**BRIEF DEFINITIVE REPORT**

**Metabolic bifunctionality of Rv0812 couples folate and peptidoglycan biosynthesis in Mycobacterium tuberculosis**

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Comparative sequence analysis has enabled the annotation of millions of genes from organisms across the evolutionary tree. However, this approach has inherently biased the annotation of phylogenetically ubiquitous, rather than species-specific, functions. The ecologically unusual pathogen *Mycobacterium tuberculosis* (*Mtb*) has evolved in humans as its sole reservoir and emerged as the leading bacterial cause of death worldwide. However, the physiological factors that define *Mtb*’s pathogenicity are poorly understood. Here, we report the structure and function of a protein that is required for optimal in vitro fitness and bears homology to two distinct enzymes, Rv0812. Despite diversification of related orthologues into biochemically distinct enzyme families, rv0812 encodes a single active site with aminodeoxychorismate lyase and D-amino acid transaminase activities. The mutual exclusivity of substrate occupancy in this active site mediates coupling between nucleic acid and cell wall biosynthesis, prioritizing PABA over D-Ala/D-Glu biosynthesis. This bifunctionality reveals a novel, enzymatically encoded fail-safe mechanism that may help *Mtb* and other bacteria couple replication and division.

**Introduction**

Despite Koch’s discovery of *Mycobacterium tuberculosis* (*Mtb*) as the causative agent of tuberculosis (TB) over 130 yr ago, TB remains the leading cause of death due to infectious disease and has emerged as the leading cause of death due to antibiotic resistance (World Health Organization, 2020). Surprisingly, knowledge of *Mtb*-specific physiology remains incomplete. High-throughput sequencing technologies have helped to overcome this barrier (Cole et al., 1998; Saghatelian and Cravatt, 2005); however, it is estimated that approximately one third to one half of all *Mtb* genes lack a functional annotation, are potentially misannotated, or encode noncanonical activities beyond their predicted functions (Hanson et al., 2009).

Current bioinformatic sequence annotations, to a great extent, are based on sequence homology. These methods derive their utility from phylogenetically conserved structure–function relationships. While powerful, these methods are associated with an underappreciated bias favoring the annotation of evolutionarily invariant functions, leaving the most specific features of a given organism’s physiology undefined (Cole et al., 1998; Lechartier et al., 2014). This bias is amplified further by its extension to genes exhibiting intermediate degrees of sequence conservation, where annotations of ancestral over species-specific functions are favored, and when functions are identified, they are often restricted to a general class level.

Here, we investigated the function of rv0812, a gene that was predicted to be essential for in vitro growth of *Mtb* and found to be upregulated within the lungs of infected mice, but that bears homology to two biochemically distinct enzymes (Dejesus and Ioerger, 2013; Dejesus et al., 2017b; Dubnau et al., 2005; Griffin et al., 2011; Zhang et al., 2012). Bioinformatic sequence analysis identified Rv0812 as a member of the type IV family of pyridoxyl-5’-phosphate (PLP)-dependent enzymes (PLPDEs), with nearly equivalent degrees of homology to bacterial aminotransferases involved in synthesis of cell wall–associated D-amino acids and enzymes involved in the synthesis of the folate precursor para-aminobenzoic acid (PABA; Cole et al., 1998; Kapopoulou et al., 2011; Lew et al., 2011; Pruitt et al., 2007; Wattam et al., 2014). Previous work implicated a role for Rv0812 in the latter (Chim et al., 2011; Thiede et al., 2016). Using a combination of biochemistry, metabolomics, structural biology, and chemical genetics, we demonstrate that Rv0812 catalyzes two enzymatic reactions in biochemically unrelated pathways. One is as an aminodeoxychorismate (ADC) lyase (ADCL) involved in folate biosynthesis. The other is as a D-amino acid...
transaminase (DAAT) involved in peptidoglycan (PG) biosynthesis. All reported ADCLs and DAATs have been found to be encoded by separate genes (Jhee et al., 2000; Mehta and Christen, 2000; Miles, 1985). The bifunctionality of Rv0812 suggests that Mtb has encoded one enzyme with both activities to mechanistically buffer against the competing metabolic demands of nucleic acid and cell wall biosynthesis in a manner that may ensure the orderly progression of Mtb replication and division.

**Results and discussion**

**Biochemical characterization of recombinant Rv0812**

Comparative sequence analysis annotates Rv0812 as a type IV PLPDE (Cole et al., 1998). PLP, the biologically active form of vitamin B6, is an enzymatic cofactor used by one of the most diverse superfamilies of enzymes and is involved in the catalysis of a wide range of transamination, decarboxylation, deamination, and racemization reactions (Christen and Mehta, 2001). The type IV family of PLPDEs is distinguished by a sterically conserved active site architecture that promotes re-face rather than si-face proton transfer relative to the C4’ of the planar pi system of the cofactor (Jhee et al., 2000; Martínez del Pozo et al., 1989a; Sugio et al., 1995; Yoshimura et al., 1993). Annotated members of the type IV family include ADCL, DAAT, branched chain amino acid transaminase, and R-stereospecific amine transaminase (Percudani and Peracchi, 2009), each of which appears to have divergently evolved distinct substrate and reaction specificities.

In contrast to most type IV PLPDEs, Rv0812 exhibits equivalent degrees of sequence homology to both ADCLs and DAATs. Published work has implicated Rv0812 in each role (Chim et al., 2011; DeJesus et al., 2017a; Kieser et al., 2015; Thiede et al., 2016; Xu et al., 2017). We sought to resolve this ambiguity by characterizing the in vitro activity of purified recombinant Rv0812. Consistent with previous phenotypic studies of a Rv0812 transposon mutant and biochemical assays of a purified preparation of recombinant Rv0812, we first confirmed Rv0812’s enzymatic activity as an ADCL (Chim et al., 2011). Owing to the intrinsic chemical instability of the substrate ADC, which precluded determination of absolute kinetic parameters, we assayed for ADCL activity using a published coupled assay in which ADC was generated in situ through the addition of chorismate to a small-molecule extract containing hundreds of potentially competing substrates (Fig. 1, A and D; de Carvalho et al., 2010). Kinetic studies revealed that Rv0812’s DAAT activity is strictly restricted to D-Ala and D-Glu, as a substrate mix containing α-ketoglutarate (AKG) and a combination of all other D-amino acids showed minimal D-Glu production over time (Fig. S1 A).

To determine the catalytic efficiency of this activity, we determined steady-state kinetic parameters for D-Ala (K_M [Michaelis constant] and k_cat [turnover number] 7.3 ± 1 mM and 1.46 s⁻¹, respectively; Fig. S1 B) and D-Glu (K_M and k_cat 0.24 ± 0.05 mM and 0.252 s⁻¹, respectively; Fig. S1 C). These values are comparable to those reported for the DAAT from the Bacillus sp. YM-1 strain (K_M and k_cat for D-Ala: 48 ± 12 mM and 129.6 s⁻¹; K_M and k_cat for D-Glu: 1.2 ± 0.2 mM and 4.3 s⁻¹, respectively), which exhibits weaker substrate affinities but higher turnover rates (Fig. 1 E; Bhatia et al., 1993). The M. smegmatis homologue of Rv0812, MSMEG_5795 (70% identity), similarly exhibited a comparable kinetic profile (Fig. 1 E; Mortuza et al., 2018). In contrast, a comparison of specificity constants (k_cat/K_M) revealed a preference of Rv0812 for D-Glu over D-Ala (1 vs. 0.2) while the Bacillus DAAT exhibited similar values for both substrates (2.7 vs. 3.6).

Substrate competition experiments were conducted to further elucidate the physiological substrate preferences of Rv0812 under conditions in which both ADCL and DAAT substrates were present. This analysis demonstrated that the catalytic efficiency of Rv0812’s ADCL activity was reduced by ~50% in the presence of saturating concentrations of D-Ala or D-Glu (Fig. S1, D and E). Both substrates mediated this inhibition in a competitive manner, as their presence increased K_M for ADC (Fig. S1 D) without affecting the turnover rate (Fig. S1 E). Consistent with the higher binding affinity of Rv0812 for D-Glu over D-Ala, the presence of D-Glu imparted a more dramatic increase in Rv0812’s K_M for ADC (Fig. S1 D). This is further supported by prior reports of D-Glu-mediated ADC inhibition in the absence of keto-acids (Magnani et al., 2013). Interestingly, in the reverse competition experiments, we found that while ADC similarly acted as a competitive inhibitor of Rv0812’s DAAT activity with D-Ala as a substrate, its presence had little impact on Rv0812’s kinetic parameters for reactions with D-Glu as the substrate (Fig. S1, F and G).

**Structural characterization of Rv0812**

To elucidate the structural basis of Rv0812’s enzymatic bifunctionality, we determined the x-ray crystal structure of recombinant Rv0812 at 2.4-Å resolution. Crystals of apo-Rv0812 belonged to the primitive monoclinic space group P2_1 with two molecules in the asymmetric unit, consistent with the behavior of Rv0812 on size exclusion chromatography and of other ADCLs
and DAATs that are functional as homodimers in solution (Table S1; Martínez del Pozo et al., 1989b; Padmanabhan et al., 2009; Ye et al., 1990). Crystals of PLP-bound Rv0812 were similarly found to belong to the P2₁ space group, with four molecules composed of two homodimers, AB and CD—in the asymmetric unit (Table S1). Molecular replacement using the type IV PLPDE CpuTA1 from Curtobacterium pusillum (Protein Data Bank [PDB] accession no. 5K3W) was used to solve and refine the structure of apo-Rv0812. The refined apo-structure was used to solve the structure of Rv0812 with PLP. There was clear electron density extending from the sidechain of Lys149 (Fig. 2 C), predicted to form the Schiff base with PLP. PLP was fit into the corresponding electron density and it was refined with good stereochemistry for all four molecules found in the asymmetric unit (Fig. 2 C). Residues common to type IV PLPDEs and involved in PLP binding, including His47, Arg50, Tyr153, Glu182, and Ser253, were similarly observed (Fig. 2 C; Marchler-Bauer et al., 2017), although residues neighboring the active site differed from those in other PLP-dependent enzymes (Fig. 2 D).

Superimposing the PLP-bound structure of Rv0812 onto representative PLP-bound DAAT and ADCL structures, we identified structural features that distinguished the Rv0812 active site from that of the DAAT from the Bacillus sp. YM-1 strain (BsDAAT; Peisach et al., 1998) and ADCL from Pseudomonas aeruginosa (PaADCL, PDB: 2Y4R; O’Rourke et al., 2011). BsDAAT and PaADCL share 23% and 27% sequence identity with Rv0812, respectively. Overlaiding all three active sites, we noted a conserved set of core catalytic residues—K149/K145/K140, E182/E177/E173, and R50/R50/R46—in Rv0812, BsDAAT, and PaADCL, respectively (Fig. 2 D); however, there were unique features of the Rv0812 active site that help explain its dual specificity. The active sites of BsDAAT and PaADCL both include a long loop of substrate-interacting residues that extend from the opposing subunit (Fig. 2 B). In BsDAAT, two residues in this loop—Arg98*
and His100*—close to the -OH of PLP, have been proposed to comprise a carboxylate trap (Peisach et al., 1998), required for correct positioning of incoming substrates. PaADCL also contains a 17-residue long loop, structurally similar to that of BsDAAT, but which is involved in stabilizing PLP-OH via a Tyr92* residue (O’Rourke et al., 2011). In contrast, Rv0812 encodes a much shorter loop that consists of only nine residues (L7: Arg95*—Pro103*), none of which appear to interact directly with substrates for either reaction (Fig. 2 B).

In light of this difference, we determined the structure of Rv0812 bound to its DAAT products in order to elucidate the structural basis of substrate binding by Rv0812. Rv0812 crystals produced after incubation with PLP and D-Glu were isomorphous with the PLP-containing crystals (Table S1) and contained electron density corresponding to pyridoxamine 5'-phosphate (PMP) and AKG in the active site, allowing the cofactor and product to be manually built in Emsley and Cowtan (2004). The PMP-AKG bound structure revealed that Arg98* in BsDAAT (Figs. 2 C and S1 N), which formed H-bonds and salt bridges with the AKG C5 (2.7 Å) and D-Ala carboxylates (3.3 Å, 3.4 Å), corresponding to Arg26* in Rv0812, while Arg115 and Arg185 donated H-bonds (2.4 Å, 2.8 Å) to the AKG C5 carboxylate, and Arg115 (2.7 Å) and Thr255 (3.0 Å) stabilized the AKG C1 carboxylate (Fig. S1, J and K). These interactions serve to identify a structurally distinct, but functionally analogous, carboxylate trap in Rv0812, while allowing for potentially broader substrate specificity (Figs. 2 and S1, J and N).

Experimental efforts to obtain crystals of Rv0812 bound to PABA were unsuccessful; however, in silico modeling of Rv0812 using the structure of PaADCL enabled manual docking of ADC into the AKG binding site of the PMP-AKG bound structure. Notwithstanding known limitations of energy minimization-based docking studies (that include protein structure rigidity and the absence of coordinating water molecules), this model revealed a series of ADC-coordinating residues that notably included the carboxylate trap residues Arg26*, Arg115, and Arg185, which appear positioned to stabilize the C9 carboxylate of ADC through a network of H-bonds and salt bridges (2.8 Å, 3.3 Å, 3.1 Å), while Arg115 (2.3 Å), Ser252 (2.7 Å), and Thr255 (2.5 Å) appear within H-bonding distance of the C5 carboxylate of ADC, and Arg26* and Thr33 appear within H-bonding distance of the oxygen next to the C1 olefin (2.3 Å) and amino group (3.1 Å) of ADC, respectively (Fig. S1 L). An overlay of the Rv0812-AKG complex structure with that of PaADCL conversely revealed a Tyr residue (Tyr22*) at the position corresponding to Arg26*, but Tyr22 was nearly 5 Å away from the ARG C5 carboxylate (Fig. S1 P).

Interestingly, ADCL enzymes from E. coli and P. falciparum, both of which contain the conserved Y22 residue, have previously been shown to act on D-Ala (Jhee et al., 2000; Magnani et al., 2013). Due to their extremely low efficiencies with D-Ala, however, these activities were proposed to be moonlighting functions derived from a shared ancestral active site. The turnover rate of D-Ala transamination by the R26A mutant compared with WT protein (Fig. S1 H). DAAT activity of the R26A variant exhibited a greater impact on k_cat

Figure 2. Structural characterization of Rv0812 reveals the basis for dual ADCL and DAAT activities. (A) Ribbon diagram of PLP-bound dimer (loops in gray, α-helices in pink, and β-sheets in light blue; panels A–D display the PLP molecule in magenta). (C) Side chains of PLP-neighboring residues within 5 Å are shown. (B and D) Structural comparison to BsDAAT and PaADCL. A shortened loop in Rv0812 (magenta) prevents interaction with the partner subunit active site, as in BsDAAT (orange) and PaADCL (cyan). (D) Active site comparison of PLP-Rv0812 (purple), PLP-bound BsDAAT (yellow), and PaADCL (green), with residues labeled as Rv0812/BsDAAT/ PaADCL. H-bonds formed between Rv0812 and PLP (C) are shown with dashed lines, and the |(2Fo)-(Fc)| electron density map, contoured at 1.2σ, of PLP and Lys149 (C) is shown as chicken wire.
(0.09 ± 0.2 s⁻¹) over $K_M$, D-Ala (2 ± 1.3 mM), suggesting a mechanistic role for the carboxylate trap in formation of a productive enzyme-substrate complex (Fig. 1E). Furthermore, the heightened impact of the R26A substitution on the DAAT activity of Rv0812, specifically, supports the notion that it is responsible for boosting DAAT activity to the extent that it is physiologically relevant.

Bioinformatic reanalysis of Rv0812 phylogeny

The structural identification of active site residues associated with the dual ADCL/DAAT activities of Rv0812 prompted a bioinformatic search for additional bifunctional orthologues (Marchler-Bauer et al., 2017). This resulted in the discovery of orthologous sequences containing the Arg26 residue across various bacterial phyla. A phylogenetic sequence analysis of type IV PLPDEs that included both ADCL and DAAT-like sequences further revealed that Rv0812 orthologues formed two different and novel clades. The clade containing Rv0812 (Clade I) clustered with a handful of annotated ADCL-like sequences but away from the majority of canonical ADCLs, while the second clade (Clade II) appeared to have more similarity to transaminase subfamilies within type IV PLPDEs (Fig. 3A; Jhee et al., 2000; O’Rourke et al., 2011). Phylogenetic mapping across bacterial phyla further revealed that the majority of Clade I orthologues were found in gram-positive species, while Clade II orthologues were only found in gram-negative species (Fig. 3B), suggesting the divergent evolution of a distinct, yet-to-be determined, activity.

Previous analyses had identified two separate subclasses of ADCL-like enzymes based on the subunit contributions of the active site Tyr92/Tyr153 residue, with one subclass deriving its active site Tyr residue from the same subunit forming the active site (Position I) and the other subclass contributing the active site Tyr to the opposite protomeric subunit (Position II; O’Rourke et al., 2011). PaADCL belongs to the former subclass, while Rv0812 is a member of the latter. Surprisingly, the two ADCL clusters observed in our analysis did not conform to this classification, as members of the Position II subclass were observed in both clusters, though all sequences in the Rv0812 cluster belonged to the Position II subclass (Waterhouse et al., 2009). We found instead that all Rv0812-like, ADCL-like sequences encoded an Arg (R26*) rather than Tyr (Y22*) at the equivalent position, in addition to several other conserved sequence differences (Fig. S2). The Rv0812-containing cluster thus appears to constitute a novel group of the Position II subclass, with sequences that share greater active site similarity to DAATs.

Awaiting future studies of this nascent subclass of bifunctional ADCL-like sequences, studies of the ADCL from Arabidopsis thaliana, which encodes the same conserved active site residues as Rv0812 (Fig. S2A), have separately reported evidence of ADCL activity in one study and DAAT activity in another.
another (Basset et al., 2004; Funakoshi et al., 2008). Moreover, superpositioning the active site of Rv0812 with that of an in silico model of ADCL/DAAT from A. thaliana (Fig. S2 B) further revealed a high degree (eight of 12 residues) of conservation of residues lining the cofactor binding site, while the spatial position of the catalytically important Arg26* of Rv0812 differed from that of Arg104* in the A. thaliana structure by less than 1.1 Å among all nearby main chain atoms (Fig. S2 C).

It is similarly interesting to note the presence of two annotated ADCL (pabC)-like sequences in the Streptomyces genome (Zhang et al., 2009). The canonical PabC-1 sequence, encoded by a gene located near pabAB, shows the conservation of the canonical ADCL Y22 and N226 residues, while the PabC-2 sequence, phylogenetically clustered with Rv0812, does not. It is unknown whether PabC-1 and -2 exhibited DAAT activity, but the unusual occurrence of two ADCL sequences in the genome and differences in their active site conservation suggest an additional DAAT function for PabC-2, rather than redundancy.

**Physiological characterization of Rv0812**

Phylogenetic predictions notwithstanding, we sought evidence of a physiological linkage between these two biochemically distinct activities. To do so, we generated a precise isogenic deletion of Rv0812. Consistent with previous reports of an ΔRv0812 mutant in H37Rv and a transposon insertion mutant in H37Ra, ΔRv0812 Mtb exhibited a growth defect in liquid culture that could be corrected by expression of an extragenic copy of the WT allele or chemical addition of PABA to the culture medium (Fig. S1 I; Thiede et al., 2016). Comparative metabolic profiling further revealed a marked and selective accumulation of D-Ala or D-Glu.

Rv0812 is a bifunctional enzyme Rv0812 https://doi.org/10.1084/jem.20191957

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M. tuberculosis bifunctional enzyme Rv0812

DCS and BCDA was due to a loss of both the DAAT and ADCL activities of Rv0812. However, we noted that the ΔRv0812 mutant exhibited an additional 1 log$_{10}$ loss of viability compared with either the WT or complemented strain for each compound, indicating an essential and specific role for the DAAT activity of Rv0812 in Mtb viability when Alr or Murl racemase activity is absent. We further showed that this enhanced susceptibility to DCS or BCDA could be rescued, in part, by the addition of exogenous D-Ala and D-Glu, or PABA, to the culture medium (Fig. 5, A and C). These results provide further physiological evidence of Rv0812’s activity as a bidirectional DAAT.

Restoration of WT levels of susceptibility to either DCS or BCDA in ΔRv0812 Mtb required the joint addition of exogenous D-Ala, D-Glu, and PABA (Fig. 5, A and C). Given the chemically and mechanistically distinct nature of DCS and BCDA and the fact that PABA alone did not cause any measurable degree of rescue from DCS or BCDA in WT Mtb, this requirement suggested that the increased susceptibility of ΔRv0812 Mtb to both DCS and BCDA was due to a loss of both the DAAT and ADCL activities of Rv0812.

Interestingly, DCS exhibited a larger impact on ΔRv0812 Mtb than BCDA. Recent work has shown that the antimycobacterial activity of DCS is mediated through the inhibition of multiple targets (de Chiara et al., 2020). We profiled the metabolomic impact of DCS on WT Mtb during the prelethal phase of

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treatment and discovered a specific impact on intermediates and downstream products of folate metabolism at bactericidal, but not bacteriostatic, concentrations (Fig. 5 D). We further observed that treatment of WT Mtb with subinhibitory concentrations of DCS combined with subinhibitory concentrations of either PAS or WR99210 (a whole-cell inhibitor of Mtb’s dihydrofolate reductase; Nixon et al., 2014) resulted in a >5 log_{10} reduction in Mtb viability (Fig. 5 B). This synergy suggests that the increased susceptibility of ΔRv0812 Mtb than BCDA is due to its additional inhibition of Mtb folate biosynthesis. Moreover, this increased susceptibility reveals a previously unrecognized biological coupling of PG and folate biosynthesis in Mtb that the enzymatic bifunctionality of Rv0812 appears poised to serve as an enzymatic failsafe defense.

**Potential implications of a bifunctional ADCL/DAAT**

Owing to their essentiality in bacteria and absence in humans, folate and PG biosynthesis pathways have served as highly validated antitubercular drug targets for decades (Gautam et al., 2011; Green and Matthews, 2007). Knowledge of specific physiological links between these two target pathways, however, has remained unaddressed. Like all cells, bacteria face the challenge
of needing to coordinate growth and division to ensure successful replication. For Mtb, this challenge is complicated by the erratic, albeit repetitive, nature of its cell cycle, in which replication often follows a prolonged, unpredictable interval of host-imposed nutritional deprivation and biochemical stress (Ehrt et al., 2018; Smith, 2003).

Our discovery of Rv0812 as a bifunctional ADCL and DAAT reveals a previously unrecognized metabolic coupling between nucleic acid and cell wall biosynthesis that appears to ensure prioritization of PABA production over D-Ala/D-Glu biosynthesis. This prioritization is evidenced by the phenotypic auxotrophy of ΔRv0812 for PABA rather than D-Ala or D-Glu. Moreover, because both ADCL and DAAT activities are catalyzed by the same active site chemistry (Percudani and Peracchi, 2009), Rv0812 appears enzymatically poised to function as a metabolic toggle that alternates between ADCL and DAAT activity, prioritizing the former over the latter in response to substrate accumulation. From a structural perspective, this bifunctionality appears to have been selected for, in part, by the loss of conserved residues Y22, Y/H112, and N236 specific to ADCLs (Nakai et al., 2000; O’Rourke et al., 2011; Parsons et al., 2002). The reaction rates of its DAAT activity additionally indicate that Rv0812 kinetically favors the production of D-Glu five times more than D-Ala. This preference is further supported by the greater degree of Rv0812-dependent D-Glu accumulation in Mtb supplemented with D-Ala than that of D-Ala and (D-Ala)2 in culture medium containing D-Glu. As all transaminase reactions are thermodynamically freely reversible, the equivalent vulnerability of ΔRv0812 to inhibition of either D-Ala or D-Glu biosynthesis, combined with its strong affinity for D-Glu and the previously reported synergy between DCS and BCDA against WT Mtb, indicate that Rv0812 has evolved to ensure balanced production of both D-Ala and D-Glu (David, 2001). Moreover, the demonstrated ability of Rv0812 to catalyze the interconversion of D-Ala and D-Glu in vivo identifies a previously unrecognized role for its DAAT activity in defense against PG-targeting drugs.

The competition between ADCL and DAAT substrates for the active site of Rv0812 further reveals a new physiological link between nucleic acid and PG biosynthesis. This link is manifested by the phenotypic auxotrophy of ΔRv0812 for PABA rather than D-Ala or D-Glu. Moreover, because both ADCL and DAAT activities are catalyzed by the same active site chemistry (Percudani and Peracchi, 2009), Rv0812 appears enzymatically poised to function as a metabolic toggle that alternates between ADCL and DAAT activity, prioritizing the former over the latter in response to substrate accumulation. From a structural perspective, this bifunctionality appears to have been selected for, in part, by the loss of conserved residues Y22, Y/H112, and N236 specific to ADCLs (Nakai et al., 2000; O’Rourke et al., 2011; Parsons et al., 2002). The reaction rates of its DAAT activity additionally indicate that Rv0812 kinetically favors the production of D-Glu five times more than D-Ala. This preference is further supported by the greater degree of Rv0812-dependent D-Glu accumulation in Mtb supplemented with D-Ala than that of D-Ala and (D-Ala)2 in culture medium containing D-Glu. As all transaminase reactions are thermodynamically freely reversible, the equivalent vulnerability of ΔRv0812 to inhibition of either D-Ala or D-Glu biosynthesis, combined with its strong affinity for D-Glu and the previously reported synergy between DCS and BCDA against WT Mtb, indicate that Rv0812 has evolved to ensure balanced production of both D-Ala and D-Glu (David, 2001). Moreover, the demonstrated ability of Rv0812 to catalyze the interconversion of D-Ala and D-Glu in vivo identifies a previously unrecognized role for its DAAT activity in defense against PG-targeting drugs.
While powerful, an underappreciated limitation of homology-based gene annotations is their inherent bias toward evolutionarily invariant functions and limited ability to reveal functions related to more phylogenetically specific selective pressures. This is because conservation is an end product, rather than a driving force, of evolution. Yet organisms evolve their genomes in response to the specific selective pressures they encounter. Existing bioinformatic methods, such as Mtb, such features correspond to a biologically ideal but untapped source of potential diagnostic biomarkers and drug targets. The discovery of ADCL-like enzymes with homology to Rv0812 across a wider range of bacteria suggests that coordination of bacterial growth and division may be a more broadly conserved, but previously unannotated, metabolic activity.

Materials and methods

Strain construction

The rv0812 gene deletion mutant was constructed by allelic exchange via homologous recombination, as previously described (Gee et al., 2012), replacing the native copy of rv0812 with a zeocin resistance cassette. Mutant candidates were isolated from Middlebrook 7H10 solid culture medium supplemented with 1 µg/ml PABA and confirmed via Southern blot analysis. The rv0812 complemented strain was constructed by reintroducing a copy of rv0812 under the control of the hsp60 promoter into the attL5 site of the Mtb genome. MtB WT (H37Rv) and mutant strains were routinely grown in Sauton’s medium with 0.04% tyloxapol, and, when necessary, zeocin and kanamycin were added to cultures at final concentrations of 25 µg/ml and 20 µg/ml, respectively.

Protein expression and purification

The sequence of the full-length Rv0812 gene was amplified from the Mtb H37Rv genome by PCR. The amplified gene was inserted into a pMCSG19B expression vector containing an N-terminal 6x-His tag using ligation-independent cloning sites (Eischenfeldt et al., 2009). The Rv0812:R26A and Rv0812:Y153A mutants were constructed by amplifying pMCSG19B-Rv0812 plasmids using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Plasmids were transformed into BL21(DE3) E. coli cells for protein expression. Cells with plasmids were grown at 37°C to an OD₆₀₀ of ~0.6–0.8 in Difco LB medium (Becton Dickinson) with 100 µg/ml carbenicillin followed by induction with 500 µM isopropyl β-D-1-thiogalactopyranoside and grown overnight at 18°C.

The sequence of the full-length EcPabB gene was amplified from a BL21(DE3) single colony by PCR. The amplified gene was inserted into the same plasmid as Rv0812 using the same method as described. The plasmids were transformed into BL21(DE3) E. coli cells for EcPabB expression. The cells with the plasmid were grown at 37°C to an OD₆₀₀ of ~0.6–0.8 in Difco LB medium with 100 µg/ml carbenicillin followed by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside and grown for 6.5 h at 37°C.

Cells were lysed via a Microfluidizer M-100P (Microfluidics) in lysis buffer (50 mM Tris [pH 7.5], 500 mM NaCl, 10% glycerol, 25 mM imidazole, 2 mM 2-mercaptoethanol, 1 mM PMSF, 10 µg/ml DNase, and 2 mM MgCl₂) and centrifuged at 27,216 × g for 1 h. The supernatant was purified over a nickel column with a 0–500-mM imidazole gradient, followed by size-exclusion chromatography using elution buffer (25 mM Tris [pH 7.5], 100 mM NaCl, 10% glycerol, 2 mM dithiothreitol). The proteins were >95% pure, as observed by SDS-PAGE, and were concentrated to 5 mg/ml, flash frozen, and stored in elution buffer at −80°C. Protein concentrations were determined by measuring A₂₈₀ using a NanoDrop 2000 UV-visible spectrophotometer (Thermo Fisher Scientific) using extinction coefficients of 37,930 M⁻¹·cm⁻¹ and 56,755 M⁻¹·cm⁻¹ for Mtb Rv0812 and EcPabB, respectively.

Activity-based metabolomic profiling (ABMP)

*Mycobacterium bovis* Bacillus Calmette-Guerin was grown in 7H9 medium supplemented with 0.2% glycerol, 0.5% BSA, 0.2% dextrose, and 0.085% NaCl. A 9-liter culture grown to OD₆₅₀ of 1.0 was harvested by centrifugation, and the resulting pellet was resuspended in 100 ml of an acetonitrile (ACN):methanol: H₂O (40:40:20) solution. Cells were lysed using an EmulsiFlex C5 high-pressure homogenizer (Avestin) and centrifuged for 20 min at 20,000 × g. Soluble extract was lyophilized and re-suspended in 10 ml 25 mM Tris·HCl (pH 7.4), yielding the final small-molecule extract (SME).

For ABMP analysis, 150-µl reactions containing 75 µl SME and 5 µl purified recombinant Rv0812 (active or heat killed for 15 min at 95°C) were incubated in 100 mM Tris·HCl (pH 8.5) at 37°C in the presence of 50 µM PLP and 1 mM D-Ala or D-Glu. At indicated time points, reaction aliquots were quenched with cold ACN containing 0.2% formic acid for a final concentration of 80% quenching solution. After centrifugation at 20,000 × g for 10 min, the resulting supernatant was separated from insoluble material and stored at 4°C for liquid chromatography-mass spectrometry (LC-MS) analysis.

Mass spectrometry

For separation and detection of metabolites, LC-MS analysis was conducted using an Agilent 1200 LC system containing a Cogent Diamond Hydride Type C silica column (150 mm × 2.1 mm; Microsolv Technologies) coupled to an Agilent Accurate Mass 6220 TOF as described (Eoh and Rhee, 2013). The mobile phase consisted of solvent A (double-distilled H₂O with 0.2% formic acid) and solvent B (ACN with 0.2% formic acid) at a flow rate of 0.4 ml/min with the following gradient: 0–2 min, 85% B; 2–5 min, 80% B; 6–7 min, 75% B; 8–9 min, 70% B; 10–11.1 min, 50% B; 11.1–14 min, 20% B; and 14.1–24 min, 5% B; followed by a 10-min equilibration period at 85% solvent B before injection of the next sample. Dynamic mass axis calibration was accomplished by continuous infusion of a reference mass solution. Electrospray ionization capillary and fragmentor voltages were set at 3,500 V and 135 V, respectively. The nebulizer pressure was set to 40 psig and nitrogen drying gas was maintained at 250°C, set to a flow rate of 10 liter/min. The MS acquisition rate was 1.5 spectra/s and m/z data ranging from 50 to 1,700 was stored. Data
were analyzed using Profinder B.08.00 software, and ions were assigned as specific metabolites based on mass accuracy within 5 ppm and retention times within 1 min of those determined for chemical standards.

**Enzymatic activity assays**

Kinetic measurements of Rv0812’s transaminase activity were obtained by means of two distinct assays, according to the substrate being consumed in the reaction. An LC-MS–based assay was used for kinetic analysis using D-Glu as the substrate. Reactions (200 µl) contained 5 mM MgCl2, 5 mM pyruvate, 50 µM PLP, 0.25 µM WT-Rv0812, and varying concentrations of D-Glu (50 µM to 10 mM) in 100 mM Tris (pH 8.5). At indicated time points, reaction aliquots were quenched with cold ACN containing 0.2% formic acid for a final concentration of 80% quenching solution. After centrifugation at 20,000 × g for 10 min, the resulting supernatant was separated from insoluble material and stored at 4°C for LC-MS analysis.

The kinetic parameters of Rv0812’s transaminase activity, using D-Ala as a substrate, were determined by using an LDH-coupled assay. Reactions (200 µl) were performed at 25°C in 100 mM Tris (pH 8.5) containing 5 mM MgCl2, 5 mM AKG, 5 U/ml LDH, 1 mM nicotinamide adenine dinucleotide (NADH), 50 µM PLP, and 0.25 µM WT-Rv0812 or mutants. The buffer was premixed and incubated for 30 min. The assay was initiated by adding 25 µl D-Ala for final concentrations ranging from 31 µM to 32 mM. Enzyme activity was monitored by the decrease in absorbance at 340 nm, representing NADH consumption, using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific).

Kinetic measurements of Rv0812’s ADCL activity were likewise obtained by using an LDH-coupled assay. ADCL activity assays were performed at 25°C in 100 mM Tris (pH 8.5) containing 5 mM MgCl2, 100 mM (NH4)2SO4, 5 U/ml LDH, 1 mM nicotinamide adenine dinucleotide (NADH), 50 µM PLP, and 0.25 µM WT-Rv0812 or mutants. The buffer was premixed and incubated for 30 min. The assay was initiated by adding 25 µl D-Ala for final concentrations ranging from 0 to 2 mM. The buffer was premixed and incubated for 30 min. The assay was initiated by adding 0.5 µM WT-Rv0812 or mutants. PABA production was monitored using a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific) at 340 nm to monitor the decrease in NADH concentration. For quantitative analysis of pyruvate production, only the 2 mM chorsamate group was used.

For in vitro DAAT and ADCL kinetics assays, experiments were performed in triplicate for each protein construct and reported as average ± SEM. Kinetic data were fitted with the Michaelis-Menten equation (Michaelis et al., 2011) using the JMP Pro 13 Software (SAS Institute).

**Substrate competition assays**

Substrate competition assays were conducted as described above in the presence or absence of varying concentrations of substrates. ADCL assays were conducted in the presence of EcPabB and 0.4 mM or 1.6 mM chorismate as a surrogate for the ADC substrate in the presence or absence of D-Ala (0, 7.5, or 30 mM) or D-Glu (0, 0.25, or 1 mM) as competing substrate. DAAT assays were conducted in the presence of D-Ala (7.5 or 30 mM) or D-Glu (0.25, or 1 mM) in the presence or absence of EcPabB and chorismate (0, 0.4, or 1.6 mM) as competing substrate. Kinetic parameters in the presence or absence of competing substrate were determined by plotting the inverse velocity against the inverse substrate concentration on double reciprocal plots and calculating the inverse of the x- and y-intercepts.

**Phylogenetic analysis**

BLAST searches using the Rv0812 amino acid sequence were conducted against various bacterial phyla and classes with a maximum output of 100 hits for each group. BLAST hits were screened to include only those sequences with conservation of Arg26 as potentially Rv0812 like. Sequences were aligned with representative members of each type IV PLPDE subfamily using Clustal W2 and were subsequently placed into a phylogenetic tree using the neighbor joining method with BLOSUM62 to predict functional annotations. Phylogenetic trees were visualized using the interactive tree of life program (Letunic and Bork, 2019).

**In vivo metabolomic profiling of Mtb strains**

In vivo metabolic profiling of Mtb strains was conducted as previously described (Eoh and Rhee, 2013). A 1-ml culture (OD580 nm = 1.0) of each strain was collected on a nitrocellulose filter and grown on 7H10 plates for 5 d at 37°C. Filters were subsequently transferred to swimming pools containing ~3 ml of Sauton’s medium in the presence or absence of PABA (1 µg/ml), DCS, PAS, WR99210, D-Ala, and/or D-Glu. Filters were collected following 24 h of exposure, and metabolic activity was quenched by placing cells in 1 ml of an ACN: methanol:H2O (40: 40: 20) solution. Cells were lysed by mechanical disruption with 0.1-mm Zirconia beads (BioSpec Products) in a Precellys tissue homogenizer (cooled to 4°C) for 3 min at 6,500 rpm three times. Centrifugation enabled the separation of soluble cellular metabolites, which were then filtered through Spin-X (0.22 µM) columns for removal from the BSL3 facility. Metabolite extracts were diluted 1:1 in LC-MS solvent B, centrifuged for 10 min at 10,000 × g, and supernatant (2 µl) was injected onto a Diamond Hydride column for LC-MS analysis as described above. For normalization of ion abundance to cell biomass, the residual protein concentration in lysates was quantified (BCA Protein Assay Kit; Thermo Fisher Scientific).

**Drug susceptibility assays**

McB cultures grown to mid-log phase in Sauton’s medium were diluted in fresh medium to a starting OD580 nm of 0.01. Varying concentrations of compounds in DMSO were added at 1% of the total volume. Following 6 d of exposure to compounds, cultures were serially diluted with 1× PBS containing 0.04% tyloxapol. Serial dilutions were streaked on 7H10 plates and allowed to recover for 21 d, at which point colonies were counted at appropriate dilutions.

**Protein crystallization**

Rv0812 was first crystallized without incubation with cofactor or compounds. Crystallization conditions (formulated by Hampton Research) were screened using a Mosquito liquid dispenser (TTP Labtech) using the sitting drop vapor diffusion technique in the Crystal Mation Intelli-Plate 96–3 low-profile
crystallization plate (Hampton Research). For each condition, 0.4 µl protein (5 mg/ml) and 0.4 µl crystallization formulation were mixed, and the mixture was equilibrated with 50 µl crystallization solution in the reservoir well. Full-length WT Rv0812 protein crystals were further optimized via hanging drop vapor diffusion by incubating 2 µl purified protein solution (5 mg/ml) with 1 µl crystallization solution (1 M sodium citrate and 0.1 M sodium cacodylate [pH 7.1]) at 18°C for 3 d. Crystals formed as clusters during the first few optimizations. The microseeding method was applied to acquire larger and well-separated single crystals (Luft and DeTitta, 1999). A crystal cluster was taken from the optimized condition along with 10 µl mother liquor. The cluster was then crushed by vortexing for 3 min with a Seed Bead kit (Hampton research). The mixture was added to 90 µl mother liquor to prepare the 1:10 seed stock, which was further diluted to make 1:100 and 1:1,000 seed stocks. New crystals were obtained via hanging drop vapor diffusion by incubating 2 µl purified protein solution (3 mg/ml) with 1 µl seed stocks. The mixture was equilibrated with optimized crystallization conditions (1 M sodium citrate and 0.1 M sodium cacodylate [pH 7.1]) at 18°C for 3 d. The best crystals appeared in 1:1,000 dilution seed stock. These crystals were cryo-protected with 23% ethylene glycol and flash frozen before data collection. For PLP complex crystals, 0.2 mM PLP (pH adjusted to 6.6) was cocryrstallized with 3 mg/ml Rv0812 using the above procedure. For PMP and AKG-bound crystals, 1 mM D-Glu and 0.2 mM PLP (pH adjusted to 6.6) were cocryrstallized with 3 mg/ml Rv0812 using the same method as for PLP-bound crystals.

**Data collection and structure determination**

Data were collected at Argonne National Laboratory using the Advanced Photon Source beamlines 19ID and 23ID-D. All data were processed and reduced using HKL2000 (Otwinowski and Minor, 1997). The structure of apo-Rv0812 was solved by molecular replacement using MOLREP (Vagin and Teplyakov, 2010) in CCP4 (Winn et al., 2011) using the coordinates for the fold IV-transaminase (CpuTA) from Curtobacterium pusillum from the PDB (accession no. 5K3W; Pavkov-Keller et al., 2016). The PLP-bound and PMP and AKG-bound structures were solved by molecular replacement using MOLREP in CCP4 with the solved apo-Rv0812 structure. Refinement and manual model building were performed with PHENIX (Adams et al., 2010) and COOT (Emsley and Cowtan, 2004), respectively.

**Molecular docking**

The simplified molecular-input line-entry system (SMILES) string of ADC was created by the JSME Structure Editor (Bienfait and Ertl, 2013) and the coordinates were generated through the CADD Group SMILES Translator (Sitzmann et al., 2008). ADC was placed into the active site of the PMP-containing Rv0812 structure—AKG, waters, and other ligands were removed from the model before docking—in COOT (Emsley and Cowtan, 2004). ADC was incorporated into the location according to the bound PMP. The active site docking model with the lowest global energy minimum was generated by the MolSoft ICM Chemist Pro Docking Program (Orry and Abagyan, 2012).

A model of the A. thaliana ADCL/DAAT was generated from the amino acid sequence of the enzyme using the Phyre2 webserver (Kelley et al., 2015). Structural superposition of Rv0812 with A. thaliana ADCL/DAAT was performed using Chimera (Pettersen et al., 2004).

**Macromolecular assembly analysis**

After acquiring Rv0812 structures, both apo- and PLP-bound structure coordinate files were uploaded to the program PDBe-PISA (Krissinel and Henrick, 2007) to analyze the macromolecular assemblies. Interactions across the interfaces were found. The buried surface area of the interfaces and the free energy of assembly dissociation (ΔGdiss) were calculated for both structures.

**Data and software availability**

The Apo, PLP-bound, and PMP and AKG-bound structures were deposited in the PDB (Berman et al., 2000) with accession nos. 6Q1Q, 6Q1R, and 6Q1S, respectively.

**Online supplemental material**

Fig. S1 provides additional evidence for the ability of Rv0812 to serve as both a DAAT and ADCL in the form of kinetic, structural, and phenotypic analysis. Bioinformatic analysis and structural homology modeling shown in Fig. S2 suggest that Rv0812 and similar sequences constitute a novel subgroup of type IV PLPDEs that are defined by their proposed ADCL/DAAT bifunctionality. Fig. S3 provides additional evidence for the ability of Rv0812 to serve as an ADCL and DAAT in vivo. Table S1 lists detailed parameters of the crystallography data and refinement statistic.

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**References**

Adams, P.D., P.V. Afonine, G. Bunkóczi, V.B. Chen, I.W. Davis, N. Echols, J.J. Headd, L.W. Hung, G.J. Kapral, R.W. Grosse-Kunstleve, et al. 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66:213–221. https://doi.org/10.1107/S0907444909052925

Basset, G.J.C., S. Ravanel, E.P. Quinlivan, R. White, J.J. Giavanonni, F. Rebelle, B.P. Nichols, K. Shinozaki, M. Seki, J.F. Gregory, et al. 2004. Folate
Figure S1. Structural, kinetic, and phenotypic analysis demonstrate the ability of Rv0812 to serve as both a DAAT and ADCL. (A) Rv0812 DAAT activity is restricted to D-Ala and D-Glu. Reaction aliquots containing 0.5 µM Rv0812, 50 µM PLP, 10 mM AKG, and 1 mM of each D-amino acid were quenched at various time points for LC-MS analysis. Activity was quantified by assessing substrate depletion over time and normalizing to a standard curve. Specific activities with D-Ala and D-Glu are plotted on the right y-axis, and specific activity (spec. act.) with other substrates is plotted on the left y-axis. (B and C) Activity profile of Rv0812 DAAT reactions. The rate of pyruvate formation (B) or D-Glu consumption (C) was determined in reactions with 0.25 µM Rv0812, 5 mM AKG or pyruvate, 50 µM PLP, and varied substrate concentrations at pH 8.5. Data were fitted to the Michaelis-Menten equation to give kinetic parameters: (A) $V_{\text{max}} = 2.8 \pm 0.16$ U/mg and $K_M = 7.3 \pm 1.06$ mM; and (B) $V_{\text{max}} = 0.49 \pm 0.019$ U/mg and $K_M = 0.24 \pm 0.05$ mM. (D–G) Substrate competition assays demonstrated the impact of DAAT substrates on ADCL activity (D and E) and vice versa (F and G). The x-axis indicates the concentration of D-amino acid or chorismate relative to the $K_M$. The y-axis indicates the kinetic constants obtained in the presence or absence of competing substrate. Blue and green bars indicate the presence of D-Ala or D-Glu, respectively. Each column represents the mean ± SD ($n = 6$; *, $P < 0.05$; ***, $P < 0.001$) compared with parameters in the absence of competing substrate. (H) Pyruvate produced in DAAT (green) and ADCL (blue) reactions. (I) Growth of WT Mtb, ΔRv0812, and ΔRv0812:Rv0812 in the presence or absence of PABA (1 µg/ml). (J–Q) Active site of AKG-bound Rv0812 and ADC-docked model. The AKG molecule and waters were removed before docking ADC into the active site. The mesh surface of AKG, ADC, and PMP is shown. H-bonds formed with protein side chains are shown as dashed lines (J–M), and the [2Fo]–[Fo] electron density map, contoured at 1.2σ, of PLP and AKG (J) is shown as chicken wire. Side chains of residues neighboring AKG and ADC (within 5 Å) are shown (K and M). (N and P) Superposition of PDA-containing BsDAAT (yellow) with AKG-bound Rv0812 (N), and superposition of PLP-containing PaADCL (green) with ADC-docked Rv0812 (coral; P). BsDAAT and PaADCL interdomain loops are shown in orange and cyan, respectively. The loop from Rv0812 containing Arg26* is shown in purple. Conserved residues forming the active site are labeled in the order of Rv0812/BsDAAT or Rv0812/PaADCL. (O and Q) Electrostatic surface of Rv0812 in AKG-bound form (O) and ADC-docked model (Q).
Figure S2. Sequence alignment of Rv0812 with ADCL- and DAAT-like proteins from selected genomes. (A) ClustalW2 multisequence alignment of Mtb Rv0812-like sequences compared with ADCL- and DAAT-like sequences from D. hafniense, P. aeruginosa, S. oneidensis, E. coli, V. cholerae, A. bacterium, C. difficile, S. aureus, C. pusillum, A. thaliani, Desulfotalea, N. cyanobacterium, S. negevensis, G. endophyticus, Bacillus YM-1, L. sphaericus, L. monocytogenes, L. pneumophila, B. parapertussis, and M. loti. Sequences within the magenta box clustered along with Rv0812 in the phylogenetic tree, while known ADCL and DAAT sequences grouped with their respective subfamilies and are thus shown above and below Rv0812-like sequences, respectively. Residues conserved across all families are shown in red boxes. Conserved ADCL-like residues are shaded in blue, while DAAT-like residues are green and Rv0812-like residues are purple. (B and C) Superposition of Rv0812 (tan) and a homology model of A. thaliana ADCL/DAAT (blue). The ribbon diagram overlay, with the conserved Arg26 (Rv0812) and Arg104 (A. thaliana ADCL/DAAT) residues as ball and sticks, shows the structural similarity between the dually functional enzymes, with a 3.88-Å root-mean-square deviation between all atoms (B). A closer view of the residues within 4 Å of PLP demonstrates the high degree of similarity between the two active sites (C).
Table S1 is provided online and lists detailed parameters of the crystallography data and refinement statistic.