

Genetic Mechanisms of D-cycloserine Resistance



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List of Abbreviations

| | |
|-------------------------------|---|
| µg | microgram |
| µL | microliter |
| <i>ald</i> | alanine dehydrogenase |
| <i>alr</i> | alanine racemase |
| BCG | Bacillus Calmette-Guérin |
| CC | critical concentration |
| CFU | colony forming units |
| <i>ddlA</i> | D-alanine:D-alanine ligase |
| DNA | deoxyribonucleic acid |
| DST | drug susceptibility testing |
| ECOFF | epidemiological cut off |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| INH | isoniazid |
| LJ | Löwenstein-Jensen |
| LOF | loss-of-function |
| MTBC | <i>Mycobacterium tuberculosis</i> Complex |
| MDR | multi drug-resistant |
| Mg | milligram |
| MGIT | mycobacterial growth indicator tubes |
| MIC | minimum inhibitory concentration |
| mL | millilitre |
| <i>M. bovis</i> | <i>Mycobacterium bovis</i> |
| <i>M. tuberculosis</i> | <i>Mycobacterium tuberculosis</i> |
| nt | nucleotide |

| | |
|-------------------------|--|
| OADC | oleic acid-albumin-dextrose-catalase |
| PCR | polymerase chain reaction |
| PZA | pyrazinamide |
| RD | region of difference |
| RIF | rifampicin |
| SNPs | single nucleotide polymorphisms |
| <i>S. aureus</i> | <i>Staphylococcus aureus</i> |
| TARGET | Tuberculosis Animal Research and Gene Evaluation Taskforce |
| TB | tuberculosis |
| TTP | time to positivity |
| WGS | whole genome sequencing |
| WHO | World Health Organisation |
| WT | wild-type |
| XDR | extensively drug-resistant |

List of Publications

1. COHEN, K. A., ABEEL, T., MANSON MCGUIRE, A., DESJARDINS, C., MUNSAMY, V., SHEA, T., WALKER, B. J., BANTUBANI, N., ALMEIDA, D., ALVARADO, L., CHAPMAN, S., MVELASE, N., DUFFY, E., FITZGERALD, M., GOVENDER, P., GUJJA, S., HAMILTON, S., HOWARTH, C., LARIMER, J., MAHARAJ, K., PEARSON, M., PRIEST, M., ZENG, Q., PADAYATCHI, N., GROSSET, J., YOUNG, S., WORTMAN, J., MLISANA, K., O'DONNELL, M., BIRREN, B., BISHAI, W., PYM, A. & EARL, A. 2015. Evolution of Extensively Drug-Resistant Tuberculosis over Four Decades: Whole Genome Sequencing and Dating Analysis of *Mycobacterium tuberculosis* Isolates from KwaZulu-Natal. *PLoS Medicine*, 12, e1001880
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Abstract

Introduction

Background: Similar to other infectious organisms, *Mycobacterium tuberculosis* (*M. tuberculosis*) is known to develop drug resistance via acquisition of mutations in its deoxyribonucleic acid (DNA). These genomic mutations often arise during the course of drug therapy as the result of non-compliance or inappropriate drug regimens, which create selective drug pressure that leads to the development of resistant strains. Although mutations that cause resistance to first-line anti-tuberculosis drugs have been well characterized, there is a significant amount of drug resistance in *M. tuberculosis* for which the genetic basis has not been defined. Knowledge of resistance conferring mutations is important for the treatment of drug-resistant tuberculosis (TB). Conventional phenotypic drug susceptibility testing (DST) in *M. tuberculosis* is a lengthy, cumbersome and requires laboratory containment. Therefore, molecular methods to determine drug resistance rapidly are highly desirable.

Method: To identify novel mutations associated with drug resistance in TB, we exploited the results of a whole genome sequencing (WGS) study of clinical *M. tuberculosis* isolates from KwaZulu-Natal (Cohen et al., 2015). We selected loss-of-function (LOF) mutations that were associated with resistance. Using isogenic strains with single genes inactivated, DSTs, growth inhibition and complementation assays we functionally tested if these LOF mutations caused resistance.

Results: LOF mutations in *ald*, encoding L-alanine dehydrogenase occurred only in drug-resistant clinical strains. The *ald* LOF mutations occurred frequently within resistant clinical isolates from Lineage 4. The *Δald* conferred a growth advantage in a single gene knockout model in the presence of D-cycloserine. Minimum inhibitory concentration (MIC) measurements of clinical and laboratory strains with mutations in *ald* displayed increased resistance to D-cycloserine when treated in vitro. With complementation analyses of the mutant with the cognate *M. tuberculosis ald* gene we confirmed susceptibility to D-cycloserine was partially restored, in this way confirming the association of the *ald* knock-out strain with D-cycloserine resistance.

Conclusion: LOF of *ald* may represent a novel mechanism of resistance to D-cycloserine. Whole genome association studies can identify novel drug resistance conferring mutations which will aid in improving rapid molecular diagnostics required for the development of tailored patient treatment regimens.

CHAPTER 1

Literature Review

1.1 Introduction

Approximately one third of the world's population is infected with *M. tuberculosis*, the etiologic agent responsible for TB (WHO, 2015). While many individuals who harbour *M. tuberculosis* never develop active disease, TB is often a lethal disease and is estimated to have been responsible for approximately 1.5 million deaths in the year 2015 (WHO, 2015).

Antibiotic therapy can be effective at treating the disease, however, the emergence of drug-resistant TB has made control efforts more difficult. Since its initial documentation in the 1940s (Schatz and Waksman, 1944, Crofton and Mitchison, 1948), drug-resistant TB has increased globally. In 2014, the World Health Organization (WHO) reported nearly 480,000 new cases of multidrug-resistant TB (MDR-TB) (WHO, 2015). MDR-TB is defined as *M. tuberculosis* with resistance to isoniazid (INH) and rifampicin (RIF). Regrettably, South Africa has one of the highest rates of TB globally, and one of the highest burdens of MDR-TB in the world (WHO, 2015).

Further along the scale of drug-resistant TB is the existence of extensively drug-resistant TB (XDR-TB). XDR-TB is defined as *M. tuberculosis* that is resistant to both INH and RIF, but also two other classes of anti-tuberculosis therapy, namely a fluoroquinolone and at least one of the injectable second-line drugs (Dahle, 2006). Second-line treatment is very expensive and more challenging to patient care as some of these drugs need to be administered intravenously (Pooran et al., 2013, Pietersen et al., 2014). TB treatment is often associated with serious medical complications due to certain drugs being very toxic and having harsh side effects (Sacchetti et al., 2008, Högberg et al., 2010).

Diagnostic limitations are a major barrier to the identification and subsequent management of drug-resistant TB. With a replication rate of approximately 24 hours, *M. tuberculosis* is a particularly slow growing bacterium (Ginsberg and Spigelman, 2007). Primary isolation using solid or liquid culture medium takes three to six weeks for isolation (Naveen and Peerapur, 2012). The slow growth rate of *M. tuberculosis* also means that standard phenotypic laboratory DST can take an additional six weeks to appropriately identify *M. tuberculosis* and determine a clinical isolate's individual drug susceptibility pattern (Garg et al., 2003, Parrish and Carrol, 2008). Clinically, these delays translate into late diagnosis of drug-resistant TB and deferred initiation

of an efficacious drug regimen, potentially leading to amplification of resistance, additional person-to-person spread of drug-resistant TB as well as increased morbidity and mortality (Garg et al., 2003).

Recently developed genotypic tools have improved diagnostic efforts. One such molecular test, the Xpert MTB/RIF assay has been recently implemented as a “point of care test” for the simultaneous detection of *M. tuberculosis* and diagnosis of RIF resistance in under two hours (Boehme et al., 2010). This test has been shown to have excellent sensitivity in smear-positive pulmonary TB (Boehme et al., 2010). These assays have improved TB case detection among both drug susceptible and MDR-TB. While Xpert MTB/RIF detects RIF resistance, it is unable to distinguish MDR from XDR-TB as well as being unable to detect INH and other mono-resistances (Evans, 2011). The Xpert MTB/RIF assay is less sensitive than culture therefore culture based assays are still required, and culture still remains the standard for TB diagnosis and DST (Evans, 2011).

Another molecular genetic tool used for the detection of resistance to first and second-line anti TB drugs of *M. tuberculosis* complex is the **GenoType MTBDR_{plus}** and **GenoType MTBDR_{sl}** (Hillemann et al., 2009, Miotto et al., 2008, Hillemann et al., 2005). These tests are based on a DNA strip technology that can be performed from pulmonary patient specimen and from culture material (Lacoma et al., 2008). Results are obtained in approximately five hours compared to one to two months with conventional methods. The **GenoType MTBDR_{sl}** test however, is only able to manipulate a limited number of resistance conferring mutations and this assay is only able to detect one in three to four cases of XDR-TB (Theron et al., 2014). The **GenoType MTBDR_{plus}** and **GenoType MTBDR_{sl}** is less sensitive in smear negative patients and requires confirmatory culture methods (Tomasicchio et al., 2016). Thus, these tools are still not optimal, and new improved therapeutic and diagnostic interventions are still urgently required to effectively halt the drug-resistant TB epidemic.

While current genotypic tests are useful, these tests are only able to identify known resistance mutations. As indicated above the **GenoType MTBDR_{sl}** has limited sensitivity for fluoroquinolone and aminoglycoside resistance indicating, there is still a significant amount of drug resistance in *M. tuberculosis* for which the molecular determinants are unknown (Zhang and Yew, 2009, Desjardins et al., 2016) even for these standard second-line drugs. Thus, it is likely that there are yet unidentified *M. tuberculosis* genes that confer drug resistance to these agents as well as to other second-line drugs where the resistance mechanisms have been less

comprehensively studied. Recent interest in WGS on sputum or on cultured isolates (Brown et al., 2015) has been increasing, this technology could potentially generate a full catalogue of all resistance conferring mutations and allow us to fully comprehend the genetic foundation of drug resistance (Köser et al., 2013, Coll et al., 2015, Starks et al., 2015, Colijn and Cohen, 2016).

The first *M. tuberculosis* genome to be successfully sequenced was the laboratory strain H37Rv by Cole and colleagues in 1998 (Cole et al., 1998). In this important undertaking, it was revealed that the *M. tuberculosis* genome contains 4,411,529 base pairs and approximately 4000 genes. While H37Rv is a fully drug susceptible strain, WGS has advanced dramatically since then and increasing numbers of studies of clinical isolates are beginning to shed additional light on the genetic diversity of drug susceptible and drug-resistant *M. tuberculosis* (Farhat et al., 2013, Köser et al., 2013, Zhang et al., 2013, Casali et al., 2014, Cohen et al., 2015, Farhat et al., 2016)

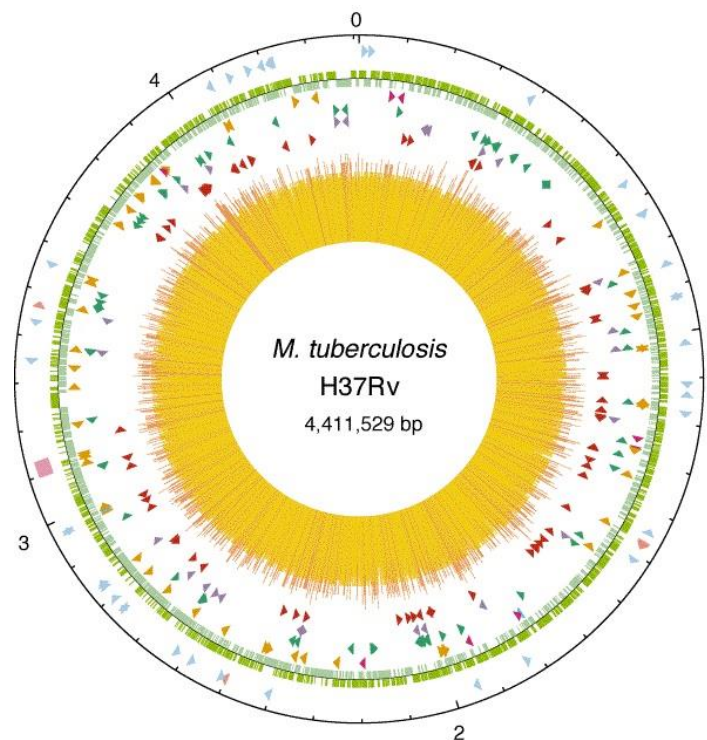


Figure 1.1: The *M. tuberculosis* H37Rv genome (Cole et al., 1998). H37Rv is GC-rich genome containing 4,411,529 base pairs. This genome was reported to encode 4057 genes, of which 4007 encoding RNA for proteins and 50 encoding for RNA molecules

Various studies using WGS have shown that for the first-line drugs INH and RIF; genome sequencing can predict a drug susceptibility phenotype determined in culture with high specificity and sensitivity (Coll et al., 2015, Walker et al., 2015). For example, a study conducted by Coll et

al., (2015) utilized WGS derived mutations as a predictor for drug resistance. In this study in silico inferred resistance phenotypes were compared to traditional DST. From their mutation analysis the authors were able to predict a 96.0% and 92.8% of resistance to RIF and INH respectively when compared to conventional methods (Coll et al., 2015). The comparison was less successful in other first-line drugs namely, pyrazinamide (PZA), as these strains possessed a resistant profile which was not recognizable by the genome analysis, although there are still reliability issues with DST for this drug. In ethambutol, however, it was found that susceptible strains possessed mutations that had previously been associated with resistance. Upon extending their analysis to second-line drugs, they found that only 39% of capreomycin resistant strains were unable to be identified by use of the authors in silico genome analysis. For the drug moxifloxacin, of the 42 strains that were tested, ten were classified as phenotypically resistant by conventional DST methods, but only six of which were identified as resistant by the mutation analysis (Coll et al., 2015).

In another study, Farhat et al., (2013) developed a phyC method to identify mutations significantly evolving more frequently on drug resistance branches of the phylogenetic tree as compared to drug sensitive mutations. This was done, utilizing a permutation test to control for phylogenetic tree topology and the distribution of phenotypic resistances (Farhat et al., 2013). The authors discovered that mutations in the gene *ponA1* conferred a modest growth advantage in the presence of low levels of RIF (Farhat et al., 2013). Zhang et al., (2013) developed a method that pre-filtered single nucleotide polymorphisms (SNPs) identified as phylogenetic i.e. inherited and not related to drug resistance (Zhang et al., 2013). The authors then identified genes with either an overabundance of non-phylogenetic mutations in general, or a greater number of non-phylogenetic mutations in drug-resistant strains than in drug sensitive ones (Zhang et al., 2013). Both studies robustly recovered known resistance mechanisms for a number of drugs, and identified novel mutations associated with resistance. However, only the *ponA1* mutation was characterised functionally to show that it contributed to RIF resistance so it is unknown whether any of the other identified mutations actually confer resistance. This illustrates how important it is to use molecular studies to characterise mutations identified in WGS association studies.

Another approach has been to use targeted sequencing of known resistance conferring loci (Farhat et al., 2016). Farhat et al., (2016), sequenced 1397 clinical strains and could predict >90% resistance to RIF and INH. However, the results of this study further illustrated that for second-line drugs a considerable proportion of drug resistance is not explained by known mutations.

Further elucidation of the genomic basis of resistance is therefore needed to develop much-needed improvements in the design of rapid molecular diagnostics. To develop these assays, we must identify all the relevant genes and genetic mutations that confer resistance. This will only be achieved by combining WGS studies with functional genomics.

In this masters study we used data from a genome wide association study to identify mutations associated with resistance. We focussed on frameshift mutations and intragenic insertions or deletions which we termed LOF mutations as these are guaranteed to inactivate the gene and result in a loss of gene function. In contrast the effects of non-synonymous SNPs on gene function are hard to predict in silico. Utilizing genomes from this completed WGS transmission study (Cohen et al., 2015), we selected LOF mutations associated with resistance. Screening isogenic strains with insertional inactivation of individual genes we were able to identify a novel mechanism by which *M. tuberculosis* gains resistance to D-cycloserine. We employed molecular techniques to determine that this *ald* LOF mutation caused resistance to D-cycloserine. To date, little is known about clinical isolates and resistance to D-cycloserine. Given the toxicities associated with D-cycloserine (Kendig et al., 1956, Murray, 1956, Bankier, 1965, Yew et al., 1993, Cunha, 2001, Torun et al., 2005, Mitnick et al., 2008) and technical difficulties (Kam et al., 2010) in performing DSTs to this drug, a rapid molecular test could provide a better avenue to determine resistance.

1.2 D-cycloserine

1.2.1 Discovery

D-cycloserine also known as oxazolidine, is an anti-tubercular drug that was initially discovered as a metabolic product of *Streptomyces* in 1952 (Kuehl Jr et al., 1955). D-cycloserine is an analogue of D-alanine, and was found to be produced by *Streptomyces lavendulae* and *Streptomyces garyphalus* (Harris et al., 1955, Kuehl Jr et al., 1955, Zawadzke et al., 1991). D-cycloserine exists in a cyclic form and is obtained naturally as a d-isomer (Lee et al., 1998).

1.2.2 Structure of D-cycloserine

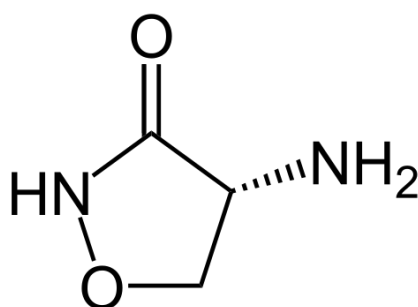


Figure 1.2: Chemical structure of D-cycloserine. This drug belongs to a group of organic compounds termed alpha amino acids (Takahashi et al., 1981), that have their amino group attached to a carbon atom, which is adjacent to a carboxylate group (Takahashi et al., 1981).

1.2.3 Mechanism of action

Early studies provided insight into the mode of action of D-cycloserine (Pittillo and Foster, 1954, Bondi et al., 1957, Morrison, 1962, Zygmunt, 1963). Pittillo and Foster.,(1954) demonstrated that D-alanine reversed the effects of D-cycloserine when added to liquid cultures of *Aerobacter aerogenes* (Pittillo and Foster, 1954).

Bondi et al.,(1957), showed in *Staphylococcus aureus* that alanine supplementation negated the effect of D-cycloserine (Bondi et al., 1957). This study deduced that D-cycloserine interferes with the alanine metabolism pathway (Bondi et al., 1957).

Further studies conducted on *Escherichia coli* (*E. coli*) and *S. aureus* demonstrated that D-alanine was more effective at reversing the effects of D-cycloserine than L-alanine (Zygmunt, 1963). Work conducted on *M. tuberculosis* and *Mycobacterium phlei* also demonstrated that growth inhibition by D-cycloserine was reversed by the addition of D-alanine (Morrison, 1962).

D-cycloserine is an analogue of D-alanine and is known to be an inhibitor of peptidoglycan cell wall biosynthesis (Caceres et al., 1997). Peptidoglycan is a composite polymer made up of glycan chains which are cross linked by short peptide chains (Typas et al., 2012, Barreteau et al., 2008). Peptidoglycan is an integral component of the bacterial cell wall including that of *M. tuberculosis* (Typas et al., 2012). In addition to providing architectural support, peptidoglycan is crucial for cell durability. Several potent antibiotics have also been known to target the synthesis of peptidoglycan, as peptidoglycan is an ideal target for drug design (Kieser and Rubin, 2014). Peptidoglycan is the backbone of bacterial cell wall, and is essential for cell wall biosynthesis and maintenance of all bacteria including mycobacteria (Morlock et al., 2003).

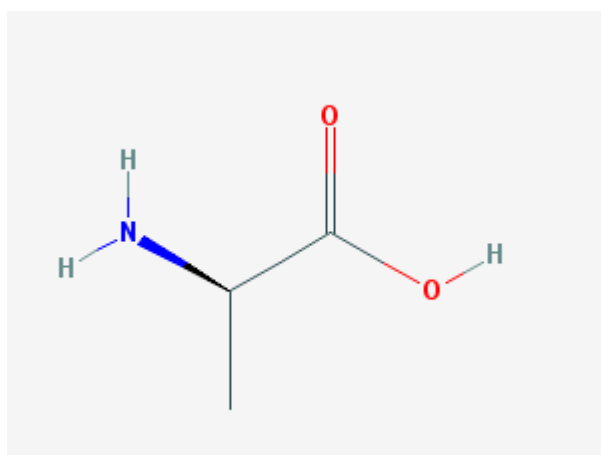


Figure 1.3: Chemical structure of D-alanine. D-cycloserine is the structural analogue of D-alanine (<https://pubchem.ncbi.nlm.nih.gov/compound/D-alanine>)

D-cycloserine is involved in the competitive inhibition of two key enzymes in the alanine metabolism pathway. These two important enzymes were also found to be targets in initial studies conducted on *E. coli* (Neuhaus and Lynch, 1964, Reitz et al., 1967, Lambert and Neuhaus, 1972). The two enzymes, alanine racemase (encoded by *alr*) (Lambert and Neuhaus, 1972) and D-alanine:D-alanine ligase (encoded by *dlla*) (Strominger et al., 1960, Prosser and de Carvalho, 2013) are essential in the synthesis of peptidoglycan (Walsh, 1989). Alanine racemase is essential for the conversion of L-alanine to D-alanine (Strych et al., 2001, LeMagueres et al., 2005). Bacteria use D-alanine for the synthesis of the peptidoglycan cell wall. Once D-alanine is created by alanine racemase, the D-alanine molecules are then joined to create a dipeptide. This D-alanine:D-alanine dipeptide is created by a ligase enzyme D-alanine:D-alanine ligase which synthesizes the dipeptides and allows for further elongation to create the peptidoglycan cell wall (Bruning et al., 2011).

In earlier studies conducted on *M. tuberculosis*, alanine racemase and D-alanine:D-alanine ligase enzymes were proposed targets of D-cycloserine in mycobacteria (Reitz et al., 1967, David et al., 1969). Studies conducted on *M. smegmatis* revealed that inactivation of the alanine racemase and D-alanine:D-alanine ligase enzymes result in susceptibility to D-cycloserine, and overexpression of alanine racemase is implicated in resistance to D-cycloserine (Zhang, 2005).

1.2.4 Clinical Uses

An oral bacteriostatic agent for the treatment of TB, D-cycloserine belongs to Group four of the World Health Organization categorization (WHO, 2008a). As a second-line agent, D-cycloserine is commonly used in the treatment of MDR and XDR-TB (Caminero et al., 2010). An advantage of using D-cycloserine in the treatment of drug-resistant TB is that it has not shown to have cross resistance to other antibiotics (WHO, 2008b, Caminero et al., 2010). Beyond its anti-tubercular activity, D-cycloserine is also known to have broad-spectrum activity against gram-positive bacteria, including *S. aureus* and some gram-negative bacteria, including *E. coli* (Roze and Strominger, 1966, Lambert and Neuhaus, 1972).

Terizidone is a derivative of D-cycloserine and structurally is formed by two molecules of D-cycloserine joined by a molecule of terephthalic dialdehyde (Zitkova and Toušek, 1974). Terizidone is rapidly metabolised to form two molecules of D-cycloserine after administration to humans. Terizidone has similar activity to D-cycloserine in that it is used to treat patients with MDR and XDR-TB and is also an oral bacteriostatic drug. Terizidone is administered to patients as it may have an improved safety profile and is a cheaper alternative in resource limited settings (Zitkova and Toušek, 1974, Galietti et al., 1991, Vora, 2010).

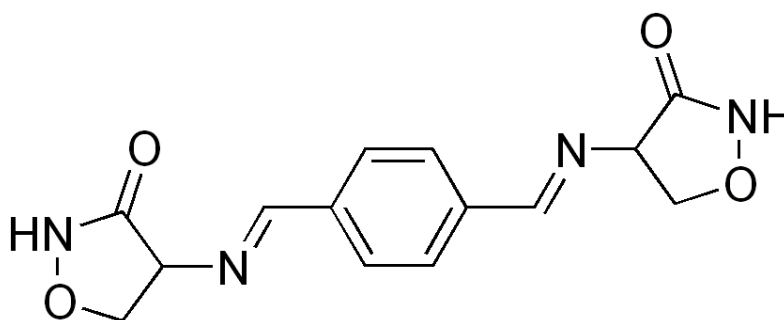


Figure 1.4: Chemical structure of Terizidone. This drug possesses double molecules of D-cycloserine that are joined by a molecule of terephthalic dialdehyde (Hwang et al., 2013).

1.2.5 Limitations of D-cycloserine chemotherapy

While D-cycloserine is an effective antimicrobial in TB treatment, its severe toxicity limits its use (Cunha, 2001, Torun et al., 2005, Mitnick et al., 2008). D-cycloserine has been known to promote increased anxiety and depression in patients (Kendig et al., 1956, Murray, 1956). Side effects include drowsiness, vertigo, memory loss, psychosis, convulsion and seizures (Yew et al., 1993, Bankier, 1965).

Phenotypic DST and current molecular testing for D-cycloserine resistance is not performed routinely by clinical laboratories due to instability of the drug (Kam et al., 2010) and due to difficulties with the assay. D-cycloserine is unstable in acidic conditions, but relatively stable under alkaline and neutral pH conditions (Woods, 2000, Kam et al., 2010). Therefore, use of freshly prepared solutions of D-cycloserine is recommended for determination of bacterial susceptibility, and complicates testing in clinical laboratories.

Given the toxicities of D-cycloserine, it would be ideal to prescribe this drug only to individuals who harbor susceptible strains (Bastos et al., 2014, Cegielski et al., 2014). Rapid diagnostics that predict resistance to these drugs do not yet exist, therefore the development of molecular diagnostics that assess D-cycloserine resistance will allow for optimization of MDR and XDR regimens. This in turn will lead to improved care of patients with drug-resistant TB.

1.3 Roles of D-alanine:D-alanine ligase and alanine racemase in the mechanisms of action and resistance to the peptidoglycan inhibitor D-cycloserine

Multiple studies in different organisms have shown that D-cycloserine interferes with the metabolism and incorporation of D-alanine into the peptidoglycan cell wall. D-cycloserine has been shown to target the two enzymes, alanine racemase and the D-alanine:D-alanine ligase. Only recently have studies been carried out to determine which of these enzymes is the principle target in mycobacteria.

1.3.1 D-alanine branch pathway of peptidoglycan biosynthesis

Alanine racemase and D-alanine:D-alanine ligase function in attaining D-alanine from L-alanine into UDP-muramyl pentapeptide. D-alanine is the central molecule which functions in the crosslinking step of peptidoglycan assembly and is a key component in peptidoglycan synthesis (Feng and Barletta, 2003). The pyridoxal phosphate-dependent alanine racemase catalyzes the conversion of L-alanine to D-alanine, while the ATP-dependent D-alanine:D-alanine ligase synthesizes the D-alanine:D-alanine dipeptide, which is incorporated into UDP-muramyl tripeptide by a D-alanine:D-alanine adding enzyme (MurF) (Feng and Barletta, 2003). These enzymes constitute the D-alanine branch pathway of peptidoglycan biosynthesis (Walsh, 1989).

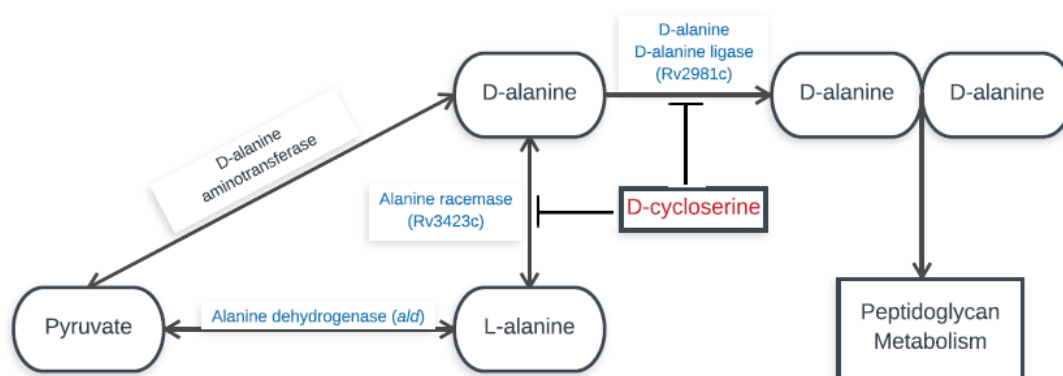


Figure 1.5: A diagrammatic representation showing the alanine metabolism pathway and targets of D-cycloserine in bacteria. Alanine racemase and D-alanine:D-alanine ligase are involved in the synthesis of peptidoglycan. Alanine racemase functions in converting L-alanine to D-alanine, which are joined to create a dipeptide. The dipeptide is formed by D-alanine:D-alanine ligase which allows for further elongation to create the peptidoglycan cell wall.

1.3.2 D-cycloserine competitively inhibits alanine racemase and the D-alanine:D-alanine ligase

The targets of D-cycloserine were initially elucidated in other bacteria. Experimental studies conducted on *S. aureus* demonstrated that D-cycloserine possessed the correct conformation that is required to bind L- and D-alanine on the enzyme surface. This was not demonstrated by L-cycloserine. The authors showed that D-cycloserine played a role in the competitive inhibition of alanine racemase (Roze and Strominger, 1966).

Studies conducted on *E. coli* showed that D-cycloserine can target both alanine racemase and D-alanine:D-alanine ligase (Lambert and Neuhaus, 1972). Research conducted on D-cycloserine resistant mutants of *Streptococcus gordonii* also showed an overproduction of the alanine racemase and D-alanine:D-alanine ligase enzymes, in this way proposing that these two enzymes play a synergistic role against D-cycloserine inhibition (Reitz et al., 1967).

These studies implicated both alanine racemase and D-alanine:D-alanine ligase as potential D-cycloserine drug targets in mycobacteria.

1.3.3 Mechanism of action of D-cycloserine in mycobacteria

The first proposal that D-alanine:D-alanine ligase was the target for D-cycloserine in *M. tuberculosis* was a study that isolated strains resistant to D-cycloserine and evaluated their uptake of alanine (David, 1971). The D-cycloserine resistant mutant strains were categorised as being either alanine permease competent or defective by measuring their uptake of D-alanine and other amino acids. The authors found D-cycloserine resistance mutants with normal uptake of D-alanine and hypothesised that these strains might harbour mutations in the *ddl* gene which encodes D-alanine:D-alanine ligase. However, there was no molecular confirmation to support the hypothesis (David, 1971).

The first formal genetic analysis of *alr* (gene which encodes alanine racemase) was conducted by Caceres *et al.*, (1997). The authors constructed a genomic library from a D-cycloserine resistant mutant of *M. smegmatis* which was transformed back into wild-type *M. smegmatis*. The clones, which were resistant to D-cycloserine, were selected and isolated for downstream analysis. In their experiments, D-cycloserine resistant mutants were observed to carry a mutation in the promoter region of *alr*, which subsequently causes its overexpression. The D-cycloserine resistant phenotype was also confirmed in *M. intracellulare* and *M. bovis* BCG by overexpressing the *M. smegmatis alrA* gene suggesting that this mechanism of resistance could also occur in slow-

growing and pathogenic mycobacterial species. They suggested that this was evidence indicating that *alrA* was the target for D-cycloserine, due to target amplification and less competitive inhibition by D-cycloserine (Caceres et al., 1997). However, if the target for D-cycloserine is actually *ddlA* then it is possible that increasing the D-alanine pool might overcome competitive inhibition at the second stage of the D-alanine branch pathway.

Further work on the characterization of mycobacterial *alr* was conducted in *M. smegmatis*. In a study where the *alr* gene was insertionally inactivated by homologous recombination, it was observed even in the absence of D-alanine, that *alr* was not necessary for growth (Chacon et al., 2002). The authors concluded that there could be an alternate pathway for D-alanine synthesis. However, in a subsequent study to re-evaluate if an *alr* knock-out could grow in the absence of D-alanine, the *alr* gene was knocked out and replaced with a kanamycin resistant cassette. Mutant colonies were grown in the presence and absence of D and L-alanine. The authors observed strains lacking the *alr* gene did in fact require D-alanine for growth in both Luria-Bertani medium and 7H11 minimal medium. This indicated that *alr* is essential for obtaining D-alanine for cell wall synthesis, compatible with it being the target for D-cycloserine (Milligan et al., 2007). This is indicative that insertional inactivation of the *alr* gene utilised by Chacon *et al.*, (2002) may not have eliminated all alanine racemase activity completely. Studies have shown that insertional inactivation can in some cases permit continued production of the active protein (Iredale, 1999, Vanhaesebroeck et al., 2004).

The role of alanine racemase was further investigated in macrophages of mice using *M. tuberculosis* H37Rv (Awasthy et al., 2012). The *alr* gene was inactivated using a two-step homologous recombination technique and tested in vivo and in vitro. In vitro growth assays showed that the *alr* knockout requires low concentrations of D-alanine for growth and removal of this amino acid resulted in no visible growth. In the macrophage model, the *alr* mutant exhibited poor growth relative to the wild-type illustrating the need for D-alanine for survival intracellularly. They went on further to infect mice intravenously with the wild-type H37Rv and the *alr* mutant. After a week of infection, mice were sacrificed and organs were plated for colony forming units (CFU). Once inside the mice *M. tuberculosis* strains lacked available D-alanine, which resulted in the *alr* mutant initially losing viability and thereafter stabilizing. This suggested that a lack or depletion of this amino acid makes cells more susceptible to D-cycloserine. The authors concluded that alanine racemase is a drug target of D-cycloserine (Awasthy et al., 2012).

To explore the relative roles of alanine racemase and D-alanine:D-alanine ligase in resistance to D-cycloserine a subsequent study showed that overexpression of the *ddl* gene from either *M. smegmatis* or *M. tuberculosis* could cause resistance to D-cycloserine in *M. smegmatis* raising the possibility that the D-alanine:D-alanine ligase could also be the target (Feng and Barletta, 2003). This was supported by the observation that a strain overexpressing both the *alr* and *ddl* genes displayed a higher level of resistance to D-cycloserine than strains overexpressing either gene alone. Belanger *et al.* (2000) engineered a temperature -sensitive mutant of *M. smegmatis* which was composed of a single amino acid substitution in D-alanine:D-alanine ligase (Belanger et al., 2000). The authors found the mutant strain to be more susceptible to D-cycloserine due to the decreased activity of the mutated D-alanine:D-alanine ligase enzyme further supporting the ligase as a target.

More recent metabolomic studies have suggested that D-alanine:D-alanine ligase is in fact the principal target for D-cycloserine (Prosser and de Carvalho, 2013). Using [²H] isotopically labelled L-alanine and a mass spectrometry approach the authors tracked alanine racemase and D-alanine:D-alanine ligase activity in *M. tuberculosis* treated with D-cycloserine in order to determine the mechanism of action. It was found that D-alanine:D-alanine ligase was more inhibited indicative of D-alanine:D-alanine ligase as a target of D-cycloserine (Prosser and de Carvalho, 2013).

Halouska *et al.*,(2013) aimed to identify the target of D-cycloserine by incorporating metabolomics and Nuclear Magnetic Resonance (NMR). They grew *M. tuberculosis* H37Rv and *M. smegmatis* mc² 155 cultures in media supplemented with ¹³C labelled alanine. The cultures were treated with D-cycloserine and the metabolome was extracted. The authors applied NMR to characterize the metabolome of *M. tuberculosis* H37Rv and *M. smegmatis* mc² 155 cultures (Halouska et al., 2013). They utilised D-alanine production as a readout and found an accumulation of this amino acid. The authors proposed that this may be as a result of the inhibition of D-alanine:D-alanine ligase by D-cycloserine. They concluded that D-alanine:D-alanine ligase is the principal target of D-cycloserine as cell growth is suppressed when the production of D-alanyl-D-alanine is stopped (Halouska et al., 2013).

1.4 Mechanism of Resistance to D-cycloserine

Resistance to D-cycloserine occurred as early as the 1950s (Steenken and Wolinsky, 1956, Bottero et al., 1958, Cohen and Dross, 1960, Grosset and Canetti, 1962). Strains resistant to D-cycloserine were first observed in *M. tuberculosis* in 1956 (Cummings, 1956). Cummings et al., (1956) showed 36% of patients receiving D-cycloserine therapy for six months developed resistance. Another early key finding was that resistant mutants selected in vitro had a range of MICs suggesting multiple mechanisms of resistance (Nitti and Tsukamura, 1957, Tsukamura et al., 1963). Despite these early phenotypic observations, the genetic mechanisms of resistance in clinical isolates has not been defined.

1.4.1 *cycA*

Like resistance to other drugs, D-cycloserine resistance occurs in steps with an additional level of resistance. Isolation and classification of step-wise D-cycloserine resistant mutants have been performed in numerous bacterial species, including *S. aureus* (Howe et al., 1964), *E. coli* (Curtiss et al., 1965), *Streptococcus gordonii* (Reitz et al., 1967) and mycobacteria (Caceres et al., 1997, David, 1971).

Further genetic studies characterised *cycA*, which encodes a permease (Curtiss et al., 1965) that transports the amino acids β -L-/D-alanine, glycine and D-serine as well as D-cycloserine. These studies led researchers to investigate *cycA* in mycobacteria. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), which originated from the infectious *M. bovis*, is used as a vaccine against TB (Brosch et al., 2007). BCG is innately resistant to D-cycloserine (Goh and Rastogi, 1991, Pelayo et al., 2009) and has a mutation in *cycA* relative to *M. bovis*. By complementing BCG with wild-type *cycA* they were able to partially restore susceptibility to D-cycloserine, suggesting *cycA* was also a D-cycloserine transporter in mycobacteria. But other mechanisms of resistance may also contribute to the D-cycloserine phenotype in BCG (Chen et al., 2012).

1.4.2 Alanine racemase and the D-alanine:D-alanine ligase

The studies described above would suggest that promoter or regulatory mutations that result in the upregulation of *alr* and *ddlA* genes result in resistance to D-cycloserine. Conceivable intragenic mutations in either or both of these genes could lead to a loss of affinity of D-cycloserine and resistance.

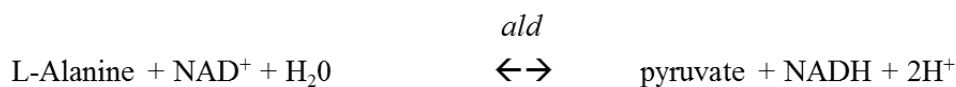
Due to the limited data available for clinical strains, of interest was a WGS study conducted on patient isolates. Here, the authors found in one particular patient the acquisition of the mutation S22L, a nonsynonymous mutation in the gene *alr* which was predicted to confer resistance to D-cycloserine (Merker et al., 2013).

1.4.3 Other mechanisms of resistance

In addition to *cycA*, *alr* and *ddlA* there may be other mechanisms of D-cycloserine resistance. Peteroy *et al.*,(2000) constructed a laboratory mutant resistant to both D-cycloserine and vancomycin of *M. smegmatis* (Peteroy et al., 2000). In pathogenic bacterial organisms resistance to D-cycloserine and vancomycin resistance is normally associated with D-alanine:D-alanine ligase which is encoded for by the *ddlA* gene (Van Heijenoort, 1996). The authors were unable to find mutations associated with this phenotype, including characterization of *ddl*, and the mechanism of resistance in their mutant model remains unknown. Work from other bacteria indicate that resistance to D-cycloserine may be more complex, and mutations in d-amino acid dehydrogenase (DadA) can cause resistance in *E. coli* (Baisa et al., 2013).

1.5 L-alanine dehydrogenase

L-alanine dehydrogenase was initially discovered in *Bacillus subtilis* (Wiame and Pierard, 1955). Rv2780 encodes L-alanine dehydrogenase (*ald*), which catalyzes the NAD-dependent interconversion between L-alanine and pyruvate. The forward reaction is necessary for aerobic utilization of alanine as a nitrogen source and the reverse reaction is involved in the reductive amination of pyruvate to L-alanine (Feng et al., 2002). The forward and reverse reaction of L-alanine dehydrogenase are as follows:



1.5.1 Significance of L-alanine dehydrogenase in pathogenesis

Alanine dehydrogenase activity has proven to be important in some bacteria as it has been associated with persistence models such as sporulation.

Bacillus subtilis has the ability to differentiate into heat-resistant spores under environmentally stressed conditions (Siranosian et al., 1993). This study revealed alanine is required for normal spore formation, and alanine dehydrogenase plays an essential role in growth when alanine is the sole carbon source, as alanine dehydrogenase is required for the catalytic deamination of alanine

to pyruvate and ammonia. The authors observed the *ald* gene is essential for normal sporulation, as the *ald* mutants showed a defectiveness in sporulation, and that pyruvate supplementation partially abrogated the phenotype suggesting that pyruvate generated by alanine dehydrogenase is an essential metabolic substrate under certain conditions (Siranosian et al., 1993).

Myxococcus xanthus, is a gram-negative bacterium which is able to produce myxospores under starvation conditions (Bretscher and Kaiser, 1978). This study confirmed that the *aldA* gene does encode a functional alanine dehydrogenase, which again is essential for the catalytic interconversion of alanine to pyruvate and ammonia. The *ald* mutants displayed reduced rates of sporulation, indicating *ald* is required for normal development (Ward et al., 2000).

In mycobacteria, the L-alanine dehydrogenase enzyme was found to be present in infectious *M. tuberculosis* strains and absent in the vaccine strains of BCG (Andersen et al., 1992). Chen et al., (2003) showed BCG strains were unable to utilise L-alanine or D-alanine as a sole nitrogen source due to a frameshift mutation in the *ald* gene (Chen et al., 2003). This could be reversed by complementing with wild-type *ald*. BCG's inability to replicate in humans has been proposed to be associated with the absence of a functional alanine dehydrogenase preventing the development of protective immunity (Scandurra et al., 2006). In *M. bovis* the *ald* gene possesses a single nucleotide deletion and consequently lacks alanine dehydrogenase (Chen et al., 2003). These studies demonstrated that there are differences in the central metabolism between *M. bovis* and *M. tuberculosis* (Chavadi et al., 2009). Regardless of changes in metabolism, *M. bovis* can cause disease in humans and BCG complemented with the *M. tuberculosis ald* gene displayed no change in survival in both macrophages and mice (Scandurra et al., 2006).

Mycobacterium marinum is the causative agent of fish and amphibian TB (Aronson, 1926, Clark and Shepard, 1963). Gene expression profiling of *M. marinum*, used as a surrogate to study the pathogenicity of *M. tuberculosis* in its persistence state in granulomas, found that *ald* was one of the genes upregulated in frog granulomas upon nutrient starvation (Chan et al., 2002). This indicates that *ald* may be important in maintaining the NAD pools under stressful conditions, or could be involved in persistence (Ramakrishnan et al., 2000, Chan et al., 2002).

In *Mycobacterium smegmatis* L-alanine dehydrogenase activity was increased during oxygen depleted conditions (Dick et al., 1998, Hutter and Dick, 1998, Raynaud et al., 1998, Usha et al., 2002). This has led to the belief that L-alanine dehydrogenase may be involved in the recycling of NADH under respiratory-inhibitory conditions such as hypoxia (Hutter and Dick, 1998). This

study also showed alanine dehydrogenase was overproduced under anaerobic conditions, which mimic dormancy (Hutter and Dick, 1998). Feng et al., (2002), further illustrating that L-alanine dehydrogenase may play an essential role in oxygen limited environments (Feng et al., 2002).

In a study conducted on *M. tuberculosis*, *ald* was found to be upregulated under hypoxic conditions (Giffin et al., 2012). Here, alanine dehydrogenase played a multi-specific enzymatic role where it was able to utilize both glyoxylate or pyruvate as substrates. In a subsequent study, alanine dehydrogenase was found to be highly expressed in the late non-replicating persistent (NRP-2) stage (Giffin et al., 2016). They also reported a growth defect in strains which possessed the alanine dehydrogenase mutant. This study in *M. tuberculosis* showed that *ald* had a role in re-aeration of hypoxic cultures.

1.6 Problem statement

The emergence of drug-resistant *M. tuberculosis* poses a huge threat to TB control strategies. To overcome this significant problem, there is need for the development of novel rapid diagnostics for the detection and identification of drug-resistant *M. tuberculosis*. Similarly, the identification of genes and mechanisms which may confer drug resistance in *M. tuberculosis* are urgently required to stem this global epidemic.

1.7 Rationale

To date little is known regarding the mechanism of resistance of D-cycloserine among *M. tuberculosis* clinical strains. By utilizing WGS we would be able to identify novel drug resistance conferring mutations, which could elucidate new mechanisms leading to D-cycloserine resistance. We incorporated the use of *ald*'s LOF in housekeeping and clinical *M. tuberculosis* strains to demonstrate D-cycloserine resistance *in vitro*.

1.8 Hypothesis

L-alanine dehydrogenase (*ald*) is involved in the alanine metabolism pathway. Genomic association studies identified LOF mutations in *ald* restricted to drug-resistant strains. We hypothesise that polymorphisms in *ald* may cause D-cycloserine resistance.

1.9 Aim

To determine the functional consequences of *ald*'s LOF and their effect on D-cycloserine susceptibility.

1.10 Specific Objectives

1. To characterise novel LOF mutations identified by genomic studies to determine their effect on drug susceptibility.
2. To perform DST's to first and second-line TB drugs on single gene knock-outs to identify genes that confer drug resistance.
3. To compare growth and viability of the sequenced clinical isolates under selective drug exposure.
4. To assess if loss of *ald* function confers increased cycloserine resistance as evidenced by longer TTP in Mycobacterial Growth Indicator Tubes (MGIT) supplemented with D-cycloserine.
5. To assess if complementation of *ald* restores growth inhibition by D-cycloserine.

CHAPTER TWO

Materials and Methods

2.1 Study design

2.1.1 Study Population

Samples for this study were obtained retrospectively and prospectively from individuals during 2008-2013 (based in Kwazulu-Natal, South Africa). Full ethical approval was obtained from the University of KwaZulu-Natal following protocols Ref:

EXP052/06- Rapid survey of drug-resistant tuberculosis in Kwazulu-Natal,

BF005/09- Treatment outcomes of Extensively Drug-Resistant (XDR-TB) at King George V Hospital, Kwazulu-Natal, South Africa

BE075/12- New diagnostics for TB Drug-Susceptibility Testing (TB-DST)

BE085/12- Analysis of any microbiological data generated from the laboratory, including bacteria, mycobacteria, fungi and parasites for publication purposes

and BE022/13- Collection of Sputum, Urine and Blood samples for research at K-RITH

Expedited ethical approval was obtained from the University of KwaZulu-Natal following protocol Ref:

BE482/16- Genetic mechanisms of D-cycloserine resistance

2.2 Procedures

2.2.1 DNA extraction

Isolation of genomic DNA using the cetyltrimethylammonium bromide (CTAB)-lysozyme method is one of the most efficient methods of extraction (Larsen et al., 2007a). This method yields high quality DNA which is useful for Southern blotting, PCR and the screening of clones (Connell, 1994). The CTAB-lysozyme method is suitable for isolation of DNA from small volume cultures (Larsen et al., 2007a).

2.2.1.1 Growth conditions of clinical strains prior to extraction

Clinical isolates were streaked on Middlebrook 7H10 agar medium. A single colony was picked for growth in Middlebrook 7H9 liquid medium supplemented with 0.5% glycerol, 0.05% Tween

80 and 10% oleic acid-albumin-dextrose-catalase (OADC) at 37°C with 5% CO₂ (Larsen et al., 2007b). DNA was extracted from the liquid culture using the (Larsen et al., 2007a) protocol.

2.2.1.2 Extraction of genomic DNA (Larsen et al., 2007a)

Procedure

Day 1

1. Preceding DNA extraction, 1 mL of 10% glycine was added to 10 mL late log culture. The samples were vortexed briefly and incubated at 37°C with a CO₂ content of ~5 % for 24 hours.

Day 2

2. Samples were removed from the incubator and transferred to individually labelled 50 mL conical tubes. Centrifugation followed at 2000 x g for 10 minutes.
3. The supernatant was discarded and samples were resuspended in 450 µL GTE solution. Samples were then transferred to individual microcentrifuge tubes holding 50 µL of a freshly prepared 10 mg/mL lysozyme solution.

Day 3

4. The samples were further incubated at 37°C, 5% CO₂ for 24 hours.
5. A 2:1 solution of 10% SDS and 10 mg/mL proteinase K was prepared. A volume of 150 µL of this freshly prepared solution was added to the samples and were mixed gently. The samples were incubated for a period of 30 minutes on a heating block at 55°C.
6. A 200 µL aliquot of NaCl was added to each sample tube and mixed gently.
7. CTAB solution was preheated to 65°C. A volume of 160 µL of this reagent was added to each sample tube. Samples were further incubated for 10 minutes at 65°C.
8. Following heating, ~1 mL of 24:1 (v/v) chloroform/isoamyl was added to the sample tubes. The tubes were shaken vigorously.
9. Tubes were centrifuged at 7000 x g for 5 minutes.
10. The aqueous supernatant (900 µL) was then transferred to a new microcentrifuge and ~900 µL of 24:1 (v/v) chloroform/isoamyl was added and the extraction process repeated.
11. Following centrifugation, the aqueous supernatant (~800 µL) was transferred to a new microcentrifuge tube.
12. To the supernatant 560 µL of isopropanol was added. Samples were mixed by inversion to precipitate the DNA.
13. Samples were incubated at room temperature for 10 minutes.
14. Following incubation samples were microcentrifuged at 7000 x g for 10 minutes.

15. The supernatant was then discarded and the pellet was washed with 1 mL of 70% ethanol.
16. Samples were again microcentrifuged at 7000 x g for 10 minutes.
17. The supernatant was discarded post ethanol wash and the pellets were allowed to air-dry for 15 minutes.
18. To the pellet 50 µL of TE buffer was added to elute the DNA. A 24-hour incubation in the 4°C refrigerator followed to dissolve the DNA.
19. Samples were then stored at 20°C for downstream applications.

2.2.2 Drug susceptibility testing (DST)

DST is critical in prescribing an effective drug regimen for appropriate patient care (Woods, 2000). Testing is traditionally conducted on cultured *M. tuberculosis* isolates from patients (Woods, 2000). Phenotypic testing involves culturing of patient isolates in the presence of TB drugs to determine resistance and susceptibility profiles (Sirgel et al., 2009).

2.2.2.1 Growth conditions for DST (Larsen et al., 2007b)

M. tuberculosis strains of interest were cultivated in Middlebrook 7H9 liquid medium supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% OADC at 37°C with 5% CO₂. Strains were grown to an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.8. Cultures were diluted to a starting inoculum of 1x 10³ CFU/mL for downstream testing. The initial inoculum was confirmed by plating onto 7H10 Middlebrook solid agar medium. Fresh cultures were used as older cultures may result in unreliable DST results. Cultures were grown from a glycerol stock and passaged once before DST set up.

2.2.2.2 Agar proportion method (Canetti et al., 1963, Canetti et al., 1969, Kent and Kubica, 1985)

DST was performed on 7H11 Middlebrook agar medium supplemented with OADC. Strains were tested on control and anti-tubercular media. The following drugs were tested at the WHO recommended critical concentrations i.e. isoniazid 0.1 µg/mL, isoniazid 1.0 µg/mL, rifampicin 1.0 µg/mL, ethambutol 7.5 µg/mL, streptomycin 2.0 µg/mL, ofloxacin 2.0 µg/mL and kanamycin 6.0 µg/mL. The critical concentration is defined as the amount of drug in the medium that prevents the growth of sensitive bacteria but not that of resistant mutants (WHO, 2008b).

When conducting the agar proportion method, resistance of a strain is established at the 1% level. A strain is considered resistant if 1% or more of the bacterial population is resistant to a specified drug. The tested cultures were incubated for 3-4 weeks. The percentage of the colonies on the drug containing agar plates is compared to the drug free medium to determine resistance.

Procedure

1. Strains grown on 7H10 agar medium were used as the source of inoculum.
2. Colonies were picked and homogenized with 3mm diameter sterile glass beads.
3. Samples were vortexed for approximately 20 seconds and the suspension adjusted to equate a McFarland standard 1.
4. Cultures were diluted ten-fold (0.5 mL of bacterial suspension diluted in 4.5 mL in sterile distilled water)
5. A volume of 100 μ L of each dilution was inoculated onto each quadrant plate.
6. Plates were incubated at 37 °C in the presence of 5% CO₂ for 3-4 weeks.
7. Result interpretation was done by comparing growth on control media and media containing drug. If more than 1 % of the test population was observed on the drug containing media, the result was interpreted as resistant to that drug.

2.2.3 *ald* complementation of CDC1551 knock-out and *M. bovis* BCG Danish 1331

2.2.3.1 PCR amplification of *ald* from H37Rv

All PCR reagents were kept on ice to prevent degradation. The Δald of *M. tuberculosis* (JHU2780-209) was obtained from the Tuberculosis Animal Research and Gene Evaluation Taskforce (TARGET) mutant library (<http://webhost.nts.jhu.edu/target/>).

A 1.116 kb fragment spanning the *ald* gene region was PCR amplified from *M. tuberculosis* (H37Rv) genomic DNA using primers from Integrated DNA Technologies (IDT) containing the *NotI* and *PciI* restriction sites.

Primer set KM_P27 (5' AAATTTGCGGCCGCATGCGCGTCGGTATTCCGACCG 3') and KM_P28 (5'GGTGGTACATGTTCAGGCCAGCACGCTGGCG 3') was used.

Procedure

1. In a sterile 0.2 mL microfuge tube, reagents were mixed in the following order:

Table 2.1 Reagents used in PCR amplification of *ald* from H37Rv

| Reagents | Amount (μL) |
|---------------------|--------------------------|
| Nuclease free water | 4 |
| Buffer(2x) | 12.5 |
| Template DNA | 0.25 |
| dNTPs mix | 5 |
| DMSO | 1.25 |
| Forward primer | 0.75 |
| Reverse primer | 0.75 |
| Taq DNA polymerase | 0.5 |
| Total | 25 |

2. The KOD Xtreme™ Hot Start DNA Polymerase kit was used (Merck Millipore, Cat. No:71975-3). The PCR reaction mix contained 1 x PCR buffer, 0.3 μM KM_P27 forward primer, 0.3 μM KM_P28 reverse primer, 0.4 mM dNTP mix, 0.5 U *Taq* polymerase, 5% DMSO and 0.025 ng total DNA.
3. All reagents were mixed gently by vortex and briefly centrifuged to collect all components to the bottom at the tube.

4. Amplification reactions consisted of an initial denaturation step of 2 minutes at 94°C, followed by 35 cycles of 15 seconds at 98°C, 30 seconds at 60°C, and 70 seconds at 68°C, and a final extension step of 5 minutes at 68°C. A final holding step was set at 4 °C.
5. PCR products were run on a 0.9% agarose gel at 150 V, 400 mA for 30 minutes with the correct size product excised.

2.2.3.2 Gel Purification of PCR product

This procedure was conducted in accordance with the protocol in the QIAquick® Gel Extraction Kit (Qiagen, Cat. No:28706) with modifications. Gel slices were excised from a 0.9% agarose gel.

Procedure

1. A 450 µL aliquot of Buffer QG was added to a micro-centrifuge tube containing a 150mg gel slice. A yellow colour of the Buffer QG is indicative of a relatively neutral pH (≤ 7.5).
2. This was followed by the addition of 150 µL 100% isopropanol to each sample tube and mixed gently.
3. A QIAquick spin column was then placed in a 2 mL collection tube. The sample was applied to the QIAquick column and centrifuged for 1 minute. In this way, DNA binding to the column took place.
4. The flow-through was discarded and the QIAquick column was inserted a collection tube.
5. An aliquot of 750 µL of Buffer PE was added to the column and centrifuged at 15000 x g for one minute.
6. The flow through was discarded. The column was centrifuged once more in the 2 mL collection tube for one minute at 15000 x g to eliminate remaining wash buffer.
7. The QIAquick column was placed into a sterile 1.5 mL microcentrifuge tube. Fifty microliters of nuclease free water (Qiagen.: Cat no 129114), was added to the middle of the QIAquick membrane to elute the DNA.
8. The column was allowed to stand for two minutes to increase the yield of purified DNA and thereafter centrifuged for two minutes. The product was stored at -20°C until further use.

2.2.3.3 Digestion of backbone vector and of PCR product

The backbone vector AYp240 contained an Emerald fluorescent protein expressed off the mycobacterial optimized promoter (MOP) (George et al., 1995) in a modified L5_integrating mycobacterial expression vector. *PciI* and *NotI* restriction sites were used to digest the chosen vector to remove the Emerald fluorescent protein as well as to digest the PCR amplified product,

to facilitate insertion of the *ald* gene to create the pMCZ-MOP_Rv2780 expression vector for complementation.

Digestion conditions for backbone vector (AYp240):

Table 2.2 Reagents used in digestion reaction of the backbone vector (AYp240)

| Reagents | Amount (μL) |
|-------------------|--------------------|
| Template DNA | 39.53 |
| Buffer | 5 |
| BSA | 0.5 |
| <i>PciI</i> | 2.5 |
| <i>NotI -HF</i> | 2.0 |
| dH ₂ O | 0.47 |
| Total | 50 |

Digestion conditions for PCR product (*ald*):

Table 2.3 Reagents used in digestion reaction of the PCR product (*ald*)

| Reagents | Amount (μL) |
|-------------------|--------------------|
| Template DNA | 22.67 |
| Buffer | 5 |
| BSA | 0.5 |
| <i>PciI</i> | 2.5 |
| <i>NotI -HF</i> | 2.0 |
| dH ₂ O | 17.33 |
| Total | 50 |

2.2.3.4 Gel Purification of backbone vector and PCR product

This procedure was performed as explained in section 2.2.4.2 using the QIAquick® Gel Extraction Kit (Cat. No.:28706).

Procedure

1. The gel purified products were run on a 0.9% agarose gel at 150 V, 400 mA for 30 minutes.
2. Gel images were viewed using the BIO-RAD ChemiDoc™ MP Imaging System.

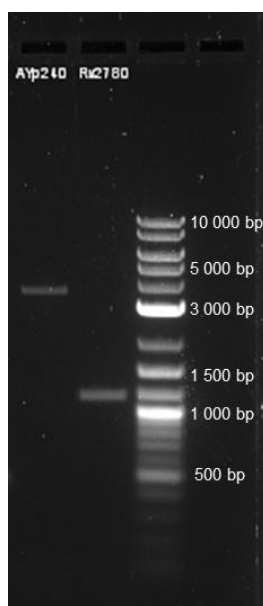


Figure 2.1: Agarose gel electrophoresis of digested and purified backbone vector and ald PCR product. AYp240 and PCR amplified *ald* (Rv2780) were restriction digested using *PciI* and *NotI*. Digested products were purified using gel extraction. Purified digests were run on a gel to confirm digests.

2.2.3.5 Ligation of *ald* fragment with mycobacterial expression vector

Procedure

1. After digestion and purification of the vector backbone and PCR product, a 5:1 molar ratio of insert to vector was incubated for 4 hours at 22°C to facilitate ligation of the *ald* to the vector backbone.
2. The ligation reaction was set up as follows:

Table 2.4 Reagents used in the ligation reaction of the PCR product (*ald*)

| Reagents | Amount (μL) |
|-------------------|--------------------------|
| Vector | 5.1 |
| Insert | 6.56 |
| Buffer | 2 |
| Ligase | 0.2 |
| dH ₂ O | 6.14 |
| Total | 20 |

3. The entire ligation reaction was then dialyzed using a 0.025 μm nitrocellulose membrane (Merck Millipore, Cat Number VSWP02500) to remove excess salts and enzymes.
4. A 2.5 μL aliquot of the dialyzed ligation mixture was added to 50 μL in house prepared DH5 α *E. coli* electrocompetent cells and gently mixed by tapping lightly. All tubes were kept on ice.
5. The mixture was then transferred to a 0.2cm cuvette and then electroporated in the BIO-RAD **Gene Pulser Xcell** using the following settings: 2.5 kV, 25 μF and 1000 Ω , following which cells were kept on ice for two minutes.
6. The cells were recovered in 1 mL SOC medium, and incubated shaking at 37°C for 1hour to allow for healing and expression of the antibiotic resistance product.
7. Healed cells were then plated on LB agar + 50 $\mu\text{g}/\text{mL}$ Zeocin and incubated at 37°C overnight.

2.2.3.6 Plasmid Mini Preparation

This procedure was conducted in accordance with the protocol in the QIAprep® Spin Miniprep Kit (Qiagen, Cat. No: 27106) with slight modifications.

Procedure

1. Six colonies from transformation plates were picked and used to inoculate 5 mL LB broth + 50 $\mu\text{g}/\text{mL}$ zeocin in 15 mL centrifuge tubes, which were incubated at 37°C overnight.
2. A 2 mL volume of the overnight cultures were centrifuged at 12000 x *g* for 3 minutes to pellet cells.
3. Supernatants were discarded and the pellets resuspended in 250 μL of Buffer P1 (Tris Buffer), and transferred to 1.5 mL microcentrifuge tubes.

4. A volume of 250 μL of Buffer P2 (NaOH and SDS) was added to the resuspended pellets containing Buffer P1 and mixed by inverting the tubes 4-6 times. A clear solution was observed.
5. Thereafter, 350 μL Buffer N3 was added (acetic acid, a neutralisation agent), and the microcentrifuge tubes were mixed thoroughly by inverting 4-6 times, at which point, the solution turned turbid.
6. Samples were then centrifuged for 10 minutes at 15000 x g.
7. Supernatants were transferred to labelled spin columns, following which the columns were centrifuged for 60 seconds at 15000 x g and the flow through from the collection tubes discarded.
8. Spin columns were then washed with 750 μL Buffer PE and centrifuged for 60 seconds at 15000 x g and the flow through discarded.
9. An additional centrifugation step was conducted for 60 seconds at 15000 x g to remove residual wash buffer.
10. Spin columns were then placed in a clean 1.5 mL microcentrifuge tubes.
11. Samples were eluted by the addition of 50 μL nuclease free water (Qiagen.: Cat no 129114) to the membrane of the spin columns. Tubes were allowed to stand for two minutes before being centrifuged for two minutes at 15000 x g to recover plasmid DNA.

2.2.3.7 Confirmatory Digest and Gel Electrophoresis

Procedure

1. Plasmid DNA preparations were then digested using restriction enzyme *SacI* to confirm successful clones.
2. The confirmatory digest was set up as follows:

Table 2.5 Reagents used in confirmatory digestion of transformants

| Reagents | Amount (μL) |
|-------------------|--------------------------|
| Template DNA | 2 |
| Buffer | 1 |
| BSA | 0.1 |
| <i>SacI</i> | 0.9 |
| dH ₂ O | 6 |
| Total | 10 |

3. Digest products were run on a 0.9% agarose gel at 150V, 400mA for 30 minutes.
4. Gel images were viewed using the BIO-RAD ChemiDoc™ MP Imaging System.

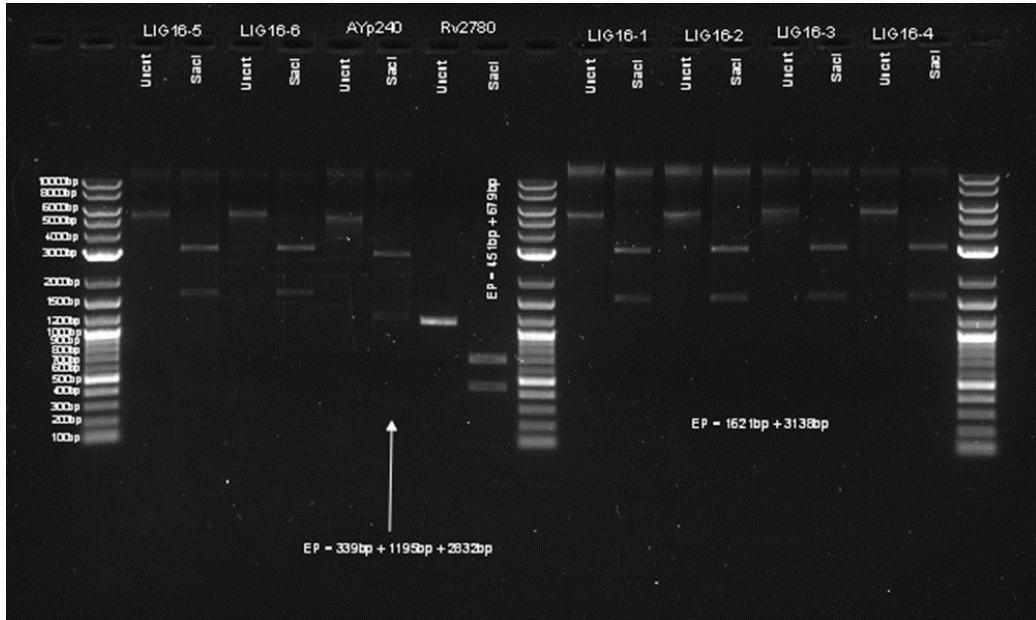


Figure 2.2: *SacI* digest confirmation of transformant colony picks

Gel image showing confirmatory digest of transformant colony picks for the pMCZ-MOP_Rv2780 complementation vector. Expected digested product sizes are 339bp and 1195bp for plasmid carrying the exogenous *ald*.

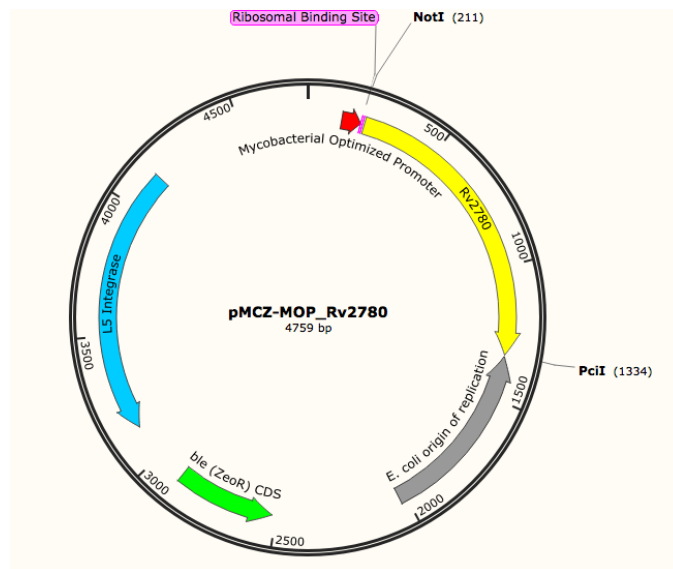


Figure 2.3: Schematic illustration of pMCZ-MOP_Rv2780. Rv2780 (*ald*) was cloned into our L5 integrating, zeocin-marked mycobacterial expression vector, under the control of MOP using the *NotI* and *PciI* sites indicated.

2.2.3.8 Transformation of *ald* into *M. tuberculosis* (Van Kessel and Hatfull, 2007, Parish and Stoker, 1998)

A successful clone of pMCZ-MOP_Rv2780 was then transformed into CDC1551 Δ *ald* and *M. bovis* BCG Danish 1331.

Procedure

1. *M. tuberculosis* strain CDC1551 Δ *ald* was grown in Middlebrook 7H9 liquid medium containing kanamycin (50 μ g/mL) supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% OADC. The *M. bovis* BCG Danish 1331 was grown in Middlebrook 7H9 liquid medium supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% OADC. These strains were incubated at 37°C with 5% CO₂ to an optical density at 600 nm (OD₆₀₀) of 0.6 to 0.8. 10 mL of starting culture was used.
2. The culture was transferred to individually labelled 50 mL conical tubes. Centrifugation followed at 4000 x g for 15 minutes.
3. The supernatant was discarded and the pellet was washed with a 5 mL volume of sterile room temperature 10% sterile glycerol. The cells were then centrifuged at 4000 x g for 15 minutes.
4. Step 3 was repeated with the pellet this time being washed with a 2.5 mL volume of sterile room temperature 10% sterile glycerol.
5. After centrifugation and discarding of the supernatant, the pellet was resuspended in 1/10th the original volume of the culture.
6. A 400 μ L volume of cells was used for each transformation. Total DNA of 100ng was mixed with the respective competent cells in a sterile microcentrifuge tube.
7. The bacteria and DNA was then transferred to a 0.2cm cuvette and then electroporated using the Biorad **Gene Pulser Xcell** using the following settings: 2.5 kV, 25 μ F and 1000 Ω . A volume of 1 mL of 7H9 containing OADC and 20% Tween 80 was added to the electroporated cells and then transferred to a 30 mL inkwell which was incubated shaking at 37°C overnight to allow for expression of antibiotic resistance.
8. Healed cells were plated on 7H10 agar containing zeocin (20 μ g/mL) for *M. bovis* BCG Danish 1331, and zeocin (20 μ g/mL) and kanamycin (25 μ g/mL) for CDC1551 Δ *ald*, with the plates being incubated at 37°C for 3-4 weeks to allow for the growth of transformants.

2.2.3.9 Small Scale DNA Extraction for confirmatory diagnostic PCR

This procedure was conducted in accordance with the protocol in the Hain **GenoLyse** Extraction Kit (Cat. No.: 51610) with modifications.

Procedure

1. Two transformant colonies for each transformation were scraped off the plate and used to inoculate 3 mL of 7H9 liquid medium, containing zeocin (20 µg/mL) for *M. bovis* BCG Danish 1331::pMCZ-MOP_Rv2780 (hereafter referred to as BCG::ald-comp), and zeocin (20 µg/mL) and kanamycin (25 µg/mL) for CDC1551Δald:: pMCZ-MOP_Rv2780 (hereafter referred to as Δald-comp), supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% OADC. These cultures were incubated shaking at 37°C for two weeks.
2. An aliquot of 500 µL cells were centrifuged for 5 minutes at 15 000 x g
3. After centrifugation the supernatant was discarded and 100 µL of Lysis Buffer (A-LYS) was added to the pellet and resuspended.
4. The samples were incubated for a period of 30 minutes on a heating block at 95°C.
5. A 100 µL aliquot of Neutralization Buffer (A-NB) was added to each tube and mixed gently.
6. The tubes were then centrifuged for 5 minutes at 15 000 x g.
7. Following centrifugation, the supernatant was transferred to a new microcentrifuge tube, with a 5 µL aliquot of the supernatant used for downstream PCR.

2.2.3.10 Small Scale DNA Extraction for confirmatory PCR

PCR primers were internal to the integrated region of the plasmid, but external in relation to *ald*, to ensure PCR amplification of our exogenously inserted *ald* gene and not the endogenous *ald* locus.

A 1.319 kb fragment spanning the MOP and *ald* gene was amplified from transformant colony picks using the ANY_P58 (5'TGGCAGTCGATCGTACGCTAGTT 3') and ANY_P59 (5'GAGCCTATGGAAAAACGCCAGCA 3') primers from Integrated DNA Technologies (IDT). All PCR reagents were kept on ice to prevent degradation.

Procedure

1. In a sterile 0.2 mL microfuge tube, reagents were mixed in the following order:

Table 2.6 Reagents used in PCR amplification of exogenously inserted *ald* gene

| Reagents | Amount (μL) |
|---------------------|-----------------------------------|
| Nuclease free water | 1 |
| Buffer(10x) | 12.5 |
| Template DNA | 2.5 |
| dNTPs mix | 5 |
| DMSO | 1.25 |
| Forward primer | 1 |
| Reverse primer | 1 |
| Taq DNA polymerase | 0.75 |
| Total | 25 |

2. The Qiagen *Taq* DNA Polymerase kit was used (Qiagen, Cat. No:201203. The PCR reaction mix contained 1 x PCR buffer, 0.4 μ M ANY_P58 forward primer, 0.4 μ M ANY_P59 reverse primer, 0.4 mM dNTP mix, 0.5 U *Taq* polymerase and 5% DMSO.
3. All reagents were mixed gently by vortex and briefly centrifuged to collect all components to the bottom of the tube.
4. Amplification reactions consisted of an initial denaturation step of 2 minutes at 94°C, followed by 35 cycles of 15 seconds at 98°C, 30 seconds at 55°C, and 90 seconds at 68°C, and a final extension step of 5 minutes at 68°C. A final holding step was set at 4 °C. A gel product of 1.319kb was confirmed by electrophoresis.
5. The PCR products were run on a 0.9% agarose gel at 150V, 400mA for 30 minutes.
6. Gel images were viewed using the BIO-RAD ChemiDoc™ MP Imaging System.

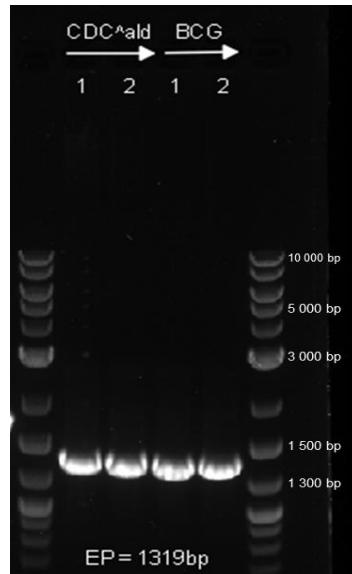


Figure 2.4: Confirmation of complementation of *ald* gene in CDC 1551 Δald and *M. bovis* BCG

Gel image showing PCR amplification of a 1.319kb fragment spanning the MOP and *ald* gene from DNA obtained from $\Delta ald-comp$ and BCG::*ald-comp* transformant colony picks confirming presence of exogenously introduced *ald*.

2.2.4 D-cycloserine growth assay assessed by Mycobacterial Growth Indicator Tube

2.2.4.1 *BD BACTEC™ MGIT™ 960 System* ((Leitritz et al., 2001, Alcaide et al., 2000, Tortoli et al., 1999, Hanna et al., 1999)

The BACTEC™ MGIT™ 960 System is an automated system which utilizes a fluorescent indicator i.e. an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate, which is able to detect oxygen depletion due to bacterial growth. As oxygen is used, the fluorochrome is no longer inhibited and results in fluorescence within the MGITs.

The system accommodates MGITs which are incubated at 37°C. The tubes are scanned into the system by a barcode, the machine scans the MGITs every hour for increased fluorescence. Viable organisms utilize the oxygen and thus produce a fluorescent signal, indicating positivity. A tube is considered instrument negative if they remain in the machine for a period of 42-56 days with no signs of growth/positivity. TTP is recorded in days and hours.

The MGIT medium consists of 7 mL modified Middlebrook 7H9 broth base medium that supports faster growth of mycobacteria. MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement is further added to the medium to support the growth of *M. tuberculosis* complex.

2.2.4.2 *MGIT Preparation* (see Appendix 1)

2.2.4.3 *Antibiotic preparation* (see Appendix 1)

The MGIT tubes were added with and without D-cycloserine to achieve final concentrations of 0, 7.5 µg/mL, 15 µg/mL and 30 µg/mL. MGITs were set up in triplicate. A drug control for each concentration of D-cycloserine was set up as a negative control.

2.2.4.4 *Growth conditions for MGIT growth inhibition assay and set up* (Larsen et al., 2007b)

M. tuberculosis strains of interest were cultivated in Middlebrook 7H9 liquid medium supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% OADC at 37°C with 5% CO₂ with agitation at 37°C. Strains were grown to an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.8. Cultures were diluted to a starting inocula of 1x 10³ CFU/mL for downstream testing. The initial inoculum was confirmed by plating onto 7H10 Middlebrook solid agar medium. Fresh cultures

were used as older cultures could result in unreliable results. Cultures were grown from a glycerol stock and passaged once before assay set up.

Procedure

1. Growth of cultures was monitored by measuring the OD₆₀₀.
2. If strains were at their desired OD i.e. ~ 0.3-0.8, they were diluted to 1×10^3 CFU/mL in 50 mL conical tubes.
3. The diluted cultures were vortexed and 500 μ L was added into each MGIT tube for each concentration in triplicate.
4. Dilutions of the starting inoculum were plated onto 7H10 Middlebrook solid agar medium to determine the CFU of the initial inoculum.
5. After inoculation, each MGIT was scanned into the BACTEC™ MGIT™ 960 System.
6. Tubes remained in the system until positive for growth. Once flagged positive, the TTP for each tube was recorded.

2.2.5 D-cycloserine minimum inhibitory concentration (MIC) determination on Löwenstein–Jensen (LJ)

Löwenstein–Jensen (LJ) is a selective egg-based medium used to culture *Mycobacterium* species for decades (Canetti et al., 1963). LJ slants incorporates inspissated eggs, malachite green and glycerol to promote the growth of mycobacteria (Canetti et al., 1969). D-cycloserine drug susceptibility has only been validated on LJ medium and is not performed routinely due to instability associated with the drug (Woods, 2000, Kam et al., 2010).

2.2.5.1 Antibiotic preparation (see Appendix 1)

2.2.5.2 LJ medium preparation (see Appendix 1)

2.2.5.3 Growth conditions for LJ MIC determination of D-cycloserine and assay set up (Larsen et al., 2007b)

M. tuberculosis strains of interest were cultivated in Middlebrook 7H9 liquid medium supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% OADC at 37°C with 5% CO₂. Strains were grown to an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.8. Cultures were diluted to a starting inocula of 1×10^3 CFU/mL for downstream testing. The initial inoculum was confirmed by plating onto 7H10 Middlebrook solid agar medium. Fresh cultures were used as older cultures may result in unreliable results. Cultures were grown from a glycerol stock and passaged once before assay set up.

Procedure

1. Growth of cultures was monitored by measuring the OD₆₀₀.
2. The strains were diluted to 1×10^3 CFU/mL in 50 mL conical tubes.
3. The diluted cultures were vortexed and 100 μ L was added into each LJ slopes for each concentration in duplicate.
4. Dilutions of the starting inoculum were plated onto 7H10 Middlebrook solid agar medium to determine the CFU of the initial inoculum.
5. The inoculated LJ slopes were incubated at 37°C with 5% CO₂ for 4 weeks.
6. Result interpretation of the LJ slopes were done after 4 weeks to report the MIC for each tested strain. MIC is defined as the lowest drug concentration, which inhibits 90% of the bacterial population when compared to the no drug control (Sirgel et al., 2009, Angeby et al., 2012).

2.2.6 Statistical tests

Statistical analysis and graphical representation of graphs were done on Graph Pad Prism (version 6.0). Statistical tests used in the analysis were the Two-Way ANOVA, to compare across groups. A p value <0.05 was considered significant. The Mann-Whitney U test was used for pairwise comparisons. One-sided t-test was used to compare mean TTP and growth inhibition in MGIT.

CHAPTER THREE

Results

3.1 Population based WGS of *M. tuberculosis* clinical isolates

We assembled a collection of drug susceptible and drug-resistant clinical isolates that were collected retrospectively and prospectively from individuals during 2008-2013 in Kwazulu-Natal, South Africa (Table 3.1). We selected isolates from different studies to have a population based sample that was enriched for drug resistance isolates and reflected the antecedents of current circulating strains.

The KwaZulu-Natal Drug Surveillance Study was a multicentre study conducted to determine the incidence of drug-resistant TB across KwaZulu-Natal, included susceptible isolates, and provided samples that represented the diversity of strains circulating in the province. The PROX, CUBS and NHLS studies were prospective studies of patients with MDR and XDR-TB, and allowed us to enrich for strains with multiple second-line resistances. The Phage study was also a contemporary prospective study that allowed us to access drug susceptible isolates. We also included three historical isolates (KZN4207, KZN1435, KZN605) that had been previously sequenced (Koenig, 2007, Ioerger et al., 2009).

For genotypic-phenotypic studies it is important to eliminate the potential confounding effects of mixed infection. We therefore streaked isolates from glycerol stocks onto 7H10 agar medium and picked single colonies for sub-culture for the majority of these strains. DNA was extracted using conventional CTAB methodology (Larsen et al., 2007a). WGS and analysis of these strains was conducted at the BROAD Institute (Cohen et al., 2015).

Table 3.1: Description of study cohorts (Cohen et al., 2015)

| Cohort Name | Cohort Description | Collection Strategy and Year of Collection |
|---|--|---|
| KwaZulu-Natal Drug Surveillance Study (KZN-SUR) | Cross-sectional study of outpatients and inpatients hospitalised with a cough. Samples were collected from each district in KwaZulu-Natal | Retrospective, 2008–2010 |
| Prospective Collection of Extensively Drug-Resistant TB (PROX) | Prospective study of patients newly commencing XDR treatment at the King DinuZulu Hospital | Prospective, 2010–2012 |
| Phage Study | Patients newly diagnosed with pulmonary TB at Prince Cyril Zulu clinic in central Durban. These samples were collected before the start of treatment | Prospective, 2013 |
| National Health Services Laboratory (NHLS) | Collection of drug-resistant clinical isolates sent for DST at the central NHLS TB Laboratory | Prospective, 2013 |
| Collection of Urine Blood Sputum Study (CUBS) | Prospective collection of patients newly initiating MDR or XDR treatment at the King DinuZulu Hospital | Prospective, 2013 |

3.2 Identification of loss-of-function mutations associated with resistance.

To identify mutations associated with resistance our sequences of clinical isolates from KwaZulu-Natal were combined with a dataset of 161 sequenced strains from China. Whole genome association analysis to identify drug resistance conferring mutations in *M. tuberculosis* is problematic. The accumulation of mutations occurs in a stepwise fashion, with resistance to first-line drugs usually preceding second-line and third-line resistance. As a result, mutations known to cause resistance to first-line drugs are often associated with second-line phenotypic resistance when association analysis are done. In addition, drug resistance is transmitted and clonal expansion of drug-resistant strains has amplified resistance in our setting (Cohen et al., 2015). Genetic drift can also occur during clonal expansion and non-resistance conferring mutations can therefore be erroneously associated with resistance. To overcome this, analysis at the BROAD Institute combined a measure of convergent evolution with the strength of association to overcome these problems. In addition, multiple mutations in single genes were collapsed to form a single variant, which increase power to detect rarely occurring variants. This analysis was then applied to strains only with unexplained resistance. The details of this analysis are beyond the scope of this Master thesis and have been described elsewhere (Desjardins et al., 2016).

We chose to concentrate on LOF mutations. These mutations included insertions or deletions (INDELS), frameshift mutations and stop codons. Unlike SNPs whose effects on protein function are hard to predict bioinformatically, LOF mutations will definitely cause inactivation of proteins. By focussing on LOF mutations we reasoned that we would have a greater chance of identifying mutations that have phenotypic consequences. From the association analysis, we therefore identified 21 genes (Table 3.2) with LOF mutations, from strains with unexplained drug resistance, that had the strongest association with resistance to any drug.

Table 3.2: Genes with loss-of-function associated with phenotypic resistance

List of genes with LOF mutations that were associated with resistance in *M. tuberculosis*. Gene number, name and putative functions were taken from the annotated H37Rv genome (<http://tuberculist.epfl.ch/>).

| Gene Number | Gene Name | Function |
|--------------------|-------------------------|--|
| Rv0157 | <i>pntB</i> | NAD(P) subunit and functions as a proton pump across the membrane |
| Rv0205 | | A membrane protein. May play a role in cell wall and cell wall processes |
| Rv0242c | <i>fabG4</i> | 3-oxoacyl- [acyl-carrier protein] which is involved in the fatty acid biosynthesis pathway |
| Rv0552 | | hypothetical protein with unknown function |
| Rv0554 | <i>bpoC</i> | hypothetically involved in detoxification reactions |
| Rv0579 | | hypothetical protein with unknown function |
| Rv0779c | | transmembrane protein with unknown function |
| Rv1157c | | alanine and proline rich protein with unknown function |
| Rv1187 | <i>rocA</i> | Involved in the arginase pathway |
| Rv1250 | | Potential transport of drug across the membrane |
| Rv1277 | | hypothetical protein with unknown function |
| Rv1375 | | hypothetical protein with unknown function |
| Rv1975 | | hypothetical protein with unknown function |
| Rv2042c | | hypothetical protein with unknown function |
| Rv2088 | <i>pknJ</i> | Involved in signal transduction (via phosphorylation) |
| Rv2780 | <i>ald</i> | May play a role in cell wall synthesis as L-alanine is an important constituent of the peptidoglycan layer |
| Rv3197A | <i>whiB7</i> | Involved in transcriptional regulation |
| Rv3436c | <i>glmS</i> | catalyzes the first step in hexosamine metabolism |
| Rv3448 | <i>eccD₄</i> | ESX-4 secretion system protein with unknown function |
| Rv3569c | <i>hsaD</i> | Catalyzes the hydrolysis of 4,9-DHSA |
| Rv3823c | <i>mmpL8</i> | A membrane transporter thought to be involved in the transport of lipids |

3.3 Functional analysis of knock-out mutant in genes associated with resistance

To determine if LOF mutations could cause resistance to selected first and second-line drugs we acquired a panel of transposon mutants. From the list of 21 genes-associated with unexplained resistance, eight genes had 11 representative strains in a single gene knock-out library; Tuberculosis Animal Research and Gene Evaluation Taskforce (TARGET) mutant library (<http://webhost.nts.jhu.edu/target/>) (Table 3.3).

The TARGET mutant library consists of strains with a single gene knock-out which is transposed with a kanamycin resistant marker.

We performed phenotypic DST using the agar proportion method at the WHO recommended critical concentration on these eleven strains against six anti-tubercular drugs. The mutants all displayed kanamycin resistance as they all possessed a kanamycin resistance marker in the transposon which was used to generate the knockout mutant.

The eleven strains from our phenotypic screen against six first and second-line anti-tuberculous drugs failed to uncover any unexpected drug resistances.

Table 3.3: Phenotypic DST of transposon TARGET mutants against anti-tubercular drugs (Desjardins et al., 2016)

Each strain was identified by the gene number (#) and the TARGET mutant library identification (ID). Critical drug concentrations listed are represented in $\mu\text{g/mL}$. S = Susceptible and R = Resistance. Drugs tested were isoniazid (INH), rifampicin (RIF), ethambutol (EMB), streptomycin (STR), ofloxacin (OFL) and kanamycin (KAN).

| Gene # | Lib ID | INH 0.2 | INH 1 | RIF 1 | EMB 7.5 | STR 2 | OFL 2 | KAN 6 |
|-----------------------|---------------|---------|-------|-------|---------|-------|-------|-------|
| Rv0157 | JHU0157-1366 | S | S | S | S | S | S | R |
| Rv0242c | JHU0242c-319 | S | S | S | S | S | S | R |
| Rv1250 | JHU1250-1 | S | S | S | S | S | S | R |
| Rv1375 | JHU1375-874 | S | S | S | S | S | S | R |
| Rv1375 | JHU1375-874 | S | S | S | S | S | S | R |
| Rv1375 | JHU1375-141 | S | S | S | S | S | S | R |
| Rv2088 | JHU2088-60 | S | S | S | S | S | S | R |
| Rv2780 (<i>ald</i>) | JHU2780-209 | S | S | S | S | S | S | R |
| Rv3197A | JHU3197A-222 | S | S | S | S | S | S | R |
| Rv3823c | JHU3823c-114 | S | S | S | S | S | S | R |
| Rv3823c | JHU3823c-1817 | S | S | S | S | S | S | R |

3.4 Association of L-alanine dehydrogenase (*ald*) with D-cycloserine resistance

The drug resistance screen to rifampicin, isoniazid, streptomycin, ethambutol and ofloxacin revealed no hits so we then looked at the function of each of the 21 genes identified in our association analysis to see if there were any pointers to which drugs the LOF mutations could be causing resistance to. We were intrigued by Rv2780 which encodes for an alanine dehydrogenase (*ald*) that interconverts pyruvate and L-alanine (Figure 1.5). We therefore hypothesised that mutations in L-alanine dehydrogenase could lead to changes in alanine flux that could ameliorate the effects of D-cycloserine and cause resistance.

3.5 Genetic variability of *ald* in clinical isolates

To determine if there was a relationship between LOF mutations in *ald* and drug resistance we first looked to see if these mutations only occurred in drug-resistant isolates. Table 3.4 shows the 11 different LOF mutations in our dataset and the drug-resistant profile of the strains harbouring these mutations. There were 7 insertions, 3 deletions and 1 stop codon. These LOF mutations occurred exclusively in MDR or XDR strains supporting our idea that *ald* was implicated in some form of resistance. The mutations were also found to have occurred in multiple different lineages indicative of convergent evolution.

Table 3.4: Loss-of-function mutations in *ald* with respect to spoligotype and DST

For each LOF mutation, the size, type of mutation, nucleotide position or codon number, number of isolates in which the mutation occurs, the spoligotype of those isolates, and the level of drug resistance of the isolate are listed.

| Polymorphism | # isolates | Spoligotype | DST |
|--------------------------|------------|-------------|-------------------|
| 1bp insertion, nt 128 | 1 | LAM4 | MDR |
| 1bp deletion, nt 132 | 4 | T1 | MDR (4) |
| 2bp insertion, nt 133 | 1 | X3 | XDR |
| 2bp deletion, nt 304 | 1 | LAM4 | XDR |
| 1359bp insertion, nt 304 | 1 | LAM4 | MDR |
| 1bp insertion, nt 317 | 3 | X2 | MDR (2), poly (1) |
| 2bp insertion, nt 433 | 2 | LAM4 | MDR (1), XDR (1) |
| Q153* | 1 | LAM4 | XDR |
| 1bp deletion, nt 459 | 8 | X3 | MDR (4), XDR (4) |
| 5bp insertion, nt 837 | 2 | LAM4 | XDR (2) |
| 2bp insertion, nt 966 | 1 | LAM4 | XDR |

3.6 Development of a growth inhibition assay to evaluate the role of *ald* mutants in D-cycloserine resistance

DST is difficult for D-cycloserine and the only proposed method is testing using LJ medium. However, we wanted to test for D-cycloserine resistance in a more controlled medium because we were not sure whether levels of L-alanine and other factors could alter metabolism in a way that might affect the alanine metabolism pathways, and influence DST. We initially tried the Trek Sensititre MYCOTB MIC plate (MYCOTB; Trek Diagnostic Systems, Cleveland, OH), a commercially available broth based assay conducted in 96 well plates to determine resistance to D-cycloserine. However, we experienced high false positive rates in our control strains. We therefore developed a growth inhibition assay using the MGIT system. The principle of the assay was to compare the TTP in the presence or absence of drug. MGIT tubes were inoculated based on OD but CFU's were also determined for all inocula to ensure tubes had roughly equivalent levels of bacteria at the start of the assay.

We initially performed a growth assay in MGIT to determine if there was a growth advantage of selected TARGET mutants in the presence of D-cycloserine. The assay was conducted on seven of the TARGET mutant strains and CDC1551 which is a fully susceptible laboratory strain. Testing was conducted with varying concentrations of D-cycloserine in triplicate. Each strain's growth was monitored for TTP, a shorter TTP suggests a growth advantage in the presence of D-cycloserine.

In comparison to CDC1551, only the *ald* LOF mutant had a significant growth advantage in the presence of D-cycloserine at 7.5 µg/mL and 15 µg/mL ($p = 0.0075$ and 0.0441 , respectively). All other transposon mutants displayed no significant difference in growth kinetics relative to CDC1551 with $p > 0.05$. This supported our hypothesis that *ald* LOF mutations cause resistance to D-cycloserine.

Table 3.5: Confirmation of growth advantage of transposon TARGET mutants in the presence of D-cycloserine is specific to *ald* loss-of-function (Desjardins et al., 2016)

| Gene number | Lib ID | 0 µg/mL | | | 7.5 µg/mL | | | 15 µg/mL | | |
|-----------------------|---------------|---------|--------|---------|-----------|--------|---------|----------|--------|---------|
| | | TTP | SEM | p-value | TTP | SEM | p-value | TTP | SEM | p-value |
| - | CDC1551 | 6.2 | 0.0212 | n/a | 16.9 | 0.8706 | n/a | 35.2 | 1.9605 | n/a |
| Rv2780 (<i>ald</i>) | JHU2780-209 | 6.3 | 0.0289 | 0.8343 | 8.5 | 0.4766 | 0.0075 | 25.0 | 0.4527 | 0.0441 |
| Rv1250 | JHU1250-1 | 6.1 | 0.0289 | 0.0880 | 19.0 | 0.2819 | 0.9740 | 41.1 | 0.5052 | 0.8844 |
| Rv1375 | JHU1375-141 | 6.2 | 0.0488 | 0.0994 | 22.0 | 0.4455 | 0.8476 | 41.6 | 0.1472 | 0.8902 |
| Rv1375 | JHU1375-874 | 6.0 | 0.0526 | 0.0617 | 22.4 | 0.3852 | 0.9627 | 41.0 | 0.3046 | 0.8951 |
| Rv2088 | JHU2088-60 | 6.0 | 0.0367 | 0.6077 | 21.6 | 0.6519 | 0.9711 | 41.3 | 0.3769 | 0.8997 |
| Rv3823 | JHU3823c-114 | 3.4 | 0.2350 | 0.0099 | 16.3 | 3.1519 | 0.4588 | 34.8 | 0.8177 | 0.4548 |
| Rv3823 | JHU3823c-1817 | 6.3 | 0.0212 | 0.9080 | 19.3 | 0.8357 | 0.8403 | 42.0 | 0.0000 | 0.9080 |

The MGIT growth assay was performed on CDC1551, the *ald* LOF mutant and single gene knockout strains in the presence of D-cycloserine. The strains were cultured in triplicate in 0, 7.5 µg/mL and 15 µg/mL D-cycloserine. The TTP for each strain was recorded in days since inoculation. The standard error mean (SEM) of the three replicates is shown above. The mean MGIT TTP and growth inhibition for the varying concentrations of D-cycloserine for each mutant strain were statistically compared (using a one-sided t-test) to CDC1551.

3.7 Growth inhibition by D-cycloserine testing of the Δald mutant and control strains

To confirm if *ald* LOF conferred resistance to D-cycloserine, we assessed growth inhibition of the Δald mutant and appropriate control strains.

We assessed CDC1551 (a fully susceptible reference strain which possessed wild-type *ald*, *ddl* and *alr* genes), the *ald* knockout (Δald) strain and the *ald* knock-out complemented with wild-type *ald* from *M. tuberculosis* ($\Delta ald-comp$). The $\Delta ald-comp$ strain was created by cloning the H37Rv *ald* gene onto an integrative vector harboring a kanamycin resistance cassette.

In addition, we included BCG as an additional positive control. BCG is naturally resistant to D-cycloserine, which may be explained by a *cycA* point mutation (Chen et al., 2012). BCG also possesses a frameshift in *ald* (Chen et al., 2003, Garnier et al., 2003). To determine the effect of the *ald* frameshift on inhibition by D-cycloserine, we performed our growth assay on *M. bovis* BCG Danish 1331 (BCG) and *M. bovis* BCG 1331 complemented with wild-type *ald* from *M. tuberculosis* (BCG:: $\Delta ald-comp$).

We performed the D-cycloserine growth assay on the CDC1551 (WT-as represented on the graph), the Δald strain, $\Delta ald-comp$ and BCG strains. (Figure 3.1). The Δald strain displayed a shorter TTP when compared to wild-type CDC1551 in the presence of D-cycloserine at 7.5 $\mu\text{g}/\text{mL}$ ($P < 0.0001$, ANOVA), indicative of a significant growth advantage at this concentration. At the 15 $\mu\text{g}/\text{mL}$ D-cycloserine concentration the Δald strain still showed a growth advantage as compared to the wild-type CDC1551 ($P < 0.0001$, ANOVA) but grew slowly relative to the BCG strain ($P < 0.0001$, ANOVA). In addition, we observed that the $\Delta ald-comp$ strain, had greater growth inhibition in comparison to the Δald strain ($P < 0.0001$, ANOVA), but was still inhibited significantly less than CDC1551 ($P < 0.0001$, ANOVA) suggesting that *ald* complementation partially reverted the growth advantage to D-cycloserine at both the 7.5 $\mu\text{g}/\text{mL}$ and 15 $\mu\text{g}/\text{mL}$ concentration. BCG showed minimal growth inhibition when exposed to both low and high dose D-cycloserine compatible with its resistant phenotype. In conclusion, the *ald* LOF strain displayed a growth advantage relative to CDC1551 compatible with a resistance phenotype and the *ald-comp* displays partial restoration of wild-type behaviour.

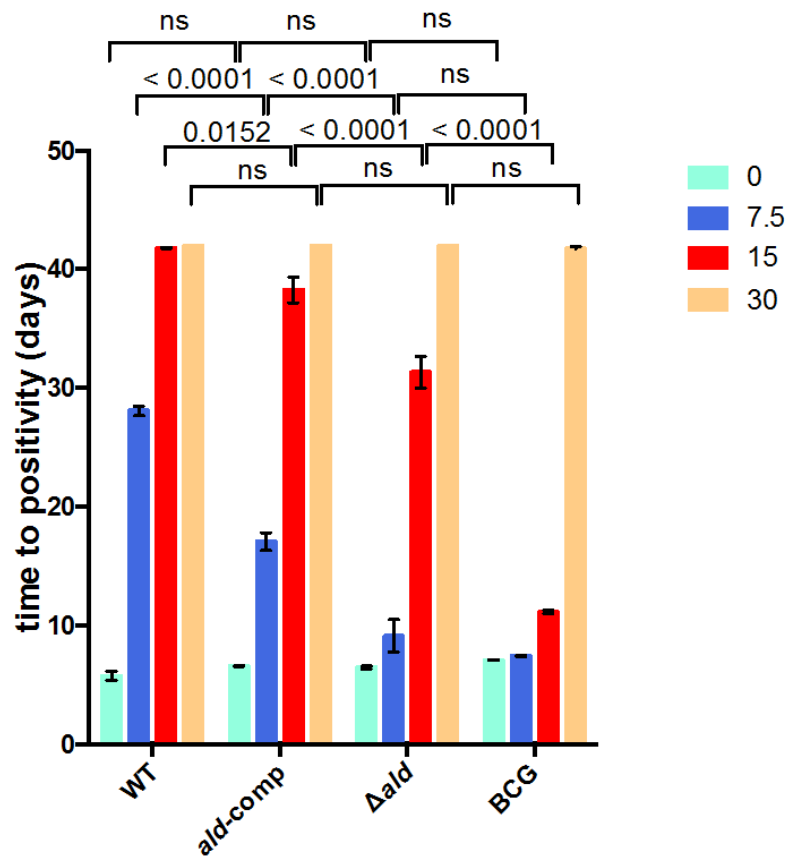


Figure 3.1: Assessment of laboratory strains TTP in the presence of D-cycloserine. The MGIT growth assay was performed on CDC1551 (WT), the $\Delta ald-comp$, Δald strain, and BCG strains in the presence of D-cycloserine. The strains were inoculated in triplicate at the 0, 7.5 $\mu\text{g/mL}$, 15 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ D-cycloserine drug concentrations. Each strains TTP was documented as days positive from the time of inoculation. Error bars represent the SEM of the mean TTP. Two-way ANOVA was used to calculate the p-values.

The D-cycloserine growth assay was then performed on the BCG and BCG::*ald-comp*, to evaluate the effect of the *ald* frameshift on growth in the presence of D-cycloserine. At the 7.5 µg/mL D-cycloserine concentration there was no statistically significant difference in growth between BCG and BCG::*ald-comp*. At the 15 µg/mL and 30 µg/mL D-cycloserine concentrations the BCG::*ald-comp* displayed significant inhibition of growth relative to BCG ($p < 0.02$ and $p < 0.0001$, respectively) (Figure 3.2).

Although we did not fully reverse the resistance phenotype this result suggests *ald* LOF does play a role in D-cycloserine resistance in *M. bovis* BCG. BCG has a *cycA* point mutation which is implicated in its resistance phenotype, although complementation with *cycA* only partially restored susceptibility suggesting other mechanisms of resistance are involved (Chen et al., 2012). The mutation in *cycA* could therefore explain the modest results of our complementation, but differences in the genetic background could also contribute. Experimentation involving the complementation of wild-type *cycA* will need to be evaluated to address this issue.

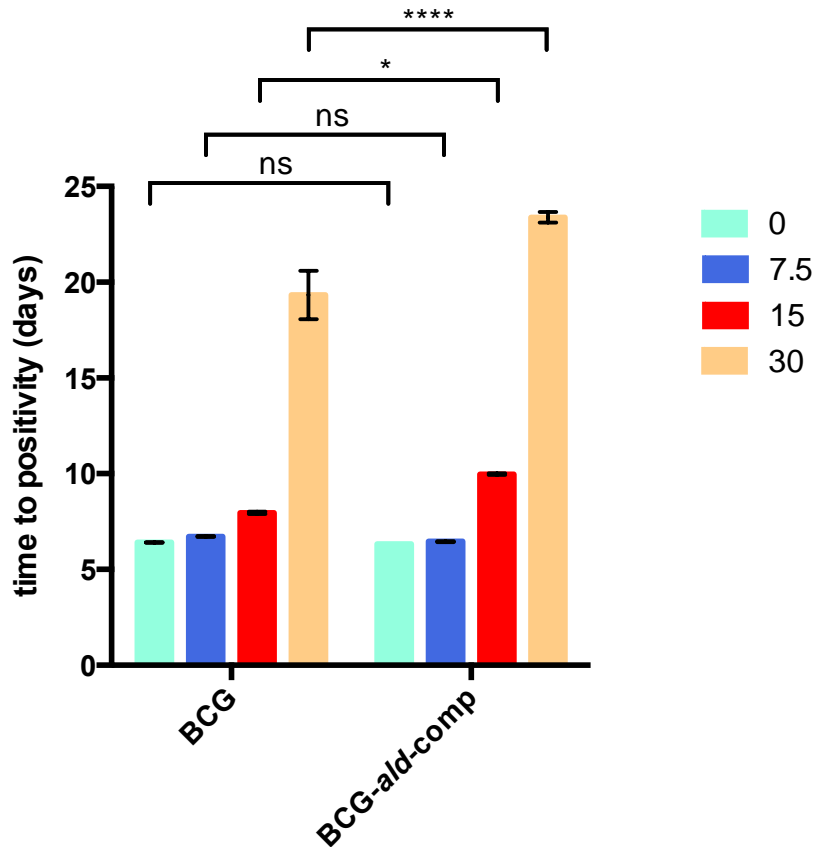


Figure 3.2: Complementation of *ald* in *M. bovis* BCG prompts slower growth in the presence of high dose D-cycloserine. The MGIT growth assay was performed on BCG and BCG::*ald-comp* strains in the presence of D-cycloserine. The strains were inoculated in triplicate at the 0, 7.5 µg/mL, 15 µg/mL and 30 µg/mL D-cycloserine drug concentrations. Each strains TTP was documented as days positive from the time of inoculation. The BCG::*ald-comp* showed inhibition of growth at higher concentrations of D-cycloserine. This indicates that *ald* LOF may contribute to D-cycloserine resistance. Error bars represent the SEM of the mean TTP. Two-way ANOVA was used to calculate the p-values.

3.8 Growth inhibition by D-cycloserine of clinical isolates with *ald* mutations

We next sought to determine whether clinical isolates with mutations in *ald* and *alr* exhibited a growth advantage in the presence of D-cycloserine. Clinical isolates with varying L-alanine dehydrogenase (*ald*) and alanine racemase (*alr*) mutations, with diverse drug susceptibility phenotypes and varying genetic backgrounds were assessed in the D-cycloserine growth assay. They are listed below with their corresponding *ald* and *alr* mutations, their DST pattern and spoligotype (Table 3.6).

Table 3.6: Clinical isolates tested by the D-cycloserine growth assay with mutations in *ald* and *alr* genotypes

| Strain | <i>ald</i> | <i>alr</i> | DST | Spoligotype |
|-------------|----------------|------------|-------------|-------------|
| TKK-04-0103 | WT | WT | XDR | X3 |
| TKK-01-0019 | WT | WT | XDR | LAM3 |
| TKK-03-0065 | WT | WT | Susceptible | Beijing |
| TKK-04-0054 | WT | WT | XDR | Beijing |
| TKK-04-0149 | WT | WT | Susceptible | Beijing |
| TKK-03-0109 | WT | WT | Susceptible | Beijing |
| TKK-02-0053 | 5bp ins at 837 | WT | XDR | LAM4 |
| TKK-02-0006 | Q153* | WT | XDR | LAM4 |
| TKK-02-0051 | 2bp ins at 433 | WT | XDR | LAM4 |
| TKK-04-0071 | 1bp del at 459 | WT | MDR | X3 |
| TKK-04-0105 | WT | Y343T | XDR | LAM4 |
| TKK-02-0004 | WT | K157E | XDR | LAM4 |
| TKK-04-0075 | 1bp del at 132 | Y388D | MDR | T1 |

The D-cycloserine growth assay was performed in MGIT on these clinical isolates with various mutations. At concentrations of 7.5 µg/mL, 15 µg/mL and 30 µg/mL of D-cycloserine, clinical isolates with the *ald* LOF mutation displayed a growth advantage when compared to strains with wild-type *ald* ($p < 0.05$ at all tested concentrations), demonstrating that the growth advantage seen in laboratory strains with *ald* LOF mutations extends to clinical strains (Figure 3.3).

All three clinical strains with SNPs in alanine racemase also exhibited a growth advantage in the presence of 7.5 µg/mL, 15 µg/mL and 30 µg/mL D-cycloserine relative to wild-type strains ($p < 0.05$ at all tested concentrations). When compared to strains with only *ald* LOF mutations, clinical strains with SNPs in alanine racemase did not have significantly different growth at the 7.5 µg/mL and 15 µg/mL of D-cycloserine but did exhibit a significant growth advantage at 30 µg/mL of D-cycloserine ($p < 0.05$). This suggests that at high concentrations of D-cycloserine, *ald* LOF mutations confer less of a growth advantage than the assayed nonsynonymous SNPs in the direct target of D-cycloserine, alanine racemase. This in turn indicates that intragenic *alr* mutations should confer higher levels of resistance than *ald* mutations.

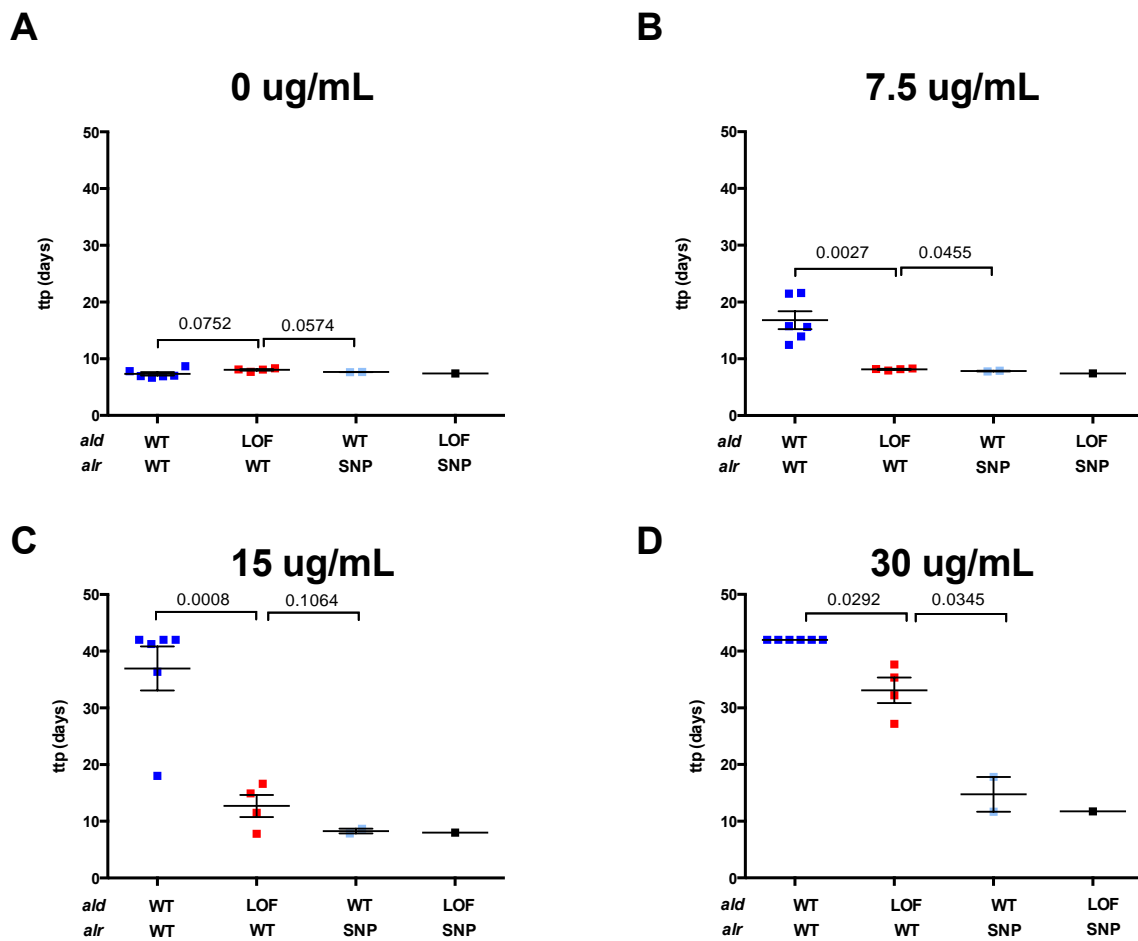


Figure 3.3: Assessment of clinical strains TTP in the presence of D-cycloserine. The MGIT growth assay was performed on clinical isolates with mutations in *ald* and *alr* in the presence of D-cycloserine. The strains were inoculated in triplicate at the 0, 7.5 $\mu\text{g/mL}$, 15 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ D-cycloserine drug concentrations. Clinical strains with LOF mutations in *ald* or polymorphisms in the target of D-cycloserine, *alr* exhibit a shorter time to positivity in the presence of D-cycloserine. Each strain's TTP was documented as days positive from the time of inoculation. Error bars represent the SEM of the mean TTP. Two-way ANOVA was used to calculate the p-values.

3.9 DST of an Δald strain and controls

Our growth inhibition assay provided a discriminatory tool to test our hypothesis that LOF mutations in *ald* result in D-cycloserine resistance. To be certain we carried out an MIC evaluation of our control strains using the only recognized method of DST using LJ medium.

According to the WHO guidelines the critical concentration for resistance on LJ medium was lowered to 30 $\mu\text{g/mL}$ (WHO, 2012). When we carried out susceptibility testing on our strains we found CDC1551 (WT as represented in Table 3.7) displayed an MIC of 15 $\mu\text{g/mL}$ and was most susceptible as compared to the other strains. The Δald strain showed an MIC of 25-30 $\mu\text{g/mL}$ greater than that of CDC1551. The *ald-comp* displayed an intermediate MIC of 20 $\mu\text{g/mL}$ relative to Δald .

The BCG (positive control) strain displayed the greatest resistance with an MIC of 40-60 $\mu\text{g/mL}$. The BCG::*ald-comp* strain MIC was 40 $\mu\text{g/mL}$, demonstrating no change to BCG. The *M. bovis* ATCC 19210 strain showed an MIC of 25 $\mu\text{g/mL}$ comparable to that of the Δald strain. We can conclude from the MIC testing conducted on these strains that the Δald strain confers a low-level of resistance to D-cycloserine.

Table 3.7: The *ald* loss-of-function mutation confers an increased level of resistance to D-cycloserine (Desjardins et al., 2016)

| Strain | D-cycloserine MIC ($\mu\text{g/mL}$) | | |
|----------------------------|--|---------|---------|
| | Assay 1 | Assay 2 | Assay 3 |
| CDC1551 (WT) | 15 | 15 | - |
| Δald | 25 | 30 | 25 |
| $\Delta ald-comp$ | 20 | 20 | 20 |
| BCG | 40 | 40 | 60 |
| BCG:: <i>ald-comp</i> | 40 | - | 40 |
| <i>M. bovis</i> ATCC 19210 | - | 25 | 25 |

MIC testing was conducted in three independent experiments to determine reproducibility. Minus (-) signs depict strains not tested for a specific assay.

3.10 Contribution of *ald*, *alr*, *ddlA* and *cycA* mutations to D-cycloserine resistance in clinical isolates from KwaZulu-Natal

Having shown that LOF mutations in *ald* can cause resistance to D-cycloserine we wanted to assess their contribution to resistance relative to other potential drug resistance conferring mutations. We therefore examined our dataset for other clinical isolates with mutations in the known targets of D-cycloserine, i.e. alanine racemase (*alr*) and D-alanine:D-alanine ligase (*ddlA*). We identified forty-four clinical strains with varying genetic backgrounds which we then assessed for D-cycloserine drug susceptibility using LJ medium. This assay included 22 strains with wild-type *ald*, *alr*, *ddlA* and *cycA* alleles, 13 strains which possessed mutations in *ald*, seven which possessed mutations in the *alr* gene and two which had mutations in both *ald* and *alr* genes. Seven strains were retested, four of which were outliers. We also assessed the level of resistance in *ald* LOF strains relative to strains which possess mutations in the *alr* gene. In our data set we observed no clinical isolates with *ddlA* and *cycA* mutations, and therefore no DST could be conducted on strains harboring mutations in these genes.

Table 3.8: List of clinical isolates with representative mutations and MIC to D-cycloserine as determined on LJ media (Desjardins et al., 2016)

| Strain Name | Spoligotype | DST | <i>ald</i> | <i>alr</i> | <i>ddlA</i> | <i>cycA</i> | LJ MIC |
|-------------|-------------|-------------|----------------|--------------------|-------------|-------------|-----------|
| TKK-04-0075 | T1 | Pre-XDR | 1bp del at 132 | Y364D | WT | WT | 40 |
| TKK-01-0050 | T1 | MDR | 1bp del at 132 | Y364D | WT | WT | 40 |
| TKK-02-0035 | X3 | XDR | 1bp del at 459 | WT | WT | WT | 15 (40) |
| TKK-04-0071 | X3 | Pre-XDR | 1bp del at 459 | WT | WT | WT | 40 |
| TKK-04-0040 | X2 | poly | 1bp ins at 317 | WT | WT | WT | 20 (20) |
| TKK-04-0038 | LAM 4 | Pre-XDR | 2bp del at 304 | WT | WT | WT | 40 |
| TKK-02-0037 | X3 | XDR | 2bp ins at 133 | WT | WT | WT | 30 (40) |
| TKK-02-0051 | LAM 4 | XDR | 2bp ins at 433 | WT | WT | WT | 40 |
| TKK-04-0060 | LAM 4 | Pre-XDR | 2bp ins at 433 | WT | WT | WT | 30 (30) |
| TKK-02-0053 | LAM 4 | XDR | 5bp ins at 837 | WT | WT | WT | 40 |
| TKK-02-0049 | LAM 4 | XDR | 5bp ins at 837 | WT | WT | WT | 25 (25) |
| TKK-02-0006 | LAM 4 | XDR | Q153* | WT | WT | WT | 40 |
| TKK-04-0122 | LAM 4 | XDR | R15Q | WT | WT | WT | 30 |
| TKK-02-0004 | LAM 4 | XDR | WT | K133E | WT | WT | 60 |
| TKK-02-0055 | Beijing | XDR | WT | L89R | WT | WT | 60 |
| TKK-04-0090 | LAM4 | mono | WT | L89R | WT | WT | 40 |
| TKK-04-0105 | LAM4 | XDR | WT | M319T ^a | WT | WT | >60 |
| TKK-02-0007 | LAM4 | XDR | WT | R373G | WT | WT | 25 (25) |
| TKK-01-0018 | CAS1-Kili | poly | WT | WT | WT | WT | 15 |
| TKK-01-0054 | X3 | mono | WT | WT | WT | WT | 20 |
| TKK-01-0084 | LAM 4 | susceptible | WT | WT | WT | WT | 40 (40) |
| TKK-03-0065 | Beijing | susceptible | WT | WT | WT | WT | 20 |
| TKK-04-0054 | Beijing | Pre-XDR | WT | WT | WT | WT | 20 |
| TKK-01-0007 | LAM 3 | susceptible | WT | WT | WT | WT | 15 |
| TKK-01-0033 | LAM 4 | XDR | WT | WT | WT | WT | 15 |
| TKK-01-0036 | LAM 4 | mono | WT | WT | WT | WT | 30 |
| TKK-01-0060 | LAM 3 | poly | WT | WT | WT | WT | 15 |
| TKK-03-0021 | T1 | susceptible | WT | WT | WT | WT | 20 |
| TKK-03-0099 | X1 | susceptible | WT | WT | WT | WT | 20 |
| TKK-04-0109 | LAM 4 | XDR | WT | WT | WT | WT | 20 |
| TKK-04-0149 | Beijing | susceptible | WT | WT | WT | WT | 15 |
| TKK-02-0018 | T3 | Pre-XDR | WT | WT | WT | WT | 20 |
| TKK-02-0062 | CAS1-Kili | Pre-XDR | WT | WT | WT | WT | 15 |
| TKK-01-0019 | LAM 3 | XDR | WT | WT | WT | WT | 15 |
| TKK-01-0035 | LAM 4 | MDR | WT | WT | WT | WT | 20 |
| TKK-01-0081 | LAM 4 | susceptible | WT | WT | WT | WT | 20 |
| TKK-01-0035 | LAM 4 | MDR | WT | WT | WT | WT | 20 |
| TKK-02-0050 | LAM 4 | XDR | WT | g-8t ^b | WT | WT | >60 (>60) |
| TKK-04-0103 | X3 | XDR | WT | WT | WT | WT | 20 |
| TKK-04-0070 | LAM 4 | Pre-XDR | 1bp ins at 128 | WT | WT | WT | 40 |
| TKK-04-0047 | LAM 3 | poly | G213S | WT | WT | WT | 30 |
| TKK-03-0026 | Unknown | susceptible | WT | A284G | WT | WT | 20 |
| TKK-03-0020 | S | susceptible | WT | WT | WT | WT | 20 |
| TKK-03-0029 | EAI1SOM | susceptible | WT | WT | WT | WT | 10 |

^a Mutation previously predicted to have high impact on *alr* function (Köser et al., 2013).

^b Mutation previously known to confer D-cycloserine resistance (Merker et al., 2013).

We found that clinical strains with the *ald* mutations exhibited a median MIC of 40 µg/mL which was significantly higher than strains that were wild-type at all of the putative D-cycloserine resistance conferring loci. In total 91% of clinical strains which possessed the wild-type *alr* and *ald* genes had an MIC of ≤ 20 µg/mL.

Two strains (TKK-04-0122 and TKK-04-0047) possessed nonsynonymous SNPs in *ald* also displayed an MIC of 30 µg/mL suggesting these mutations also result in LOF. Taken together these results indicate that LOF mutations and nonsynonymous SNPs in *ald* can result in resistance to D-cycloserine at or above the WHO recommended critical concentration of 30 µg/mL.

In our assessment of clinical strains with *alr* mutations, we found these strains displayed a high level of resistance to D-cycloserine in comparison to strains that possessed wild-type *alr* and *ald* genes. One strain (TKK-02-0050), which possessed a promoter mutation in *alr* displayed an MIC of > 60 µg/mL, which is compatible with a study conducted on *M. smegmatis*, where it was observed that a mutation in the promoter region of *alr* conferred resistance to D-cycloserine (Caceres et al., 1997).

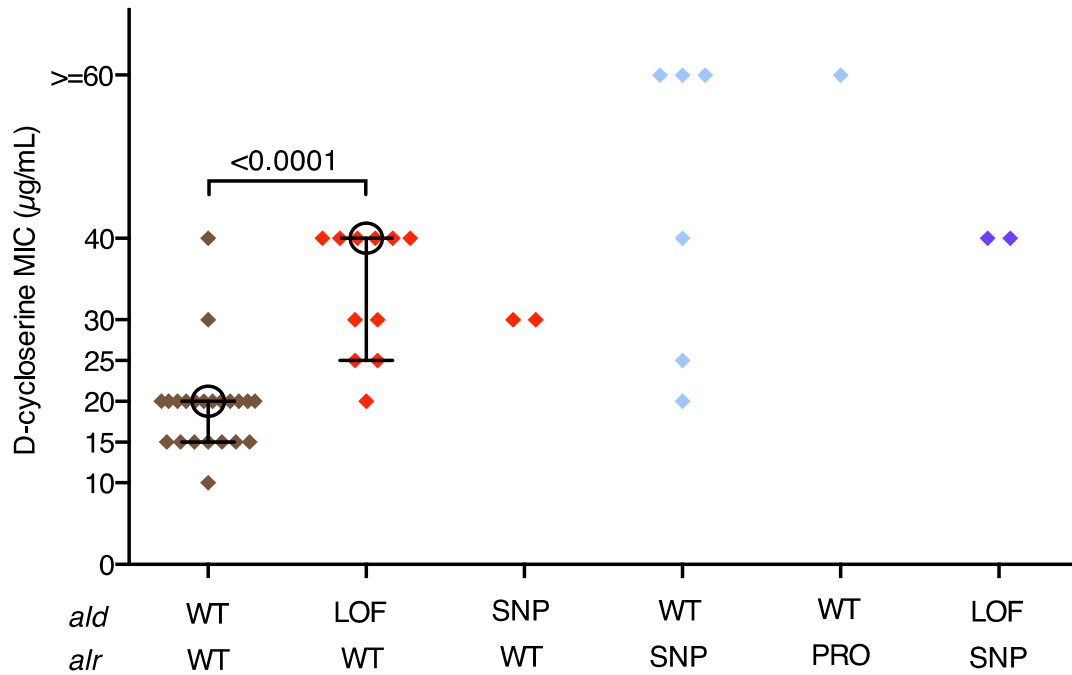


Figure 3.4: Distribution of D-cycloserine MIC of *M. tuberculosis* clinical strains with diverse drug susceptibility phenotypes and varying genetic backgrounds (Desjardins et al., 2016). D-cycloserine MIC testing was performed on clinical isolates with mutations in *ald* and *alr* using LJ medium. This analysis includes the strains with wild-type *ald* and *alr* genes, LOF mutations in *ald*, nonsynonymous mutations (NSY) in *ald* and *alr* genes and promoter mutations (PRO) in *alr*. Strains which possessed *ald* and *alr* mutations displayed a significantly greater resistance to D-cycloserine in comparison to strains which possessed WT *ald* and *alr*. The median of wild-type and *ald* LOF strains are represented as open circles and quartiles are represented as connected bars. The strains with *ald* LOF displayed greater resistance when compared to wild-type strains ($P < 0.0002$, Mann–Whitney U test).

CHAPTER FOUR

Discussion

The objective of this study was to identify novel genes and mechanisms implicated in drug resistance in clinical isolates of *M. tuberculosis* in KwaZulu-Natal. Focussing on LOF mutations we were able to provide new insights into the genetic basis of D-cycloserine resistance in clinical isolates. Due to the toxicities (Cunha, 2001, Torun et al., 2005, Mitnick et al., 2008) and severe side effects (Kendig et al., 1956, Murray, 1956, Bankier, 1965, Yew et al., 1993) associated with the administration of D-cycloserine to patients, it would be particularly worthwhile to develop a rapid molecular based assay to detect D-cycloserine resistance. In this way, it would be possible to prescribe this drug to patients which actually harbor susceptible *M. tuberculosis* strains, thereby avoiding unnecessary severe drug associated side-effects. In addition, due to technical difficulties, phenotypic D-cycloserine DST is not routinely performed (Woods, 2000, Kam et al., 2010, WHO, 2014). The development of molecular diagnostics would greatly expand the detection of resistance to this still widely used second-line anti-tuberculosis drug. Our results will therefore contribute to improved diagnostics for D-cycloserine.

Based on an association and correlation evolution analyses performed (Desjardins et al., 2016), we identified genes with LOF mutations which may be associated with phenotypic resistance. From this screening, we focused on *ald* LOF, which encodes for L-alanine dehydrogenase. Eleven different *ald* mutations were observed in drug-resistant *M. tuberculosis* clinical strains (Table 3.4). Distinct *ald* LOF mutations occurred independently multiple times consistent with convergent evolution. This suggests a positive selective pressure. We do not have direct evidence that this was D-cycloserine as we do not have detailed drug histories for these patients and many of them were primary TB infections. However most of the mutations occurred within resistant clinical isolates from Lineage 4 which is associated with the evolution of XDR-TB (Chihota et al., 2012, Muller et al., 2013, Cohen et al., 2015). (Figure S1). Patients infected with strains within this lineage will have definitely received D-cycloserine therapy. It is therefore highly likely that exposure to D-cycloserine was the antibiotic pressure that selected for *ald* mutations, and our experimental validation confirmed mutations in *ald* confer resistance to D-cycloserine *in vitro*.

An important part of this study was the development of a reliable assay to assess susceptibility to D-cycloserine. Testing on LJ medium is cumbersome and not amenable to more high-throughput testing. Our attempts using simple culture in broth were not reliable. So, we optimized a growth assay utilizing the BD BACTEC™ MGIT™ 960 system, with titered inocula, which allowed us

to evaluate the effects of D-cycloserine on growth in a carefully controlled, sensitive and quantitative fashion. This enabled us to show that the *Δald* possessed a growth advantage in the presence of D-cycloserine relative to the wild-type and complemented controls (Figure 3.1). We were then able to validate our assay using classical MIC determination on LJ slopes confirming *ald* contributes to D-cycloserine susceptibility *in vitro* (Table 3.7).

We hypothesised that the mechanism by which *ald* LOF confers D-cycloserine resistance in *M. tuberculosis* is through the prevention of the conversion of L-alanine to pyruvate, which in turn leads to the accumulation of intracellular L-alanine (Figure 1.5). If *ald* LOF increases available L-alanine, then this could reduce the competitive inhibition of alanine racemase and D-alanine ligase activity by D-cycloserine through increased substrate availability, which would allow for continued cell wall synthesis. At increasing D-cycloserine concentrations drug would eventually overwhelm the moderate level of resistance provided by the increased L-alanine pool which is what we observed. Metabolomic experiments would be able to test this hypothesis.

Previous work has shown that the innate resistance of *M. bovis* BCG to D-cycloserine is in part due to a SNP in the alanine transporter *cycA*, but additional genes may be involved (Chen et al., 2012). Here we hypothesised that the frameshift in *ald* present in *M. bovis* BCG (Chen et al., 2003) might also contribute to D-cycloserine resistance. While complementation of functional *ald* into *M. bovis* BCG did not affect the MIC to D-cycloserine (Table 3.7) we did see at higher concentrations in our growth assay, the growth of complemented strains in the presence of D-cycloserine was significantly more inhibited than that of *M. bovis* BCG (Figure 3.2). This suggests that *ald* LOF also contributes to D-cycloserine resistance in *M. bovis* BCG. It is possible that the contribution of *ald* to D-cycloserine resistance would have greater penetrance in a genetic background with the wildtype *cycA*, and further experimentation could address this. For example, the permease *cycA* probably also transports alanine and mutations in this gene and could therefore alter the intracellular flux of L-alanine. The reason the phenotype was not restored may also be partly due to an integrative vector being used from which sub-optimal expression is achieved. This could also explain why complementation of the *ald* into the *ald* knockout strain only partially restored a growth advantage with D-cycloserine (Figure 3.1). Further experimentation evaluating the overexpression of *ald* needs validation.

The muted complementation of BCG led us to test *M. bovis* which also has the same *ald* mutation as BCG. It is generally thought that only BCG is resistant to D-cycloserine but a previous study has reported *M. bovis* to have an elevated MIC (Rist et al., 1967) for D-cycloserine which we

confirmed for the reference *M. bovis* strain ATCC 19210. This prompted us to further evaluate other strains of the MTBC, which like *M. bovis* lack RD9 (Brosch et al., 2002) and which share a frameshift mutation in *ald*. However, experimentation is ongoing and has proven to be difficult to conduct as supplementation of pyruvate (required for growth of these strains) was found to increase the MIC of D-cycloserine (data not shown). As the *ald* LOF mutation emerged in the RD9 lineage prior to antibiotic therapy, it suggests that the mutation could be selected for in the absence of drug. It is however unclear what the adaptive advantage of *ald* in these strains might be, however *ald* mutations do not lead to a loss of fitness in a mouse model in an *M. tuberculosis* genetic background. Further experimentation is warranted to obtain conclusions.

Having observed *ald* LOF causes a moderate level of resistance to D-cycloserine in laboratory strains we wanted to determine if this phenomenon was also reflected in clinical strains which possessed the *ald* LOF.

Our data showed, clinical strains (Figure 3.4, Table 3.8) with *ald* LOF mutations displayed a low-level of resistance to D-cycloserine. In contrast strains, which harbored non-synonymous and promoter mutations in *alr* displayed a high level of resistance when compared to strains with only *ald* LOF mutations. Clinical isolate, TKK-02-0050 was of particular interest due to this strain possessing a promoter mutation in *alr*, and was predicted to cause resistance from studies conducted with *M. smegmatis*. This mutation most likely causes an upregulation of the *alr* gene and therefore increases the available enzyme which would reduce competitive inhibition by D-cycloserine.

Testing of our clinical isolates indicated that the majority of the strains with *ald* LOF mutations displayed a median MIC of 40 µg/mL (Figure 3.4, Table 3.8) and it is unclear if this represents a clinically significant MIC or not. There is a dearth of studies evaluating how low-level resistance conferring mutations effect TB treatment outcomes. In the case of INH resistance the *katG* mutation, S315T, confers high level INH resistance (Wengenack et al., 1997, Saint-Joanis et al., 1999, Pym et al., 2002, Dalla Costa et al., 2009, Ando et al., 2010) and up to 94% of INH resistant clinical strains are known to harbor this mutation in some settings (Musser et al., 1996, Telenti et al., 1997, Mokrousov et al., 2002, Morlock et al., 2003, Guo et al., 2006, Muller et al., 2011). The *inhA* promoter mutations are known to confer low-level INH resistance and this mutation is observed in up to 35% of INH resistant clinical strains (Brossier et al., 2006, Dalla Costa et al., 2009, Ando et al., 2010, Muller et al., 2011). However, it is still unknown if increasing INH concentrations are necessary or can overcome this low-level resistance although a study is

currently evaluating this. Similarly, our data show that mutations in *ald* are associated with low-level resistance to D-cycloserine and we cannot predict the clinical importance, but increasing D-cycloserine levels will not be possible due to toxicity.

Two clinical isolates (TKK-04-0075 and TKK-01-0050) harbored both *ald* and *alr* mutations. In our investigation of D-cycloserine resistance these strains displayed an elevated MIC of 40 µg/mL to D-cycloserine (Figure 3.4, Table 3.8). It is possible that *ald* LOF mutations may represent a “stepping stone” mutation to higher level resistance conferred by alterations in the direct targets of D-cycloserine.

In two strains which possessed the wildtype *ald*, *alr*, *ddlA* and *cycA* alleles (TKK-01-0084 and TKK-01-0036) we observed an increased MIC to D-cycloserine. These two strains revealed MICs of 40 µg/mL and 30 µg/mL (Figure 3.4, Table 3.8) respectively. We propose resistance in these strains is not caused by mutations in *ald*, *alr*, *ddlA* and *cycA*, indicating alternate mechanisms of D-cycloserine resistance could exist in *M. tuberculosis*.

D-cycloserine is an inhibitor of the peptidoglycan biosynthesis pathway, but very little is known about its targets. Previous studies have shown that overproduction of alanine racemase and D-alanine:D-alanine ligase may play a role in D-cycloserine resistance (Chacon et al., 2002, Milligan et al., 2007, Chacon et al., 2009, Awasthy et al., 2012). In BCG, resistance to D-cycloserine may be caused by the polymorphism in the alanine transporter *cycA* (Chen et al., 2012). However, none of our characterized clinical strains carried the *ddlA* and *cycA* mutations that are normally associated with D-cycloserine resistance. This led us to assume that mutations in *ddlA* and *cycA* may not be of clinical relevance in D-cycloserine resistance. This observation shows the value of more accurate genetic characterization of drug targets in a laboratory to predict clinically relevant mutations.

In 2008 the recommended WHO D-cycloserine critical concentration was set at 40 µg/mL, as verified on LJ medium (WHO, 2008a). The critical concentration was later lowered to 30 µg/mL (WHO, 2012). Based on our data, we established a tentative epidemiological cut-off (ECOFF) value of 20 µg/mL in our assessment of wild-type strains (Figure S2). ECOFFs are determined from analysing MIC distributions of a large number of wild-type strains which possess no known drug resistance mechanisms and from patients who have not been exposed to drug (Kahlmeter et al., 2003, Kahlmeter and Brown, 2004, Turnidge et al., 2006, Angeby et al., 2012, Kahlmeter, 2015). Defining ECOFF values is especially important in cases where strains exhibit elevated

MICs that do not reach the critical concentration breakpoints (which should be defined on the basis of predicting clinical success) (Kahlmeter, 2015). ECOFF values may also be useful in circumstances where clinical breakpoints are not known and these values can then be used to detect and compare resistance (Kahlmeter et al., 2003, Kahlmeter and Brown, 2004). We observed clinical isolates which possess *ald* LOF mutations are likely to have D-cycloserine MICs close to the critical concentration breakpoints that define D-cycloserine resistance, even if they appear to be D-cycloserine susceptible according to standard DST methods. This necessitates considering ECOFF values in redefining the parameters of resistance, more especially to drugs which exhibit low-level resistance mechanisms as observed in our study of D-cycloserine.

However, testing on more wild-type strains in South Africa and other geographical areas is required to establish a more concrete ECOFF for D-cycloserine. Our data also suggests the current critical concentration of D-cycloserine by WHO standards be re-evaluated (Angeby et al., 2012, Torrea et al., 2015). This is necessary as conventional DST methods alone may result in the exclusion of isolates exhibiting low-level resistance.

The combination of D-cycloserine or terizidone (a D-cycloserine analogue) with other TB drugs, encompasses an important component of the MDR and XDR treatment regimen in South Africa (<http://www.health-e.org.za/wp-content/uploads/2014/06/MDR-TB-Clinical-Guidelines-Updated-Jan-2013.pdf>). Further examination of the global distribution of *ald* LOF and other D-cycloserine resistance conferring mutations will enable an evaluation of the extent of D-cycloserine resistance in the absence of phenotypic drug resistance testing. Ultimately the identification of these mutations will allow studies to determine if they impact on clinical outcomes, and provide critical information for the development of rapid genetic based tests for D-cycloserine resistance.

Conclusion

Our data is representative of diverse drug resistance profiles of clinical isolates in KwaZulu-Natal. We were able to identify potential drug resistance conferring mutations by utilizing whole genome sequencing (Cohen et al., 2015, Desjardins et al., 2016).

Our results identified that *ald* LOF mutations represent a novel resistance mechanism to D-cycloserine albeit at a low-level of resistance. These strains should therefore be treated with attentiveness for resistance, even if they appear to be D-cycloserine susceptible on conventional testing methods. They may represent a stepping stone to higher level resistance. The clinical implication of these mutations is unknown, but given that D-cycloserine can cause severe neurological side effects their presence could be sufficient to exclude D-cycloserine treatment where other options are available.

Although our data are from a single province of a single country we have also expanded the knowledge of genetic mechanisms of resistance. We have also shown that both *alr* promoter and intragenic mutations are associated with higher levels of resistance to D-cycloserine. We did not find *ddl* or *cycA* mutations in our data which suggests these genes may be of lesser importance in conferring D-cycloserine resistance.

Due to limitations of drug resistance testing in the presence of pyruvate we are currently unable to determine if all the RD9 strains of the MTBC have an elevated MIC to D-cycloserine. But this seems highly likely in which case *ald* LOF is a rare example of emergence of a bacterial drug resistance conferring mechanisms in the absence of antibiotic selective pressure.

Our work will provide data for the development of novel molecular diagnostics which will allow individualized use of D-cycloserine and reduction MDR-TB associated drug toxicities.

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Appendix 1

Media Formulation

7H9 Broth

1. 4.7 g powdered Middlebrook 7H9 broth base was dissolved in 900 mL dH₂O.
2. 5 mL of 40% glycerol was added to the mixture.
3. Autoclaved at 121°C for 15 minutes.
4. Media was allowed to cool and 100 mL OADC and 2.5 mL 20% Tween 80 added.
5. Stored at 4°C until use, three-week expiration post preparation.

7H10 Agar

1. 19 g powdered Middlebrook 7H9 broth base was dissolved in 900 mL dH₂O.
2. 12.5 mL of 40% glycerol was added to the mixture.
3. Autoclaved at 121°C for 15 minutes.
4. Media was allowed to cool to ~65°C and 100 mL OADC added.
5. 25 mL media was poured per petri dish and allowed to cool.
6. Stored at 4°C until use, three-week expiration post preparation.

LB Broth

1. 25 g powdered LB broth base (Sigma L3522), was dissolved in 1000 mL dH₂O.
2. Autoclaved at 121°C for 15 minutes.
3. Media was allowed to cool and stored at 4°C until use, three-week expiration post preparation.

LB Agar

1. 37 g powdered LB agar base (Sigma L3147), was dissolved in 1000 mL dH₂O.
2. Autoclaved at 121°C for 15 minutes.
3. Media was allowed to cool to ~65°C and desired drug added at this interval if required.
4. 25 mL media was poured per petri dish and allowed to cool.
5. Stored at 4°C until use, three-week expiration post preparation.

SOC medium

1. 20 g powdered Bacto Tryptone and 5 g Bacto Yeast Extract was weighed out.
2. The weighed out powders and 2 mL of 5 M KCl and 10 mL of 1 M MgCl₂ was dissolved in 970 mL dH₂O.
3. The medium was autoclaved at 121°C for 15 minutes.
4. Media was allowed to cool to ~ 65°C and sterile 10 mL of 1 M MgSO₄ and 20 mL of 1 M glucose was added.
5. Stored at 4°C until use, three-week expiration post preparation.

1 M Sodium phosphate buffer (pH 8) (Sambrook and Russell, 2001)

1. 138 g powdered NaH₂PO₄.H₂O (monobasic; m.w.=138) was dissolved in 1 L dH₂O using a magnetic stirrer to achieve a 1 M stock concentration.
2. To a separate flask 142 g powdered Na₂HPO₄ (dibasic; m.w.=142) was dissolved in 1 L dH₂O using a magnetic stirrer to achieve a 1 M stock concentration.
3. To prepare a 50 mL of sodium phosphate buffer at pH 8, add 4.66 mL of 1 M Na₂HPO₄ and 0.34 mL of 1 M NaH₂PO₄ to 45 mL dH₂O.
4. pH was adjusted to 8 with concentrated HCl or NaOH.
5. Prepared working solutions were filter sterilized using a 0.22-micron filter system.
6. Stored at room temperature, one-month expiration post preparation.

D-cycloserine antibiotic preparation

D-cycloserine (Sigma C6880), was prepared freshly in 0.1M Sodium phosphate buffer (pH 8) on the day of LJ preparation testing due to instability of the drug. The stock of D-cycloserine was prepared and filter sterilised with a 0.22-micron filter.

MGIT supplementation

The MGIT (Mycobacteria Growth Indicator Tube), contains 7 mL of Middlebrook 7H9 broth base (**BD BBL™ MGIT™** Cat. No. 245122)

1. To the 7 mL MGIT tubes 800 µL growth supplement which contains OADC (Oleic acid Dextrose Albumin Catalase) was added. (**BD BACTEC™ MGIT™** Cat. No. 245124).

Preparation of Löwenstein-Jensen (LJ) medium

LJ medium is required for mycobacterial culture (Jensen, 1954) and is recommended by the WHO (Woods, 2000, Kam et al., 2010). Fresh eggs were used for LJ preparation.

1. To a sterile flask dissolve 2.4 g monopotassium dihydrophosphate (KH_2PO_4), 0.24 g magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), 0.6 g magnesium citrate, 3.6 g L-asparagine in 600 mL dH_2O .
2. Autoclaved at 121°C for 20 minutes.
3. To avoid contamination, eggs were cleaned by washing them thoroughly with plain soap and rinsed with water. Thereafter the eggs were wiped with 70% ethanol and cracked with a sterile knife. A 1000 mL egg homogenate was used.
4. The egg suspension and 600 mL sterile LJ medium were aseptically mixed and cooled to approximately $50\text{-}60^\circ\text{C}$.
5. The appropriate volume of D-cycloserine was added to the LJ tubes prior to inspissation to achieve final concentrations of 0, 10, 15, 20, 25, 30, 40, 60, and $120\ \mu\text{g/mL}$.
6. The drug containing medium was dispensed in 6 mL amounts into screw cap 15 mL conicals and placed in a slanted position on the inspissator at 85°C for approximately one hour to set.
7. To check for sterility, the prepared batch of media was incubated at 37°C for 24 hours.

Appendix 2

Supplementary Figures

The *ald* LOF mutations occurred within drug-resistant clinical isolates from Lineage 4. This mutation occurred in 25 MDR and XDR *M. tuberculosis* strains, indicating that this LOF occurred in patients receiving second-line treatment.

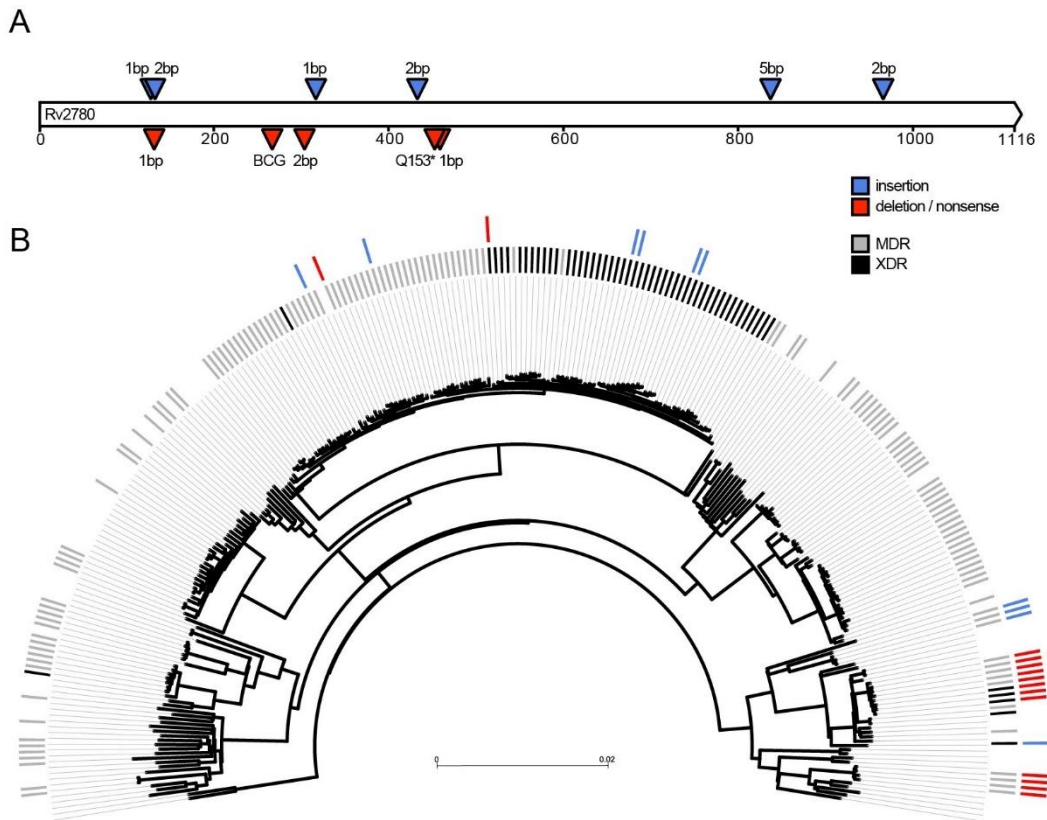


Figure S1: Pattern of convergent evolution in drug-resistant *M. tuberculosis* strains (Desjardins et al., 2016). **A) Distribution of mutations across full length of the *ald* gene.** Position of insertions (blue triangles), deletions and nonsense (red triangles) mutations in *ald* of *M. tuberculosis* drug-resistant strains. Mutations in *ald* which occur in *M. bovis* BCG are also displayed. **B) Phylogenetic representation of *ald* LOF mutation in Lineage 4 drug-resistant strains.** Inner Grey ticks represent MDR *M. tuberculosis* strains. Inner Black ticks represent XDR *M. tuberculosis* strains. Outer blue ticks represent insertion mutations in *ald*, and red triangles represent deletion and nonsense mutations in *ald*.

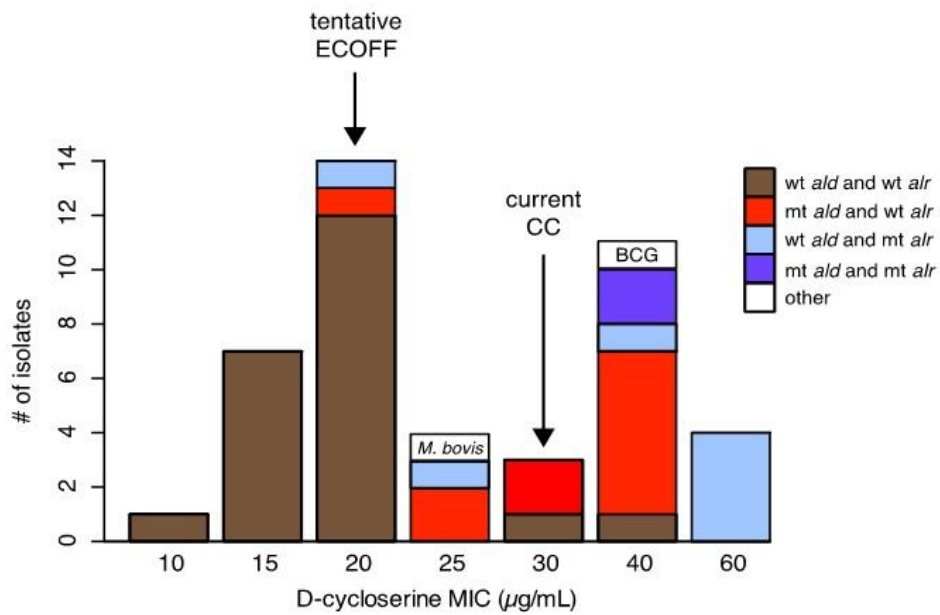


Figure S2: Distribution of D-cycloserine resistance in clinical *M. tuberculosis* strains (Desjardins et al., 2016) This histogram represents distribution of D-cycloserine MICs of the respective *M. tuberculosis* clinical strains according to the different mutations possessed. The current critical concentration (CC) for D-cycloserine and tentative epidemiological cut off (ECOFF) are shown. The MICs of BCG and *M. bovis* are also represented on this histogram.