Role of the kdpDE Regulatory Operon of *Mycobacterium tuberculosis* in Modulating Bacterial Growth in vitro

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Bacteria use K⁺-uptake transporters differentially for adaptation in varying growth conditions. In *Mycobacterium tuberculosis*, two K⁺-uptake systems, the Trk comprising the CeoB and CeoC proteins and the Kdp consisting of the two-component system (TCS), KdpDE and KdpFABC, have been characterized, but their selective utilization during bacterial growth has not been completely explored. In the current study, the roles of the *M. tuberculosis* KdpDE regulatory system alone and in association with the Trk transporters in bacterial growth were investigated by evaluating the growth of *M. tuberculosis* KdpDE-deletion and KdpDE/Trk (KT)-double knockout mutant strains in planktonic culture under standard growth conditions. The KT-double knockout mutant strain was first constructed using homologous recombination procedures and was evaluated together with the KdpDE-deletion mutant and the wild-type (WT) strains with respect to their rates of growth, K⁺-uptake efficiencies, and K⁺-transporter gene expression during planktonic growth. During growth at optimal K⁺ concentrations and pH levels, selective deletion of the TCS KdpDE (KdpDE-deletion mutant) led to attenuation of bacterial growth and an increase in bacterial K⁺-uptake efficiency, as well as dysregulated expression of the kdpFABC and trk genes. Deletion of both the KdpDE and the Trk systems (KT-double knockout) also led to severely attenuated bacterial growth, as well as an increase in bacterial K⁺-uptake efficiency. These results demonstrate that the KdpDE regulatory system plays a key role during bacterial growth by regulating K⁺ uptake via modulation of the expression and activities of both the KdpFABC and Trk systems and is important for bacterial growth possibly by preventing cytoplasmic K⁺ overload.

Keywords: *Mycobacterium tuberculosis*, two-component system KdpDE, KdpFABC system, Trk system, K⁺-uptake systems, K⁺ concentration, pH level, gene expression

INTRODUCTION

Growth of *Mycobacterium tuberculosis* is optimal in artificial culture media supplemented with potassium (K⁺) concentrations of around 14–15 millimolar (mM) at a pH of about 6.8 (Piddington et al., 2000; Cholo et al., 2015; Baker et al., 2019; Salina et al., 2019). *Mycobacterium tuberculosis* uses K⁺ to support cellular metabolic activities, such as cell wall biosynthesis, protein synthesis,
lipid metabolism, and aerobic respiration, which are associated with logarithmic growth (Salina et al., 2014). However, in K’-limiting (Salina et al., 2014) and low pH environments (pH 5.5; Cholo et al., 2015), this bacterial pathogen alters its metabolic rates, transitioning to slow-growing, persistent-to-non-growing, and dormant phenotypes and allowing for bacterial survival in these unfavorable growth conditions.

Other studies have shown that bacteria adapt to these adverse growth conditions through differential utilization of K’-uptake transport systems (Epstein, 2003). For example, *Escherichia coli* utilizes the Trk system at elevated K’ concentrations in near-neutral pH, while the K’-uptake permease (Kup: TrkD) is used at low pH. However, at K’-limiting conditions at neutral pH, *E. coli* utilizes the Kdp system (Roe et al., 2000; Epstein, 2003). In this context, *M. tuberculosis* possesses two active K’-uptake transporters, namely the Trk and Kdp systems (Cole et al., 1998). The Trk consists of two TrkA proteins, CeoB and CeoC, encoded by highly homologous *trk* genes (*ceoB* and *ceoC*) of the *ceoBC* operon. We have previously shown that the Trk system is constitutively expressed in *M. tuberculosis* (Cholo et al., 2015), and has a lower affinity for K’ than the Kdp (Cholo et al., 2006). It plays a role in slowing bacterial growth in optimal conditions in standard 7H9 broth medium (15 mM K’, pH 6.8; Cholo et al., 2006). The Trk system has also been implicated in bacterial dormancy, with the CeoB protein being expressed at low K’ concentrations in biofilm cultures (Kerns et al., 2014; Hegde, 2020), while both *trk* genes are upregulated at low extracellular pH levels in planktonic culture (Cholo et al., 2015).

The *M. tuberculosis* Kdp system, on the other hand, is an inducible two-component system (TCS) comprised of the KdpDE sensor-regulator and the high-affinity K’-uptake transporter complex, KdpFABC (Cholo et al., 2006). The two Kdp components consist of six proteins encoded by a cluster of six genes, arranged in two operons, *kdpDE* and *kdpFABC*. These operons are divergently transcribed on the bacterial genome; being separated by an intergenic region (~234 bp) located between the *kdpD* and *kdpE* genes (Cole et al., 1998; Cholo et al., 2008). Activation of the Kdp system is mediated by the KdpDE, consisting of the sensor kinase KdpD and response regulator KdpE proteins (Steyn et al., 2003; Freeman et al., 2013). The KdpD and KdpE proteins interact with one another under basal conditions, with KdpD sensing the environmental stimulus and undergoing autophosphorylation at the histidine-64 residue, transferring the phosphoryl moiety to the KdpE subunit at the aspartate-52 residue, resulting in its phosphorylation, and leading to induction of the *kdpFABC* operon (Steyn et al., 2003; Agrawal and Saini, 2014). Low extracellular pH levels (Cholo et al., 2015), as well as K’-limiting conditions (Salina et al., 2014), are environmental stressors that lead to the induction of both the *kdpDE* and *kdpFABC* operons. These adverse growth conditions also result in acquisition of a dormant phenotype, seemingly implicating the involvement of the Kdp system in bacterial survival.

As with growth in adverse conditions of low extracellular K’ and pH, information on the role of the Kdp system during mycobacterial growth in optimal K’ concentrations and pH levels *in vitro* is also limited. In this context, we have previously reported that in the setting of optimal growth conditions, the Kdp system is repressed when the Trk system is functional, being induced and activated as a back-up when the Trk system is inactive (Cholo et al., 2006). Despite the limited information on growth, a few studies have identified that only the *kdpE* among the *kdp* genes, is necessary for optimal growth of *M. tuberculosis* (Sassetti et al., 2003; Griffin et al., 2011), as well as that of *Mycobacterium smegmatis* (Ali et al., 2017).

The issue of the differential utilization of these high- and low-affinity K’-uptake systems of *M. tuberculosis* during planktonic growth of the pathogen, including the seemingly modulatory role of KdpDE, have been explored in the current study. The research strategy used was based on a comparison of the growth of a selective KdpDE-deletion mutant strain and a recently constructed KdpDE/Trk (CeoBC; KT)-double knockout mutant strain with that of the wild-type (WT) strain of *M. tuberculosis* in standard growth conditions.

**MATERIALS AND METHODS**

**Antimicrobial Agents and Chemicals**

Unless indicated, all chemicals were purchased from the Sigma Chemical Co (St. Louis, MO, United States). Kanamycin and hygromycin antibiotics were used at 10 and 50 mg/L, respectively, for the selection of antibiotic-resistant colonies; 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-Gal) at 0.24 mg/L for blue colonies and sucrose at 2% (20 g/L) as a counter-selectable marker for *sacB*-expressing clones.

Rubidium-86 chloride (**86Rb**) was purchased from PerkinElmer Life and Analytical Sciences, Du Pont-NEN Research Products, Boston, MA, United States.

**Strains and Growth Media**

All plasmids and bacterial strains used in this study are as shown in **Table 1**. The *E. coli* DH5α competent cells were used for cloning procedures for plasmid transformation. The pSOU42 suicide-delivery vector (SDV) carrying the mutated *M. tuberculosis* *kdpDE* fragment and the KdpDE-deletion mutant strain of *M. tuberculosis*, were kindly provided by Professor N. Stoker, Royal Veterinary College, United Kingdom (Parish et al., 2003). The *M. tuberculosis* Trk-deletion mutant strain, which we had constructed previously (Cholo et al., 2006) was used for the construction of the KT-double knockout. The *M. tuberculosis* KdpDE-deletion (Parish et al., 2003) and the KT-double knockout (current study) mutant strains were compared with the WT strain for phenotypic and genotypic characteristics.

The Psi (Ψ) broth medium (5 g Bacto yeast extract, 20 g Bacto tryptone, 5 g MgSO₄/L, and pH 7.5) was used for preparation of competent *E. coli* cells and Luria-Bertani (LB) broth for growing plasmid-carrying *E. coli* bacteria, while the *E. coli* colonies were developed on Luria agar (LA) medium. For *M. tuberculosis* cultures, 7H9 broth and 7H10 agar (Difco) media supplemented with 10% oleic acid, dextrose, catalase (OADC), and 2/5% glycerol with/without 0.05% Tween 80
TABLE 1 | Plasmids and bacterial strains used in the study.

| Strain | Feature or relevant genotype | Source |
|--------|-----------------------------|--------|
| **Plasmids** | | |
| pSOUP42 | PacI fragment from pGOAL19, unmarked 1,691-bp SphI kdpDE deletion in kdpD and kdpE genes. | Parish et al., 2003 |
| pSOUP43 | PacI fragment from pGOAL17, unmarked 1,691-bp SphI kdpDE deletion in kdpD and kdpE genes. | This study |
| **Bacteria** | | |
| DH15a | Wild type Escherichia coli strain. | Parish et al., 2003 |
| H37Rv | Wild type laboratory M. tuberculosis strain ATCC 25618. | Parish et al., 2003 |
| KdpDE-deletion | Deletion of 1,691-bp SphI kdpDE fragment in kdpD and kdpE genes. | Parish et al., 2003 |
| Trk-deletion | Marked mutant, deletion of 348-bp cesO, insertion of 1746-bp hyg-resistance gene cassette at cesO gene. | Cholo et al., 2006 |
| KdpDE/Trk-double knockout mutant | Marked mutant, combined mutations of kdpDE and cesO operons. | This study |

respectively, were used for liquid-based growth assays and colony development, respectively.

**Construction of a KdpDE/Trk (CeoBC)-Double Knockout Mutant**

The KT-deletion mutant, which is characterized by inactivation of both the kdpDE and trk (cesBC) operons, was constructed using homologous recombination following a two-step strategy (Parish and Stoker, 2000). Prior to electroporation, the hyg, P_{cesO}-lacZ, P_{cesO}-sacB PacI cassette in the pSOUP42 SDV was replaced with the P_{cesO}-lacZ, P_{cesO}-sacB PacI cassette from pGOAL17 to form pSOUP43 (Table 1). Briefly, approximately 5 μg of UV-pretreated pSOUP43 was electroporated into the M. tuberculosis Trk-deletion mutant, carrying the mutation of the cesO operon (Cholo et al., 2006) and plated onto 7H10 agar medium supplemented with hygromycin, kanamycin, and X-Gal for the isolation of blue single crossovers (SCO) clones, followed by isolation of white double-crossovers (DCO) on non-selection media. The DCO clones were characterized for absence of the plasmid phenotypically using sucrose and kanamycin sensitivity testing procedures. DNA samples from white sucrose-resistant, kanamycin-sensitive M. tuberculosis clones were extracted and mutations at the kdpDE and cesBC operons were confirmed by a non-radioactive Southern blotting procedure using a digoxigenin (DIG)-labeled PCR-synthesized probe as described in the PCR DIG Probe Synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany).

**Bacterial Inoculum Preparation**

A bacterial inoculum of each strain was prepared as described, with minor modifications (Cholo et al., 2015; Mothiba et al., 2015). Briefly, a seed culture of M. tuberculosis cells was inoculated into 50 ml of 7H9 broth and grown to the mid-log phase at 37°C under stirring conditions. The bacterial cells were harvested by centrifugation at 2851 × g at room temperature (RT) for 15 min and the supernatant discarded. The pellet was washed twice and re-suspended in 7H9 broth, followed by adjustment of the optical density (OD) to 1.2 at 540 nm, yielding ca. 10^5–10^6 colony-forming units (cfu)/ml. An inoculum of ca. 10^5 cfu/ml was used in all of the assays.

**Preparation of Bacterial Cultures at Logarithmic Phases**

For each strain, the bacterial cultures were prepared by inoculating approximately 10^5 cfu/ml cells into 7H9 broth followed by incubation of the culture at 37°C under stirring conditions until the early-, mid-, and late-log phases were reached, corresponding to ODs of 0.1–0.3, 0.4–0.6, and 2.0–2.3 at 540 nm, respectively (Cholo et al., 2015).

**Rates of Growth**

Cultures for determination of the rates of growth were prepared by inoculating ca. 10^6 cfu/ml of cells of the WT and mutant strains of M. tuberculosis into 7H9 broth. The cultures were thoroughly mixed followed by incubation at 37°C for 15 days in the dark with continuous stirring. The cultures were sampled every 3 days beginning at day 0 (D0) to day 15 (D15) and growth was determined spectrophotometrically at a wavelength of 540 nm. The rates of growth of each strain were determined as the time taken by the bacteria to reach the different logarithmic growth phases.

**Uptake of Rubidium (**^{86}\text{Rb}^*)**

Uptake of K⁺ by the WT and mutant strains was determined at the mid- and late-log phases using ^{86}\text{Rb}^* as a surrogate tracer for K⁺. Briefly, the bacteria were harvested from cultures grown to the two logarithmic growth phases and resuspended to ca. 10^6 cfu/ml in K⁺-free buffer (KONO) containing 2 mCi/L ^{86}\text{Rb}^* and uptake of the radioisotope determined as absolute counts per minute (cpm) as previously described (Steel et al., 1999; Cholo et al., 2015).

**Extracellular Potassium and pH**

The K⁺ concentrations and pH levels were determined at the early-, mid-, and late-log phases for each strain. Following culture preparation, the supernatants were harvested by centrifugation (2,851 × g, 15 min) followed by decontamination by heat treatment at 95°C for 60 min. The K⁺ concentrations and pH levels were measured in the undiluted samples by indirect potentiometry utilizing a K⁺-selective electrode in conjunction with a Na⁺-reference electrode using the Beckman Coulter Synchro LX 20 System (Beckman Coulter, Ireland Inc., Gateway, Ireland) and the Crison micropH2001 pH meter (Crisson Instruments, Barcelona, Spain), respectively. These measurements were determined at the initial (D0), intermediate and final (early-, mid-, and late-log) growth phases, as well as in the processed and unprocessed bacteria-free 7H9 broth medium.
Gene Expression Using the Reverse-Transcriptase-PCR

Gene expression was performed at the early-, mid-, and late-log growth phases in standard 7H9 liquid culture medium as described previously (Cholo et al., 2015). Briefly, RNA was extracted following the Trizol method, and complementary deoxyribonucleic acid (cDNA) was synthesized using the Sigma Enhanced Avian HS reverse transcriptase-PCR (RT-PCR) kit and amplified by quantitative (q)PCR using the LightCycler FastStart DNA Master SYBR Green I kit with the LightCycler 2.0 instrument (Roche Molecular Biochemicals, Mannheim, Germany). The quantities of the individual genes were determined using absolute (AQ) and relative (RQ) quantifications with sigA as the reference gene. The relative quantifications were determined based on quantification cycles (Cq) using the $2^{-\Delta\Delta Cq}$ method.

Statistical Analysis

All statistical analyses were performed using the INSTAT program and the unpaired and paired Student $t$-test/Mann–Whitney U-test for analysis of growth rates and gene expression data, respectively. The results are expressed as the means ± SDs. Significance levels were taken at a $p \leq 0.05$.

RESULTS

Construction of the KT-Double Knockout Mutant

In order to investigate the roles of the TCS KdpDE system on bacterial growth, alone and in association with the Trk system, acquisition of the K’-uptake mutant strains lacking the single KdpDE regulatory system (KdpDE-deletion mutant strain), as well as a combination of both the KdpDE and Trk systems (KT-double knockout mutant strain), was necessary.

The TCS KdpDE, together with the KdpFABC transporter, are the main components of the Kdp system, encoded by separate kdpDE and kdpFABC operons, transcribed in opposite directions, with the start codon of kdpD separated by 234 bp from the start codon of kdpF. This genomic arrangement of the kdp operons is similar to that of Mycobacterium bovis, but is different from those of other bacterial and mycobacterial species, which show both operons having similar transcriptional orientations with the kdpD being adjacent to the kdpC (Cole et al., 1998; Cholo et al., 2008; Agrawal and Saini, 2014).

The construction of the single KdpDE-deletion mutant of M. tuberculosis was as previously reported (Parish et al., 2003). Both the kdpD (Rv1028c: 2583 bp) and kdpE (Rv1027c: 681 bp) genes are transcribed in the negative direction with kdpE located at position 1149.104–1149.197 (681 bp) with its start codon overlapping the stop codon of kdpD found at position 1149.104–1051.686 on the chromosome. Mutation of the kdpDE operon was achieved by deletion of a 1,691-bp SphI fragment that spans both the kdpD and kdpE genes, resulting in inactivation of the sensor kinase/response regulator of the KdpFABC system. However, due to separation of the kdpDE and promoter-carrying kdpFABC operons on the M. tuberculosis genome, the promoter region of the kdpFABC operon remained genotypically intact.

In the case of the KT-double knockout strain of M. tuberculosis, we used the Trk-deletion mutant strain, which we had constructed previously (Cholo et al., 2006). The trk genes comprising the ceoB (Rv2691: 684 bp) and ceoC (Rv2692: 663 bp) genes are found on the ceoBC operon, with the ceoB located at position 3009.344–3010.027, with its stop codon overlapping the start codon of ceoC found at position 3010.024–3010.686 on the chromosome. Both trk genes are transcribed in the positive direction. The Trk-deletion mutant strain was characterized by inactivation of both the trk genes, resulting in a 348-bp deletion at ceoB gene and insertion of the 1746-bp BamHI-BglII hyg resistance cassette, derived from the plp963 vector, at the Nhel site of the ceoC gene. The KT-double knockout mutant was constructed by introducing a kdpDE-deletion fragment-carrying plasmid, psOUP43, constructed as described (Parish et al., 2003; Table 1) into the Trk-deletion mutant (Cholo et al., 2006; Figure 1). Successful mutagenesis of the kdpDE operon in the KT-double knockout mutant strain was evident by detection of the 1996-bp Xhol-kdpDE fragment with the 1,010-bp PCR-synthesized kdpDE probe (Figure 1A; Table 2; Parish et al., 2003). For the ceoBC operon, mutation was revealed by detection of the 2,231-bp BclI-ceoBC fragment using the 714-bp PCR-synthesized ceoB probe (Figure 1B; Table 2; Cholo et al., 2006).

Rates of Growth

We used the WT and mutant strains to determine the role of the TCS KdpDE alone and in association with the Trk system on bacterial growth. This was achieved by assessing the rates of growth of the WT and the K’-uptake mutant strains in 7H9 broth medium (15 mM K+, pH 6.7) under aerobic conditions, sampled every 3 days beginning at D0 to D15 for OD determination.

The numbers of bacteria were determined at D0 and were shown to be $8.8 \times 10^{4} \pm 7.5 \times 10^{4}$, $2.1 \times 10^{5} \pm 1.8 \times 10^{5}$, and $9.7 \times 10^{5} \pm 1.3 \times 10^{6}$ cfu/ml for the WT, KdpDE-deletion, and Trk-deletion mutant strains, respectively (Cholo et al., 2015). The rate of growth of each strain was determined as the time, measured in days, taken by the bacteria to reach each growth phase, and the results are shown in Figure 2.
Despite the KdpDE-deletion and KT-double knockout mutant strains reaching the early-, mid-, and late-log phases at the same time points (i.e., at D9, D12, and D15, respectively), the growth levels of the two mutant strains determined by comparing their OD measurements at the different time points, were nevertheless different, as shown in Figure 2, with the rate of growth of the KT-double knockout mutant strain at D6, D9, and D12 being significantly slower than that of the KdpDE-deletion mutant strain \( p < 0.05 \).

As we have previously shown that \( M. \text{tuberculosis} \) utilizes the Trk \( K^+ \)-uptake transporter exclusively when cultured during standard growth conditions in 7H9 medium, suppressing the activity of the KdpFABC transporter (Cholo et al., 2006, 2015), these observations appear to implicate the KdpDE system in harmonizing the activities of both the Trk and KdpFABC systems to achieve optimum growth.

As\( ^{86} \)Rb\(^+ \) Uptake

Following determination of growth rates, we then assessed the magnitudes of \( K^+ \)-uptake by measuring the uptake of \( ^{86} \)Rb\(^+ \) by the WT and mutant strains at the mid- and late-log phases of growth as described previously (Steel et al., 1999; Cholo et al., 2015) and the results are shown in Figure 3.

Using the \( ^{86} \)Rb\(^+ \)-uptake model we have, previously shown that the Trk system has a low-affinity for \( K^+ \) being responsible for \( K^+ \) influx during bacterial growth at the mid- and late-log phases. On the other hand, the Kdp system has been characterized as the high-affinity \( K^+ \) transporter, which is suppressed during
the logarithmic phases of growth, being induced as a backup when the Trk system is not active (Cholo et al., 2006, 2015).

Somewhat surprisingly, the results revealed that the $^{86}$Rb$^+$-uptake efficiencies of the mutant strains, were higher than those of the WT strain at both the mid- and late-log phases being highest at the mid-log phase for the KT-double knockout mutant (Figure 3). At the late-log phase, the $^{86}$Rb$^+$-uptake efficiencies of the mutant strains were again higher than that of the WT strain, but not significantly different from one another (82,738 ± 8,309 and 98,569 ± 9,212 cpm for the KdpDE-deletion and KT-double knockout mutants, respectively, p = 0.126). These findings indicate that deletion of the KdpDE system results in K$^+$ overload, presumably due to dysregulation of the Trk and KdpFABC K$^+$ transporters, an event that may disrupt mycobacterial cytoplasmic pH, cellular metabolism, and growth.

For all the strains, the $^{86}$Rb$^+$-uptake efficiencies were significantly reduced at the late-log phase, being 46, 62, and 57% of the corresponding efficiencies noted at the mid-log phase for the WT, KdpDE-deletion and KT-double knockout mutant strains, respectively, most probably due to decreased extracellular pH (Cholo et al., 2015).

Extracellular Potassium Concentrations and pH Levels

Alterations in the extracellular K$^+$ concentrations and pH levels that occur during the early-, mid-, and late-log phases of growth of the WT and mutant strains of Mycobacterium tuberculosis under optimal culture conditions in 7H9 broth (15 mM K$^+$ and pH 6.8) were determined. In the case of the extracellular K$^+$ concentrations of the culture supernatants, these remained unchanged (14–15.5 mM) with respect to all three strains of M. tuberculosis and were comparable during the three log phases (Supplementary Table S1). The lack of significant alterations in extracellular K$^+$ may reflect recycling of K$^+$ during bacterial growth.

As shown in Figure 4, the extracellular pH values of the growth media increased at the early- to mid-log phases and decreased dramatically during the late-log phase, achieving statistical significance in comparison with the corresponding D0 pH value (pH 6.711) for all three strains. The extracellular pH levels of the two mutant strains were comparable at the three log phases of growth, but were higher than those of the WT strain.

**Gene Expression**

The effects of the KdpDE regulatory system alone and in the presence of the Trk system on the expression of the K$^+$-uptake genes during bacterial growth were explored by determining the expression levels of all six kdp and two trk genes in the WT and both mutant strains at the early-, mid-, and late-log
growth phases in standard 7H9 liquid culture medium using RT-PCR. We confirmed mutations of the kdpDE (kdpD and kdpE) and ceoBC (ceoB and ceoC) genes by formation of non-specific fragments, with different melting temperatures from those of targeted gene-specific fragments using melting curve analysis data (Supplementary Table S2).

The results were analyzed as absolute amounts (μg/ml; AQ) and relative quantifications (RQ) using sigA as the reference gene (Figure 5; Supplementary Table S3). As shown in our previous study under similar conditions (Cholo et al., 2015), the expression levels of the sigA gene were comparable between the WT and the KdpDE-deletion and the KT-knockout mutants between the early- and mid-log phases. However, at the late-log phase, expression levels of sigA were significantly increased in all the strains, possibly indicative of low pH-induced stress. Similarly, all the K'-uptake genes in all the strains were elevated during the late-log phase of growth, highlighting responses to alterations in the environmental conditions in the growth medium. Despite this, the levels of increased expression of the sigA gene in the WT were much lower than those of the K'-uptake genes illustrating the constant expression of the sigA gene at the logarithmic phase. However, the levels of sigA were excessively high in the mutants, increasing by 2–2.5-fold relative to those of the WT at the late-log phase due to low pH stress (Cholo et al., 2015), which are clearly shown by AQ data (Figure 5A; Supplementary Tables S3.1–S3.3).

For each strain, the expression of all the measured K'-uptake genes during growth was determined by comparing the expression levels of each gene at the mid- and late-log phases relative to those at the early-log phase.

WT Strain
Absolute Quantification
The AQ data for the WT strain were the same as we have previously reported (Cholo et al., 2015). In summary, during growth, at the early- and mid-log phases, the kdp and the trk genes were expressed at minimum levels and were upregulated at the late-log phase, with the ceoB gene being the most prominently induced gene among all the K'-uptake genes, followed by kdpD and kdpF (Figure 5A; Supplementary Table S3.3). Despite upregulation of the K'-uptake genes, bacterial growth was slow at the late-log phase, due presumably to low extracellular pH levels (Cholo et al., 2015). Similar findings have been reported in E. coli, revealing that expression levels of the trk and the kdp genes are dependent on the extracellular pH (Epstein, 2003).

Relative Quantification
The RQ values of the genes in the WT strain were determined during growth at the mid- and late-log phases in relation to the early-log phase (Figure 5B; Supplementary Table S3.4). Expression of the kdpDE and kdpBC genes was significantly increased at the mid-log phase, while all the kdp genes were upregulated at the late-log phase. In the case of the trk genes, expression levels of both genes were unchanged at the mid-log phase, while both genes were upregulated at the late-log phase, particularly the ceoB gene.

KdpDE-Deletion Mutant Strain
Absolute Quantification
During growth, expression levels of all the kdpFABC and trk genes were minimal at the early- and mid-log phases. However, the kdpFABC genes were upregulated at early-log phase probably as a response to the decrease in extracellular pH (6.7), while they were downregulated at mid-log phase at elevated pH level of 6.9. These genes were significantly increased by ≥1,000-fold at the late-log phase at the lower pH of 6.5 (Figure 3; Supplementary Tables S3.1 and S3.3). In relation to the WT (Cholo et al., 2015), the kdp genes were increased, while the trk genes were decreased at early-log phase. Both kdpFABC and trk genes were downregulated at mid-log phase. However, with the exception of the ceoB gene,
and similar to those of the WT, the expression levels of the ceoC and kdpFABC genes were significantly increased by at least 1.5 and up to 7-fold relative to those of the WT at the late-log phase.

**FIGURE 5** | Gene expression measured during the various phases of bacterial growth for the WT and mutant strains. (A) Ratio of gene expression of absolute quantities of the K⁺-uptake genes and the sigA gene in the mutant strains during growth at mid- and late-log phases in relation to early-log phase, (B) relative quantification of K⁺-uptake genes in the mutant strains during growth relative to early-log phase (2^{−ΔΔCq}), and (C) relative quantification of K⁺-uptake genes in mutant strains relative to the WT (2^{−ΔΔCq}). *represents mutations of the individual genes.

Relative Quantification
During growth, all the kdpFABC and trk genes were downregulated at the mid-log phase and upregulated at the late-log phase (Figure 5B; Supplementary Table S3.4). In relation
to the WT strain (Figure 5C; Supplementary Table S3.5), all the kdpFABC and trk genes were increased at the early-log phase, decreased at mid-log phase, and upregulated at the late-log phase, showing dependency on extracellular pH levels for their expression.

The AQ and RQ results appear to show that inactivation of the KdpDE system leads to dysregulation of both the kdpFABC and trk genes, resulting in constitutive expression of both operons.

**KT-Double Knockout Mutant**

Only the kdpFABC genes were evaluated as the kdpDE and trk genes were deleted in this mutant strain as shown by Tm of non-specific fragments due to mutations of the targeted genes (Supplementary Table S1).

### Absolute Quantification

The expression levels and patterns of the kdpFABC genes were similar to those of the KdpDE-deletion mutant at the three growth phases (Figure 5A; Supplementary Tables S3.2 and S3.3).

### Relative Quantification

During growth, the kdpA and kdpB genes were downregulated, while expression of the kdpC and kdpF genes remained unchanged at the mid-log phase (Figure 5B; Supplementary Table S3.4). All the kdpFABC genes were upregulated at the late-log phase. In relation to the WT strain (Figure 5C; Supplementary Table S3.6) and similar to the KdpDE-deletion mutant, all the kdpFABC genes were increased at the early-log phase, decreased at the mid-log phase, and upregulated at the late-log phase.

**DISCUSSION**

*Mycobacterium tuberculosis* is able to grow and adapt to varying environmental conditions. We have previously demonstrated that this bacterial pathogen grows exponentially at the mid-log phase at optimum K⁺ concentrations and pH levels (14–15 mM K⁺, pH 6.8–7.0), but is attenuated for growth, acquiring slow growth-to-dormant status at low pH levels (pH 5.5–6.0), despite maintaining elevated K⁺ concentrations at the late-log phase (Cholo et al., 2015).

Most bacteria adapt to varying extracellular K⁺ concentrations and pH levels by utilizing different K⁺-uptake transporters. Bacterial species, such as *E. coli* and *Salmonella* species, in which these K⁺ transporters have been extensively studied, utilize the Trk at elevated K⁺ concentrations and neutral pH levels and the Kup at low pH levels (pH 5.5–6.0), despite maintaining elevated K⁺ concentrations at the late-log phase (Cholo et al., 2015).

Utilizing the **Rb⁺** uptake procedure to determine bacterial K⁺-uptake efficiency, we demonstrated that during growth in optimal conditions, *M. tuberculosis* uses the KdpDE system to regulate K⁺ uptake by the KdpFABC and Trk transporters. In our previous studies, using the Trk-deletion mutant strain
Cholo et al. (2006, 2015), we demonstrated that at elevated K⁺ concentrations, bacteria utilize the low-affinity Trk system for K⁺ uptake, while the high-affinity Kdp system is suppressed, being induced as a back-up in the absence of Trk, conferring high K⁺-uptake efficiency on the Trk-deletion mutant (Cholo et al., 2006, 2015). The increase in K⁺-uptake efficiency of the KdpDE-deletion mutant strain in the presence of both KdpFABC and Trk K⁺-uptake transporters observed in the current study appears to demonstrate failure of the bacteria to differentially regulate the utilization of the two transporters, resulting in both systems being simultaneously operative. Excessive uptake of K⁺ is likely to result in dysregulation of bacterial cytoplasmic pH creating an intracellular environment unfavorable for cellular metabolism for growth.

However, at late-log phase (15 mM K⁺, pH 6.5), despite bacterial requirements of both the Kdp and Trk systems, the Trk has been shown to be the main K⁺-uptake transporter responsible for uptake of the cation. This contention is supported by the ⁸⁶Rb⁺ uptake data, showing low K⁺-uptake efficiency of the WT in relation to the Trk-deletion mutant, together with gene expression data (AQ) in the WT strain showing that the ceoB gene is the most highly induced gene among all the K⁺-uptake genes at late-log phase (Cholo et al., 2015). In the current study, we have shown that in these conditions, as with optimal growth, the bacteria use the KdpDE regulatory system to regulate the activities and expression of both K⁺-uptake transporters. This has been demonstrated by the findings of increased uptake of ⁸⁶Rb⁺ consistent with dysregulated, simultaneous, excessive functioning of both the Kdp and Trk K⁺-uptake transporters, in the absence of the KdpDE regulatory system.

These findings illustrate the constitutive activation of the KdpFABC transporter in the absence of its inducer KdpDE (Epstein, 2003; Steyn et al., 2003; Freeman et al., 2013; Agrawal and Saini, 2014). Similar findings have been demonstrated in previous studies in KdpDE mutant strains of E. coli (Asha and Gowrishankar, 1993; Sardesai and Gowrishankar, 2001; Epstein, 2003). These suggest a spontaneous induction of this operon in the absence of its inducer, or alternatively, the presence of an additional mechanism(s) of induction. While these have not been identified in M. tuberculosis, such mechanisms that bypass KdpDE have been described in E. coli and involve utilization of the histone-like nucleoid-structuring (H-NS) protein, thioredoxin 1, and thioredoxin reductase.
(Cole et al., 1998; Sardesai and Gowrishankar, 2001; Epstein, 2016). Although present in *M. tuberculosis*, the involvement of these mechanisms in activation of KdpFABC has not been described (Cole et al., 1998).

We do concede that our findings on the expression of these two mycobacterial K⁺-uptake transporters at various stages of growth in the absence of the TCS KdpDE system, are based on quantitation of their mRNA expression, which represents a potential limitation of our study. Nevertheless, we do believe that our findings demonstrate a novel and potentially important dual regulatory role of the KdpDE system in harmonizing the activities of the Trk and Kdp K⁺ transporters to ensure stringent control of cytoplasmic pH and growth. We also believe that these findings represent a platform that enables progression to additional confirmatory studies that would include target gene promoter expression using the β-galactosidase assay in this difficult and slow-growing pathogen.

These findings of the current study highlight the critical roles played by the K⁺-uptake transporters of *M. tuberculosis* during infection of the host. For example, in macrophages, the primary targets of the pathogen, in high intracellular K⁺ concentrations (phagosomal vacuolar K⁺ concentration: 19–50 mM; Wagner et al., 2005) and pH levels (pH 6.8; Piddington et al., 2000; Vandala et al., 2009), are probably conducive to bacterial growth. Presumably in this setting, the KdpDE and Trk systems are utilized by *M. tuberculosis* to establish infection (Haydel and Clark-Curtiss, 2004; Rengarajan et al., 2005; MacGilvary et al., 2019), possibly playing a role in bacterial virulence. However, at low pH levels, *M. tuberculosis* bacteria may utilize both the Kdp and Trk systems for survival (Figure 6). In this context, absence of the KdpDE system alone, and particularly in combination with the Trk system, is clearly detrimental to bacterial growth, underscoring the potential of these K⁺ transporters to serve as potential targets for development of anti-TB drugs.

In conclusion, in *M. tuberculosis*, the KdpDE system plays a key regulatory role in controlling the activities of the KdpFABC and Trk K⁺ uptake transporters to regulate growth.

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**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

MC and RA contributed to the conception and design of the study and wrote, edited and reviewed the manuscript. MC constructed the mutant strain. MC, MM, and AO performed the phenotypic experiments. MC, MM, AO, and RA contributed to interpretation of the data. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.698875/full#supplementary-material
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