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Identification of novel ADP ribosylation sites in Mycobacterium tuberculosis isocitrate lyase by mass spectrometry

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***Abstract*---**Mass spectrometry is a powerful proteomics tool for the identification of therapeutic targets and disease biomarkers on one hand and for characterization of post-translationally modified (PTM) and alternatively spliced protein isoforms on the other. PTMs are the covalent modifications of certain proteins either by the addition of functional groups or by proteolytic cleavage. These modifications result in the structure-function regulations of target proteins and generate protein isoforms which may be associated with a particular disease. The prior knowledge of PTMs in a specific drug target aid in designing novel therapeutics. Isocitrate lyase (ICL) of *Mycobacterium tuberculosis* (*Mtb*) is one such drug target, which exists in two isoforms - ICL1 (Rv0467) and ICL2 (Rv1915 and Rv1916) and the activity of the former was reported to be regulated by lysine acetylation and succinylation. While reviewing the major PTMs in *Mtb*, this review also brings up the plausible role of reversible post-translational modifications such as ADP-ribosylation and acetylation Rv1915 and Rv1916 in assisting persistent *Mtb* to survive and respond to environmental cues respectively.

***Keywords*---**Mycobacterium tuberculosis, isocitrate lyase, ICL1, ICL2, Rv1915, Rv1916, post-translational modifications.

Introduction

Post-translational modifications (PTMs) are the key mechanism of protein alterations that affect the localization, structure and function of the important drug targets. There are ~200-400 different types of characterized PTMs in literature out of which the phosphorylation, acetylation, ubiquitination, succinylation, methylation glycosylation, biotinylation, SUMOlyation, ADP-ribosylation account for the major ones. PTMs can be categorized into reversible and irreversible based on their stability or transience. Irreversible PTMs such as protein deamination, N-methylation, N-terminal acetylation, S-thiolation, glycation of proteins or lipids, and proteolysis can permanently affect the molecules not be reversed. In contrast, phosphorylation, ADP ribosylation, acetylation, succinylation etc., are reversible PTMs, involved in signaling and regulatory processes, whose effects can be undone by specific deconjugating enzymes (Liddy et al., 2013; Macek et al., 2019). PTMs were found to be associated with several serious physiological diseases as well as microbial infections. A freely accessible human disease associated PTM database (PTMD) identified 1950 PDA, 749 proteins were modified by 23 types of PTMs causing 275 human diseases where phosphorylation was majorly linked with human diseases. The construction of a disease-gene network further demonstrates that most of the PTMs were associated with breast cancer including acetylation, phosphorylation, ubiquitination etc. Furthermore, AKT1 kinase, a protein found in a variety of cell types across the human body and is involved in several signalling pathways, has the largest PTM sites which were linked to various diseases (Xu et al., 2018).

Apart from physiological diseases, PTMs play critical roles in the virulence of human pathogens including fungi, bacteria, and viruses. For long, proteomic studies in eukaryotes have identified well-defined PTMs which control or modulate the functionality of different virulence factors of pathogenic organisms. Pathogenic fungi rely on PTMs for stress response and host adaptation (phosphorylation, acetylation, ubiquitination, and methylation), host cell manipulation and immune system evasion (glycosylation and phosphorylation) and antifungal resistance (acetylation and phosphorylation) (Retanal et al., 2021). Likewise, Viral PTMs were known to regulate viral assembly as well as the enzymatic activity of the viral protein. In the Influenza virus, lysine acetylation of viral nucleoprotein at K77 and K229 are known to enhance the polymerase activity and hence positively regulate viral replication (Giese et al., 2017). The genome of Coronavirus has also been identified to have various PTMs including phosphorylation and ADP-ribosylation linked with nucleocapsid protein, N- or O-linked glycosylation modify membrane proteins and other PTMs which are associated with non-structural and accessory proteins (T. S. Fung & Liu, 2018). Initially believed to be eukaryotes specific, recent advancements in PTM detection techniques, reveal the process to be widespread in prokaryotes as well. In fact, a recent review on “Protein post-translational modifications in bacteria” provides ample research evidence that PTMs have a vital role in almost all cellular processes be its cell division and spore formation, signal transduction, protein synthesis or degradation, biofilm formation, etc. [2]. Commonly, phosphorylation, acetylation, methylation and glycosylation modify about half of the bacterial proteome. For instance, phosphorylation of DevR (*Mycobacterium smegmatis*) and WalR (*B. subtilis*) modulate the DNA binding abilities of these response regulators.

In *Streptomyces coelicolor*, both phosphorylation and acetylation of GlnR regulate its binding to target DNA albeit on different environmental cues. Similarly, phosphorylation of mycobacterial NAD^+-dependent deacetylase exhibited its reduced deacetylase activity as opposed to the un-phosphorylated form providing evidence for cross-talk between phosphorylation and deacetylation PTMs in bacteria. In addition to phosphorylation, glycosylation modifies a broad range of membrane and secreted proteins and is emerging as a key player in infection-causing ability of many bacterial pathogens. Exhaustive proteomics analysis of *Campylobacter jejuni* demonstrates N-glycosylation of bacterial proteins as an important virulence determinant (Cain et al., 2019). Similarly, *Acinetobacter baumannii*, a causative agent of nosocomial infections, with disrupted O-glycosylation machinery, shows decreased virulence and biofilm formation in the infection models namely, *Dictyostelium discoideum* and *Galleria mellonella* (Iwashkiw et al., 2012).

Due to the importance of PTMs in basic cellular homeostasis as well as in disease states, it is becoming important to identify and characterize these for the development of preventive and therapeutic strategies. Conventional techniques for detecting PTMs include (i) immunoprecipitation-based techniques which further consist of affinity-based purification technology, Western blotting, (ii) 2D gel electrophoresis (iii) immunofluorescence for global PTM and (iv) mass spectrometry (MS) (Aslam et al., 2017). The basic requirement for the first is the availability of PTM specific antibody, but the associated PTMs may alter or blocks the antibody binding site of the targeted protein and non-specific interaction may occur. The shortcomings of the second technique include the requirement of a large amount of sample, limited reproducibility and the inability to separate proteins with the same pI. Immunofluorescence methods need optimization of fixation techniques (Hoffman et al., 2008). In contrast, MS, an analytical technique, is routinely used for the identification of unknown analytes in a high-throughput manner. PTMs can generate multiple isoforms from a single native protein by the addition of various low mass functional groups. The high resolving power and determination of accurate mass, make it possible to detect these low mass additions on the proteins due to PTMs. With advancements in MS configurations and data analysis which allow the determination of exact sequence identity as well as the molecular weight of the targeted peptides or proteins, it is being increasingly adopted in clinical medicine (A. W. S. Fung et al., 2020).

This work is an illustration of usefulness of this technique in the identification of ADP ribosylation PTM in Isocitrate Lyase (ICL), a novel drug target for persistent *Mycobacterium tuberculosis* (*Mtb*), the causative agent of life threatening Tuberculosis (TB) disease, accounting for 1.5 million deaths in 2020 worldwide (*GLOBAL TUBERCULOSIS REPORT 2020,* 2020). ICL of the glyoxylate shunt pathway, is the important drug target for the persistent phase of *Mtb*. This is a bi-functional enzyme that catalyses acetyl-CoA and propionyl-CoA moieties from β-oxidation of fatty acids into carbohydrates via two pathways namely-glyoxylate shunt pathway and methylcitrate cycle respectively. During the activation of the glyoxylate cycle ICL specifically cleaves the carbon-carbon bond of isocitrate to generate glyoxylate and succinate whereas, in methylcitrate cycle, 2-methylisocitrate is converted into pyruvate and succinate by the enzyme (Gould et al., 2006). These cycles are the well-illustrated pathways where ICL play a vital

role in providing metabolic flexibility to the pathogen for persistence inside the lipid rich environment of human macrophages (Graham & Clark-Curtiss, 1999). The essentiality of ICL during the persistence phase of *Mtb* is explicit in mice models and inflammatory macrophages where the disruption of the *icl* gene in the virulent Erdman strain of *Mtb* results in the reduction of bacterial virulence and persistence (McKinney et al., 2000). Furthermore, most of the pathogenic *Mtb* strains including Erdman strain and CDC1551 encode two isoforms ICLs i.e. ICL1 and ICL2 which are together required for pathogenesis and lipid metabolism in *Mtb.* The situation is even more complicated in the case of *Mtb* H37Rv where during transcription, the *icl2* gets separated into two genes *rv1915* and *rv1916* which have been recently characterized to have dual activities as other *Mtb* ICLs (M. Antil et al., 2019; Monika Antil et al., 2020). The determination of crystal structures of *Mtb* ICL1 (H37Rv) (Sharma et al., 2000) and ICL2 (from CDC1551) (Bhusal et al., 2019) and the possibilities of ICL as a target for the treatment of persistent *Mtb* make it critical to explore the regulation of ICL to find possible ICL inhibitors. Given this, it is important to understand the mechanisms that *Mtb* manages to alter the drugs and their drug targets to acquire resistance. This review focuses on the significance of reported PTMs (phosphorylation, acetylation, succinylation and ADP-ribosylation) and a novel PTM namely, ADP-ribosylation identified recently through Mass Spectrometry, in *Mtb* ICL.

PTMs in *M. tuberculosis* H37Rv ICLs

Recent literature provides ample evidence that a significant fraction of *Mtb* proteome is modified post-translationally via phosphorylation, ADP-ribosylation, acetylation, pupylation, acylation, thiolation, lipidation and glycosylation which play vital roles in survival, metabolism, host-pathogen interactions, drug resistance and persistence of the pathogen (Arora et al., 2021; Macek et al., 2019). Some of the important PTMs involved in the virulence, persistence and alteration of *Mtb* drug targets resulting in drug-resistant strains are discussed below.

Phosphorylation

Phosphorylation is the reversible PTM where protein kinases transfer the phosphate group (PO_4^{3-}) from adenosine triphosphate and covalently bind it to the specific amino acid residues of the target protein. The main target sites of phosphorylation are Ser, Thr, Tyr and His residues but it may occur on Cys, Pro, Asp and Arg residues [11, 12]. Phosphorylation is one of the major post-translational modifications that mediates signals throughout the cell for achieving cross-talk between various players and regulating cellular processes such as growth, apoptosis and adaptation to stress, host-pathogen interactions in several microorganisms etc. For example, RecA/LexA is a well-studied system involved in drug tolerance, resistance and persistence of *Mtb* where phosphorylation of a Serine residue in RecA loop 2 after DNA damage, is critical for regulation of DNA-damage response via inhibition of LexA coprotease function and development of antibiotic-resistant strains [13]. Similarly, phosphorylation of *Mtb* elongation factor Ef-Tu at Thr118 reduced its binding with the specific inhibitor kirromycin [14]. Studies also reveal the phosphorylation of various *Mtb* transcription factors

such as EmbR and EthR by multiple serine/threonine kinases confer the resistance from ethambutol and ethionamide respectively [14–16].

Furthermore, phosphorylation is known to regulate the ICL activity differently in different organisms. For example, phosphorylation negatively controls the activity of ICL from pathogenic yeast *Paracoccidioides brasiliensis* (*Pb*ICL). The study demonstrates the constitutive expression of the *Pbicl* gene and protein production which is not dependent on the carbon sources. Quantitative RT-PCR analysis revealed that the yeast cells grown in glucose-rich medium exhibited the higher transcript levels of *Pbicl*, than the cells cultured in an acetate carbon source. However, a seven times lower specific activity of the enzyme was observed in the cell-free extract of the former media than in the latter one, illustrating the *Pbicl* expression and activity are independent on carbon sources. The higher abundance but lower activity of the *Pb*ICL in the presence of glucose was suggested to be regulated by phosphorylation in addition to the lower stability of the enzyme in glucose-rich medium. The authors further confirm this hypothesis, by treating the cell-free extract using calf intestinal phosphatase enzyme which leads to dephosphorylation of the enzyme resulting in the eightfold increase in the ICL activity of *Pb*ICL. Furthermore, the inhibition of phosphatase by o-vanadate was not observed to reverse the activity of the enzyme indicating its negative regulation by phosphorylation. In contrast to *P. brasiliensis*, the expression of *A. fumigatus* ICL was not induced by glucose with an undetectable specific activity in the presence of glycerol. On the other hand, the enzyme shows significant activity when extracted from acetate source and remained unchanged in the presence of phosphatase enzyme (Da Silva Cruz et al., 2011).

Apart from eukaryotic system, histidine phosphorylation of *E.coli* ICL seems to be essential for its catalytic activity. The study identify that, *E.coli* ICL from partially purified cell extracts get phosphorylated with an endogenous kinase when incubated with [ɣ-^{32}P]ATP. The evidences for histidine phosphorylation were collected by treating the enzyme with (i) dipotassium phosphoramida which specifically phosphorylate histidine residues and (ii) diethyl pyrocarbonat which modified histidine residues. The former slightly decreasing (acidic) its isoelectric point as happened in case of phosphorylation event whereas the later blocks the incorporation of ^{32}P from [ɣ-^{32}P]ATP. Further, the treatment *E.coli* ICL with potato acid phosphatase results in the inactivation of the enzyme and an increase in the isoelectric point was observed similar as dephosphorylation (Robertson et al., 1988). Despite the widespread role of phosphorylation in the regulation of ICLs from different organisms its functionality in case of *Mtb* ICL still needs to be explored.

Acetylation

Acetylation involves the transfer of the acetyl group (C_2H_3O) from acetyl coenzyme A (acetyl-CoA) to the specific amino acid of the target protein by the action of the enzyme acetyltransferases. The three types of acetylation are Nε-acetylation, O-acetylation and Nα-acetylation where the former two are reversible PTMs whereas the latter is an irreversible modification. Lys is the most targeted residue for acetylation along with Cys, Gly, Ala, Arg, Glu, Asp, Val, Pro, Ser, Thr and Met residues. Acetylation is widespread in various cellular processes such as Protein

stabilization and localization, protein-protein interaction, cell cycle control and different aspects of cellular metabolism. In *Mtb*, acetylation plays a crucial in the development of drug-resistant strains by inactivating various antibacterial drugs such as kanamycin, capreomycin, and amikacin. The enhanced intracellular survival protein of *Mtb* is known to acetylate these drugs and hence affecting their function. Furthermore, the high-throughput dynamic imaging technique showed the association of lysine acetylation (Lys86) of *Mtb*'s HupB in the development of isoniazid-resistant subpopulations of mycobacteria (Sakatos et al., 2018).

The comparison of *Mtb* H37Ra lysine acetylome studied under enriched and hypoxic conditions represented an altered pattern of lysine acetylation. Liquid chromatography coupled to tandem mass spectrometry in enriched conditions, identified a total 441 peptides from 268 proteins which were modified by acetylation and particularly 75 proteins were found to have acetylated lysine residues. In contrast, under hypoxic conditions out of 111 peptides, only 56 acetylated peptides were detected. This differential pattern of acetylome under normal and stress condition demonstrate the adaptation of *Mtb* to variable environment inside the host by the aid of this dynamic, rapid and reversible PTM. Being a persistent factor during hypoxic condition, *Mtb* ICL1 has also been identified to have acetylation of lysine residues (K322, K331, and K392) which can modulate its activity in several way. Acetylation of K392 resulted in enhanced ICL activity, whilst acetylation of K322 resulted in lower ICL and MICL activities of ICL1 (Bi et al., 2017). Till date no PTMs are reported for the *Mtb* ICL2, however in our recent work, we have found lysine acetylation at K221, K225, K241, and K257 positions of recombinant Rv1916 like ICL1 (Monika Antil & Gupta, 2022).

Succinylation

Succinylation is the addition of succinyl group ($CO\text{-}CH_2\text{-}CH_2\text{-}CO_2H$), to protein residues mainly on lysine by succinyl transferases or without enzyme. The succinyl group is a bulky group which increases the molecular weight of the protein to 100 Daltons resulting in large conformational changes and thus affecting their functions. The first succinyl proteome profiling of extensively drug-resistant *Mtb*, reveal that out of 686 succinylated proteins, 13 proteins were linked with resistance of *Mtb* from various first line drugs including rifampin, ethambutol and isoniazid. Succinylation KatG, KasA, AhpC, FadE24, Nat, and acpM were found to be associated with isoniazid resistance whereas EmbR and rpoB succinylation results in the development of *Mtb* resistance against ethambutol and rifampin (Xie et al., 2015).

Furthermore, Lysine succinylation in *Mtb* ICL1 has been detected at three sites - K189, K322, and K334, however, only the succinylation of the former is reported to drastically reduce the activity of ICL1. Loss of activity is attributed to the introduction of a negative charge that keeps the enzyme in “open conformation” and in the catalytically inactive form. Mutation of K to E also mimics the same effect and renders the enzyme non-functional (L et al., 2015; M et al., 2017). Interestingly, recombinant *Mtb* ICL1-K189E mutant, heterologously expressed in *M. smegmatis*, is not only an inactive variant but also imparts higher susceptibility towards rifampicin and streptomycin to *M. smegmatis* as opposed to

the wild type, reinforcing the role of succinylation in antibiotic resistance (L et al., 2015; M et al., 2017).

ADP-Ribosylation

ADP-Ribosylation involves the addition of ADP-ribose moiety on the targeted proteins by enzymes ADP-ribosyltransferases (ARTs). ADP-ribosylation has been recognized as an important PTM of nucleic acids (Groslambert et al., 2021; Weixler et al., 2021), proteins (Mikolčević et al., 2021) and small molecules (Baysarowich et al., 2008; Selwyn Quan et al., 1999) across all life domains (D et al., 2014). It influences various cellular processes linked to common stress responses like defence against the host, cell growth and differentiation, persistence, DNA repair mechanisms, secondary metabolite synthesis, gene regulation, biofilm formation, apoptosis and cell signaling. Several pathogenic microorganisms encode ARTs which reversibly modify important target proteins of the host cell by transferring the ADP-ribose moiety from NAD^+. PTMs structural-functional studies reveal that the binding of ADP-ribose moiety can activate or deactivate the functions of the target proteins by different mechanisms. For instance, in the case of positively charged amino acids (lysine or arginine), the attachment of negatively charged large ADP-ribose moiety increases the size and molecular weight of the acceptor molecule. Further, after ADP-ribosylation, the target protein may undergo conformational changes that either create new binding grooves or cause steric hindrance to the interaction of other cellular partners (Laing et al., 2011). The DarT (toxin) and DarG (antitoxin) system of *Mtb* is an illustrious example where DarT acts as an ART and reversibly ADP ribosylates the thymidine bases of *Mtb*'s single-stranded DNA with high substrate specificity. The role of the DarTG system has been speculated in inducing *Mtb* persistence as it arrests bacterial growth by interfering with DNA replication (Jankevicius et al., 2016). ADP-ribosylation has also been demonstrated as a major factor for the inactivation of rifampicin by a 600 bp long DNA sequence of *Mycobacterium smegmatis* DSM 43756 (S Quan et al., 1997), which was further characterized as a mono ART (Selwyn Quan et al., 1999).

Furthermore, eukaryotic Sirtuins or SIRTs are NAD^+-dependent lysine deacetylases, most of which also possess intrinsic ART activity but require an acetylated peptide as substrate (Kowieski et al., 2008; Rack et al., 2015). Although not many reports can be found of Sirtuins counterparts in prokaryotes, a human SIRT4 homologue MSMEG_4620, essential for metabolism and growth of non-pathogenic *M. smegmatis* in the natural environment, has been found to be an auto ADP-ribosyltransferase (Tan et al., 2016). This SIRT4 homologue is transcriptionally upregulated under malnutrition stress and hence believed to be important for metabolism of the species when faced with shortage of nutrients. It is noteworthy that *M. smegmatis* also has a SIRT5 homologue (MSMEG_5175), however, pathogenic *Mtb* has no SIRT4 but has one SIRT5 (Rv1151c) homologue, which is reported to possess the auto ADP-ribosyltransferase activity (Gu et al., 2009). Identification of a unusual PTM, ADP-ribosylation at K96 of Rv1915 is riveting as well as a novel finding (Monika Antil & Gupta, 2022) since it has not been reported to date for any ICL. Sequence alignment of Rv1915 with Rv1151c and MSMEG_4620 displayed 23% and 24% identity, respectively. To further investigate in this direction, a DALI search

(http://ekhidna2.biocenter.helsinki.fi/dali/index.html#tabs-1) was performed with the modeled structure of Rv1915 (Holm, 2020) which, despite having low sequence identity, reveals the structural similarity with Poly-β-1,6-N-acetyl-D-glucosamine N-deacetylase [PDB ID: 3VUS] and Adenine phosphoribosyltransferase [PDB ID: 4X44] (Fig.1 A). Comparative structural analysis revealed Rv1915 and related DALI hits have either Rossmann, or Rossmann-like folds which is the structural basis for the function of sirtuins (Fig. 1B). Rossmann, or Rossmann-like folds are typically βαβ fold which bind to various enzymatic cofactors such as NAD^+, $NADP^+$ and FAD (Sanders et al., 2010). The above indications provide framework for future investigations into involvement of Rv1915 and Rv1916 in SIRT-mediated ADP-ribosylation of acetylated substrate with the latter serving as an acetylated substrate for the auto ADP-ribosylation of former akin to sirtuins.

Conclusion and future prospect

ICL is the main enzyme of glyoxylate pathway which maintain the carbon flux not only in *Mtb* but in other pathogens, under the conditions where TCA cycle is not active. The essentiality of ICL as an important persistent factor and hence important drug target against *Mtb*, make it significant to study all cellular aspects which may influence its structure and functions. PTMs are among the key mechanisms for the modifications of important drug targets that contributes to metabolic shifting, pathogenesis, virulence, persistence and drug tolerance or resistance of *Mtb*. This study deliberates on the biological significance of ADP-ribosylation and acetylation identified recently in Rv1915 and Rv1916 respectively. Although further investigations are required, it appears that in *Mtb* H37Rv these two halves of ICL2 may be involved in the phenomenon of acetylation-dependent ADP ribosylation known to affect important cellular processes such as fundamental metabolism and growth, drug resistance, DNA damage response and biofilm formation.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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A)

PROTEIN	PDB ID	RMSD	%IDENTITY
Poly-beta-1,6-N-acetyl-D-glucosamine N-deacetylase	4p7l-A	3.3	11
	4f9j-A	3.5	13
	4p7o-A	3.4	10
	1o12-A	3.5	10
	3vus-B	3.3	8
Quinolinate phosphoribosyltransferase	4i9a-A	3.3	12
Orotate phosphoribosyltransferase	2yzk-A	3.7	9
Adenine phosphoribosyltransferase	4x44-A	2.7	8

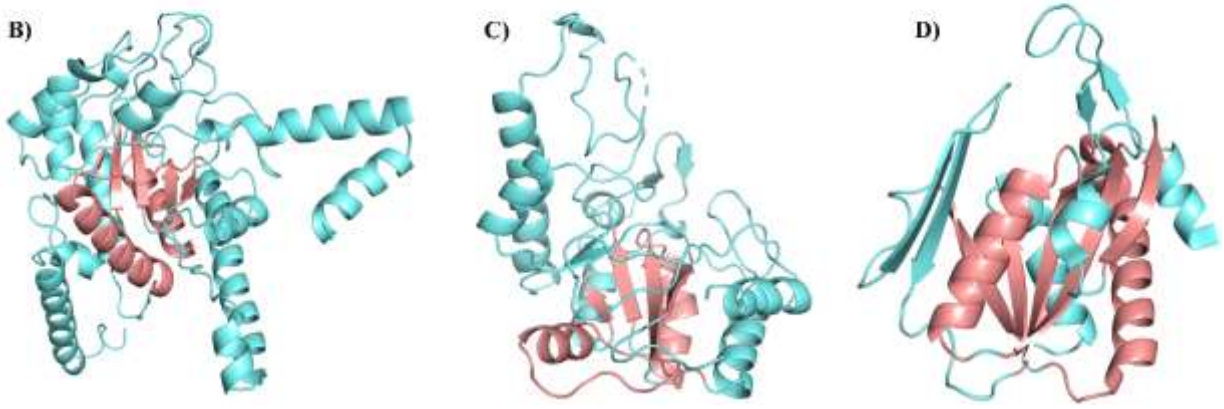

Figure 1. The DALI search results in (A) identification of deacetylases and APD ribosyltransferases. The comparison of (B) modeled structure of Rv1915 and crystal structures of (C) Poly-β-1,6-N-acetyl-D-glucosamine N-deacetylase [PDB ID: 3VUS] and (D) Adenine phosphoribosyltransferase [PDB ID: 4X44] represents the similar Rossmann fold (salmon).