, and the lowest similarity (31%) with the *M. leprae* pseudogenes.

The deduced TrkA proteins of MTB also share some degree of sequence homology with those of other bacterial genera. The CeoB shares 52% and 25% amino acid sequence identity with the TrkA of *Streptomyces coelicolor* and *E. coli,* respectively, while the CeoC fragment shows 24% amino acid identity to the TrkA proteins of both strains. Both proteins possess the NAD^+-binding motif, which is also found in the TrkA proteins of various other bacterial genera, including the Gram-negative bacteria, *Streptomyces coelicolor* and *Azorhizobium caulinodans* and archaebacteria.[15-17]

The second K^+-uptake system of MTB, the Kdp, is an inducible, high-affinity two-component, P-type, ATP-driven regulatory system, comprised of six proteins, KdpA (60 kDa), KdpB (75 kDa), KdpC (20 kDa), KdpD (93 kDa), KdpE (25 kDa) and KdpF (3 kDa). The genes encoding these proteins are found at position 1148.427 on the bacterial chromosome and are arranged in *kdpDE* and *kdpFABC* operons. These are transcribed in opposite directions and are separated by a region of approximately 234 bp between *kdpF* and *kdpD.*[16]

The genomic organisation and sizes of the *kdp* genes of MTB in relation to those of other mycobacteria are illustrated in Figure 2. As is the case with other bacterial genera, the mycobacterial *kdp* genes are comprised of *kdpDE* and *kdpFABC/ABC* operons. A high degree of sequence similarity has been shown to exist between the *kdp* genes of MTB and *M. bovis/BCG;* in addition to the separation of the two operons, the individual genes are of equal sizes, and have the same orientation, with the stop codons of *kdpD* and *kdpA* genes being the start codons of *kdpE* and *kdpB,* respectively. In contrast to MTB, the *kdpDE* and *kdpFABC/ABC* operons in other mycobacterial species are similarly transcribed, with the *kdpD* being separated from the *kdpC* by a few base pairs. Of these, the *kdpF* gene has been sequenced in *M. avium* while no *kdp* genes have been reported in *M. leprae* and *M. ulcerans.*

The MTB *kdp* genes also share some degree of sequence homology with the corresponding genes of *E. coli.*[16] However, unlike the MTB *kdp,* the *E. coli kdpFABC* and *kdpDE* operons are sequentially transcribed, with *kdpDE* adjoining the *kdpC* gene at the 3' end.[18]

While the precise cellular locations of the CeoB and CeoC proteins of MTB are unknown at this stage, those of the Kdp proteins have been determined.[5] The KdpD protein, which has a cytosolic sensor domain and C-terminal histidine kinase activity, spans the bacterial cell membrane, while the other Kdp proteins have an exclusively cytosolic location.

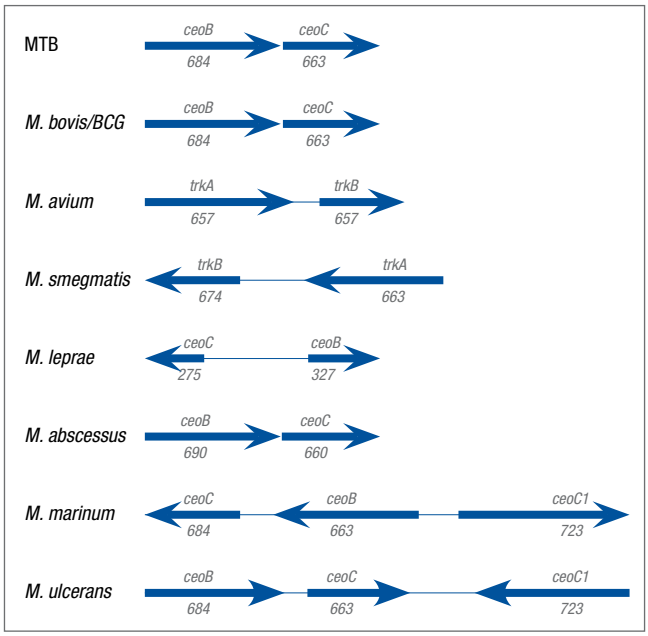

Figure 1: The genomic arrangements of the *trk* genes of MTB and other mycobacteria. The diagram was not drawn according to scale. In some organisms, *ceoB* is named *trkA* while *ceoC* is *trkB.* The number below each gene represents the gene size in base pair and the line connecting the individual genes represents the presence of interspace sequences.

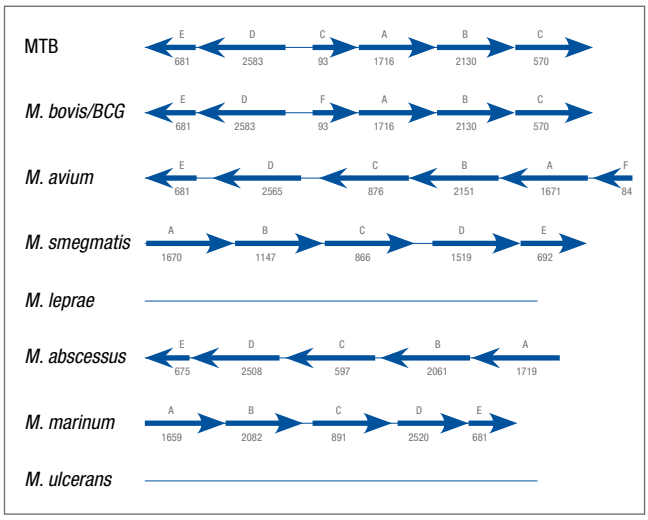

Figure 2: The genomic organisation of the *kdp* genes of MTB and other mycobacteria. The diagram was not drawn according to scale. A, B, C, D, E and F denote *kdpA, kdpB, kdpC, kdpD, kdpE* and *kdpF,* respectively, while the number below each gene represents the gene size in base pair. The straight lines shown for *M. leprae* and *M. abscessus* indicate the absence of genes, while those connecting individual genes represent the presence of interspace sequence.

Properties and functions of MTB potassium transporters

The properties of the K^+ transporters of MTB have been partially characterised. The Trk system operates during the logarithmic stage of growth in a medium with high K^+ concentration, has a lower affinity for K^+, and is dispensable for *in vitro* growth if the Kdp system is intact.[19, 20]

On the other hand, the Kdp system of MTB is repressed during the *in vitro* logarithmic phase of growth, when the K^+ concentration is high, as is the case with other bacteria. The system is induced as a back-up when the osmolarity is low.[5] Interestingly, the Kdp is expressed when the Trk system is inactivated, even when the K^+ concentration is high.[20]

The activation of most bacterial Kdp systems follows a response to several stimuli including ionic and non-ionic solutes, pH, growth temperature and low concentrations of K^+. These alter the expression of the *kdp* genes by affecting signal strength.[21,22] This is also likely to be the case with mycobacteria. Steyn *et al* have reported that a low K^+ concentration is also a stimulus for Kdp expression in MTB.[5]

In contrast to *E. coli,* in which, in response to an appropriate signal the N-KdpD and C-KdpD domains interact with each other, the N-KdpD and C-KdpD domains of MTB form ternary complexes with the membrane lipoproteins, LprF and LprJ. The N-KdpD/C-KdpD/LprF and N-KdpD/C-KdpD/LprJ complexes in turn dephosphorylate KdpE, with resultant transcription of the *kdpFABC* operon. No other known functions of LrpF and LprJ have been reported previously.[5]

The concept that the expression of Kdp is controlled by the *kdpDE* operon is well-documented.[18] To ascertain if activation of the Kdp system is critically dependent on an intact *kdpDE* operon, we are currently studying the expression profile of the *kdp* genes in a KdpDE-deletion mutant strain of MTB. It has been reported[23] that inactivation of the *kdpDE* operon increased the virulence of MTB in a murine model of experimental infection, while growth *in vitro* was unaffected.

We are also characterising the K^+-uptake systems of MTB in relation to their preferential utilisation by the organism during varying stages of growth *in vitro,* their relative efficiencies in transporting the cation and the expression profiles of the individual genes encoding the Trk and Kdp systems during different stages of growth.

Involvement of the potassium transporters of MTB in intracellular survival and dormancy

Rengarajan *et al* using transposon site hybridisation (TraSH) technology, have reported that *ceoB* (Rv2691) is required for survival of MTB in macrophages.[24] We have proposed that the uptake of K^+ by the Trk system of MTB may favour virulence by promoting intracellular survival. This contention is based on the presence of NAD^+-binding motifs on the CeoB and CeoC proteins, as well as the degree of sequence homology with the Trk proteins of *E. coli,* which require both ATP and a protonic potential, compatible with a phosphorylation-regulated K^+/H^+ symporter.[15,25] Given the structural similarities between the Trk systems of MTB and *E. coli,* it is conceivable that the Trk of MTB, by functioning as K^+/H^+ symporter, may antagonise vacuolar acidification in MTB-infected macrophages. This, in turn, may contribute to delayed phagosome maturation, preventing phagosome/lysosome fusion, favouring intracellular survival of MTB.[26-28] This proposed mechanism is summarised in Figure 3.

The Kdp system has been associated with persistence of the organisms in the host.[29-31] During persistence the organisms experience nutrient deprivation[32,33] and are in a state of little or no replication, exhibiting a low respiration rate, but maintaining long-term viability.[34,35] An upregulation of the Kdp regulatory genes during persistence in the host has been demonstrated in the *in vitro* nutrient starvation model developed by Betts *et al.*[35] Of the 11 two-component regulatory systems present in MTB, only the *kdpE* gene was induced and only 96 hours after starvation. Other upregulated genes associated with the Kdp transporter, included the *lprJ*, which was constitutively expressed from four to 96 hours, and the *lprF* and *hns* which were induced within 4 hours of nutrient starvation.[35,36]

In addition to their involvement in microbial persistence in the *in vitro* model, a requirement for the *kdp* regulatory genes has been demonstrated in murine models of experimental TB. Parish *et al,* as mentioned above, have demonstrated that elimination of the *kdpDE* operon increased the virulence of the organism in mice, while Sassetti and Rubin have shown that *kdpD* is required for survival of MTB in this animal model.[23, 37]

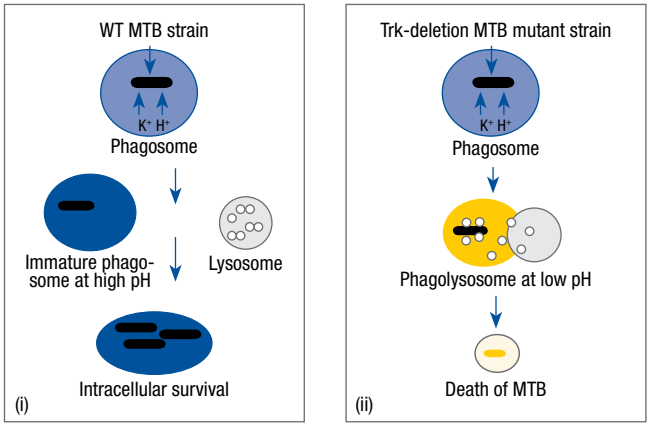

Figure 3: Schematic illustration of possible scenarios in macrophages infected with (i) the wild-type (WT) MTB strain and (ii) the Trk-deletion mutant strain of MTB. The presence of the Trk system in the organism in (i) may contribute to the decrease in $[H^+]$ leading to high vacuolar pH, which will interfere with phagosome maturation. In (ii) the macrophage infected with the Trk-deletion mutant of MTB is presumed to utilise the Kdp system, which may take up K^+ in exchange for H^+, thereby releasing the proton into the vacuole and decreasing the pH. This in turn will promote phagosome maturation, leading to phagosome/lysosome fusion and ultimately elimination of the bacteria.

MTB potassium transporters as potential drug and vaccine targets

The essential requirement for K^+ for bacterial metabolism and growth is underscored by the presence of two distinct K^+ transporters in MTB. MTB K^+ transporters have been reported to serve as potential targets for several anti-tuberculosis agents. We have demonstrated that decreased uptake of K^+ is one of the earliest detectable changes following exposure of MTB to clofazimine, the prototype of the riminophenazine group of antimycobacterial agents.[38] More recently, the effects of clofazimine, on the different K^+-uptake systems of MTB were investigated using a Trk-gene knockout strain of MTB. Both the Trk and Kdp systems were found to be sensitive to the inhibitory actions of clofazimine. Importantly, maximum inhibition of K^+ influx was observed at clofazimine concentrations of 1.25 -2.5 µg/mL, which were close to the minimum inhibitory concentration (MIC) values of this agent for MTB, compatible with a mechanistic relationship between inhibition of uptake of K^+ and bacterial growth.[20]

Although the molecular/biochemical mechanism of clofazimine-mediated inhibition of mycobacterial K^+ transporters has not been established, two possibilities exist. Clofazimine may interact directly with, and inactivate K^+-uptake systems, or, alternatively, it may simply function as a membrane-destabilising agent, dismantling membrane architecture, with consequent secondary dysfunction

of membrane transporters. The range of bacterial K^+ transporters which are sensitive to clofazimine, including the MTB K^+ transporters, as well as the Kup system of *E. coli,*[39] suggests that a membrane disruptive mechanism, as recently proposed by us[40] and others,[41,42] appears most likely. In support of this contention, exposure to clofazimine is accompanied by a rapid increase in phospholipase activity in MTB.[40,43] Although increased phospholipase activity is not primarily linked to inhibition of uptake of K^+, it is, nevertheless, compatible with membrane destabilization.[40] Given the differences in phospholipid composition between the outer membranes of eukaryotic and prokaryotic cells, the development of novel membrane-destabilising agents for antimicrobial chemotherapy may be a viable strategy. The advantage of such agents is that they may target several different membrane transporters simultaneously, thereby minimising development of resistance. Possible approaches include the design of membrane-disruptive agents which selectively target prokaryotes, or agents which selectively inhibit the synthesis of fatty acids in bacteria.[44,45]

Isoniazid (INH), one of the essential drugs in the treatment of tuberculosis, has been reported to bind tightly to the CeoB protein of the Trk system,[46] suggesting that this K^+ transporter is a potential target of INH, in addition to the known primary targets, enoyl-ACP reductase (InhA) and dihydrofolate reductase (DfrA). The MTB *ceoC* gene was not identified among the targets of INH in this study. Interestingly, however, insertion of the *ceoC* gene has previously been shown to increase INH resistance in an *E. coli* OxyR strain.[15] Although we have been unable to detect any inhibitory effect on uptake of K^+ following of short-duration (60 minutes) exposure of MTB to INH at a concentration of 0.25 µg/ml, which is approximately five-fold greater than the MIC value,[38] the possibility exists that the Kdp system may compensate for INH-mediated inactivation of Trk.

Although mycobacterial K^+ transporters represent alternative and novel targets, validation of these as potential, selective targets for anti-mycobacterial chemotherapy will require construction of a dual Trk/Kdp-knockout mutant of MTB and demonstration of lethality. Such studies are currently in progress. Even if target validation is achieved, however, successful pharmacotherapy based on the selective inactivation of a single K^+ transporter is unlikely because of the ability of these systems to compensate for one another. Nevertheless, selective targeting of the Trk and Kdp systems, as strategies to decrease virulence, may represent an adjunctive pharmacological approach to complement chemotherapy with conventional anti-mycobacterial agents, but will require detailed knowledge of the molecular structures and functions of these K^+ transporters.

Conclusion

We have reviewed the characteristics of the K^+ transporters of MTB and their potential involvement in microbial pathogenesis, as well as their potential to serve as novel drug targets. Both the Trk and Kdp systems seem to have varying involvement during different growth phases in *in vitro* and changing environments in the host. The Trk system has been demonstrated to be important during early intracellular infection (Figure 3). Targeting this system, may favour intra-vacuolar elimination of the bacilli. The Kdp system on the other hand, seems to operate during microbial dormancy such that targeting of this system represents a potential strategy to eliminate microbial persistence.

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