Role of protein repair enzymes in oxidative stress survival and virulence of Salmonella

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Abstract

Purpose: Proteins are the principal biomolecules in bacteria that are affected by the oxidants produced by the phagocytic cells. Most of the protein damage is irreparable though few unfolded proteins and covalently modified amino acids can be repaired by chaperones and repair enzymes respectively. This study reviews the three protein repair enzymes, protein L-isoaspartyl O-methyl transferase (PIMT), peptidyl proline cis-trans isomerase (PPIase), and methionine sulfoxide reductase (MSR).

Methods: Published articles regarding protein repair enzymes were collected from Google Scholar and PubMed. The information obtained from the research articles was analyzed and categorized into general information about the enzyme, mechanism of action, and role played by the enzymes in bacteria. Special emphasis was given to the importance of these enzymes in Salmonella Typhimurium.

Results: Protein repair is the direct and energetically preferred way of replenishing the cellular protein pool without translational synthesis. Under the oxidative stress mounted by the host during the infection, protein repair becomes very crucial for the survival of the bacterial pathogens. Only a few covalent modifications of amino acids are reversible by the protein repair enzymes, and they are highly specific in activity. Deletion mutants of these enzymes in different bacteria revealed their importance in the virulence and oxidative stress survival.

Conclusion: PIMT repairs isoaspartate residues, PPIase catalyzes the conversion of cis-trans forms of proline residues, while MSR repairs oxidized methionine (Met) residues in the proteins. These repair enzymes maintain the activities of the target protein(s), thus aid in bacterial survival and virulence. The interventions which can interfere with this mechanism could be used for the development of novel therapeutics.

Keywords: Oxidative stress, Protein damage, Protein repair, Salmonella, Poultry

Introduction

Phagocytes constitute a very important part of host innate immunity. After sensing microbial ligands, phagocytes produce various reactive oxygen species (ROS), reactive nitrogen species (RNS), antimicrobial peptides, chemokines, and cytokines with an overall goal to contain or kill the invaders and help other immune cells to generate adaptive immunity. Upon activation, NADPH oxidase (NOX) gets assembled on the phagosomal membrane and pumps the electrons from NADPH to oxygen by generating superoxide anions (O$_2^-$). O$_2^-$ is then metabolized into a variety of other toxic ROS, like hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (’OH) (Miller and Britigan, 1997). Further, myeloperoxidase catalyzes the production of highly toxic reactive chlorine species (RCS), hypochlorous acid (HOCl) from H$_2$O$_2$, and chloride ions (Fig. 1). It has been demonstrated that phagocyte-generated NADPH oxidase plays a very vital role in controlling bacterial pathogens, including Salmonella enterica serovar Typhimurium infection. Simultaneously, inducible nitric oxide synthase (iNOS) gets...
activated and catalyzes the oxidation of one of the guanidine nitrogens of L-arginine to generate nitric oxide (NO). NO gets autooxidized to produce other reactive nitrogen species like NO$_2^−$, N$_2$O$_3$, and S-nitrosothiols which are more reactive and have enhanced cytotoxic abilities (Fang, 1997). Further, the combination of NO and O$_2^−$ forms peroxynitrite (ONOO$^−$), which is one of the most potent RNS. Phagosomal oxidase and iNOS knockout mice were found to be more susceptible to Salmonella infection (Felmy et al. 2013). Almost all macromolecules, including DNA, RNA, lipids, and proteins, are susceptible to ROS- and RNS-mediated damage (Fig. 1).

The genus Salmonella includes two species, Salmonella enterica and Salmonella bongori. Salmonella enterica is further divided into six subspecies I (enterica), II (salamae), IIIa (arizonae), IIIb (houteaume), and VI (indica) (Le Minor L & Popoff MY 1987; Popoff et al. 1998). These subspecies are further classified into various serovars. Based on host preference and adaptability, the Salmonella enterica subspecies enterica are categorized into two serovars, i.e., typhoidal (includes S. Typhi and S. Paratyphi) and non-typhoidal (NTS, includes S. Enteritidis and S. Typhimurium) (Hohmann, 2001; Parry et al. 2002; Gal-Mor et al. 2012). Typhoidal Salmonellae are the causative agents of “enteric fever,” which is characterized by prolonged fever, fatigue, and severe gastroenteritis. Most pathogenic species of Salmonella which are the leading cause of foodborne gastroenteritis across the globe causing illness in humans belong to the species Salmonella enterica. Poultry serves as reservoir of Salmonellae and acts as major source of human infection (Bailey et al. 2002, Velge et al. 2005). Poultry meat and eggs have been implicated in the large outbreaks of foodborne Salmonellosis (Leach et al. 1999; Humphery, 2000). In 2015, WHO in its 10 year report of global burden of foodborne diseases identified Salmonellosis as the most common foodborne illness and estimated approximately 230,000 deaths in the USA alone due to non-typhoidal Salmonella enterica (NTS) (WHO, 2015). In the same year, the eggs contaminated with Salmonella spp. were associated with the highest number of foodborne outbreaks reported and were among the top five foodborne pathogens in terms of overall illness (EFSA and ECDC, 2016). Normally, NTS causes self-limiting, mild to moderate gastroenteritis in healthy adults. However, young, old, and immuno-compromised individuals are at a higher risk, wherein NTS causes severe gastroenteritis to invasive, extra-intestinal disease culminating in bacteremia and infections in multiple organs (Mandal and Brennand, 1988; Lê-Bury and Niedergang, 2018).

Salmonella can replicate in a variety of host cells. One important feature of this bacterium is its ability to infect and replicate inside the phagocytic cells. Recent studies suggest that Salmonella can survive and replicate in neutrophils, which are considered a dead-end for most bacterial pathogens (Geddes et al. 2007). Virulence of S. Typhimurium has been correlated with its survival in the phagocytic cells, whereas mutants that are unable to survive inside the macrophages are considered avirulent (Fields et al. 1986).

Salmonella handles oxidants in three ways. First, it injects Salmonella pathogenicity island 2 (SPI2) effectors...
into the host cell that interfere with the assembly of phagosomal oxidase, thus modulating the production of \( \text{O}_2^− \) (Vázquez-Torres et al. 2000; Gallois et al. 2001) and consequently other ROS and RNS. Second, the primary antioxidants of Salmonella directly detoxify the oxidant species. Third, macromolecular (DNA and protein) repair enzymes mend damaged DNA and proteins and restore their functions without de novo synthesis.

Among primary antioxidants, Salmonella encodes four superoxide dismutases (SodA, SodB, SodCl, SodCII), three catalases (KatE, KatG, KatN), and three peroxiredoxins (AhpC, TsaA, Tpx). SODs neutralize \( \text{O}_2^− \); KatE scavenge \( \text{H}_2\text{O}_2 \); peroxiredoxins reduce organic hydroperoxides and \( \text{H}_2\text{O}_2 \) (Fang, 2011; Slauoch, 2011; Aussel et al. 2011). AhpC and KatG degrade ONOO\(^−\) (McLean et al. 2010; Henard and Vázquez-Torres, 2011) (Fig. 1). During the respiratory burst, the quantity of phagocyte-generated oxidants can be much higher than the scavenging capacity of primary antioxidants (SOD, catalases, peroxiredoxins, etc.) of Salmonella. Furthermore, microbial enzymes that can degrade host-generated toxic oxidants like ‘OH and HOCl which results in macromolecular damage are not known yet. Therefore, DNA and protein repair enzymes help Salmonella to cope with oxidative insult and ensure its propagation in the host.

Due to their abundance and reactivity, proteins are highly prone to oxidative damage. Two types of protein damage are known including covalent modifications to amino acids and changes in the secondary structure (Mahawar et al. 2011). Degradation of damaged proteins to amino acids followed by ribosomal synthesis is an obvious and well-studied way to replenish damaged proteins. On the other hand, protein repair is a rapid and energy-efficient approach to reactivate damaged proteins without de novo synthesis (Brot et al. 1981; Zhang and Weissbach 2008). Under stress conditions, when limited resources are available to the cell, the repair of vital protein(s) becomes indispensable for cellular survival (either the cell repairs them or it dies). Chaperones can refold unfolded proteins. However, even though various covalent modifications have been described (Hawkins et al. 2003), only three types of repair enzymes are known: (1) protein L-isoaspartyl O-methyltransferase (PIMT), (2) peptidyl prolyl cis-trans isomerase (PPlase), and (3) methionine sulfoxide reductase (MSR) which repair damaged aspartate or asparagine (isoaspartate), isomerized proline, and oxidized methionine residues respectively (Li and Clarke, 1992; Boschi-Muller et al. 2008; Ünal and Steinert, 2014).

**Protein L-isoaspartyl O-methyl transferase**

Aspartyl (Asp)/asparaginyl (Asn) residues in proteins spontaneously get converted into iso-aspartate (iso-Asp) as a part of normal post-translational modification which decides the half-life of the protein (Güttler et al. 2013). But under the condition of certain stresses, their rate of formation has been shown to accelerate. Iso-Asp formation leads to distortion of protein structure resulting in unfolding and aggregation of the proteins. Thus iso-Asp formation has been linked to compromised protein function (Kern et al. 2005; Shimizu et al. 2005; Dimitrijevic et al. 2014) which consequently affects cellular survival. Protein L-isoaspartyl O-methyl transferase (PIMT) (EC 2.1.1.77), a product of \( \text{pcm} \) gene in bacteria, methylates the α-carboxy group on iso-Asp residues by using the methyl group of S-adenosyl-L-methionine (AdoMet), thus producing methyl esters. By repairing iso-Asp to Asp, PIMT restores the protein function(s) partially and thereby enhances cellular survival under stress conditions (Dimitrijevic et al. 2014).

**Mechanism of PIMT-mediated repair**

Asp/Asn residues in proteins, spontaneously or under stress, get converted into succinimide. The hydrolysis of succinimide yields isoAsp and Asp in a ratio of 3:1 (Vigneswara et al. 2006). The PIMT transfers methyl group from S-adenosyl methionine to isoAsp residues resulting in the formation of iso-aspartyl methyl esters which are unstable and rapidly hydrolyzed to form succinimide (Fig. 2). With multiple cycles of such reactions, the aspartyl residues can be salvaged and thereafter proteins regain their functions (DeVry and Clarke, 1999; Dimitrijevic et al. 2014).

**Effect of pcm gene deletion on the survival of various organisms**

The \( \text{pcm} \) gene knockout strain of \( \text{E. coli} \) (Li and Clarke, 1992; Visick et al. 1998; Hicks et al. 2005) showed hypersensitivity to oxidative, temperature, and other stresses. On the other hand, the PIMT overexpressing \( \text{E. coli} \) cells showed enhanced tolerance to oxidative and temperature stresses (Kindrachuk et al. 2003; Verma et al. 2010). The enhanced survival capabilities of PIMT overexpressing cells under temperature stress were shown to be due to the methyltransferase independent activities of PIMT (Kindrachuk et al. 2003). The structure crystallographic study of PIMT in \( \text{E. coli} \) has revealed the presence of 2 highly conserved Glu\(^{81}\) (E81) and Glu\(^{104}\) (E104) in the binding site of PIMT for AdoMet (Kindrachuk et al. 2003). The same study undertook the in situ mutagenesis of glutamine residues to alanine in PIMT of \( \text{E. coli} \) cells (E81A and E104A mutants) to analyze the specific effects of PIMT on cellular survival. It was observed that upon exposure to temperature stress, overexpressed wild and inactive PIMT (E81A) led to increased but comparable survival rates, while the E104A inactive mutant showed the highest cellular survival. Since, E104A inactive mutant had no methyltransferase activity, the study postulated a different role of...
PIMT in enhancing cellular survival other than its stereotypical role of enzymatic repair. Further, the western blot study revealed the overexpression of DnaK chaperone protein in E104A inactive mutant under temperature stress. Therefore, the study highlights the importance of a methyltransferase independent role of PIMT in increasing cellular survival through the induction of heat shock proteins. Thus, this study suggested methyl-transferase dependent as well as the independent role of PIMT in the survival of *E. coli* against temperature stress (Kindrachuk et al. 2003).

**PIMT in *S. Typhimurium***

PIMT contributes to the resistance of *S. Typhimurium* against hydrogen peroxide, hypochlorous acid, and temperature stresses in vitro (Kumawat et al. 2016; Pesingi et al. 2017). The survival of Δ*pctm* mutant strain inside interferon-γ (IFN-γ) stimulated macrophages was found to be 10-folds less as compared to the parent WT strain (*p* < 0.001), and it also showed attenuated virulence to mice (Kumawat et al. 2016). Further, the *pcm* gene is required for colonization in poultry cecum and dissemination to the spleen and liver (Pesingi et al. 2017). The expression of PIMT protein was about three-fold higher following exposure of *S. Typhimurium* at 42 °C (Pesingi et al. 2017). On the other hand, HOCl exposure induced PIMT by 1.5-fold (Kumawat et al. 2016), suggesting a greater role of this protein under thermal than oxidative stress.

**Peptidyl proline cis-trans isomerase**

Among the various known post-translational modifications of proteins, the dynamics of polypeptide chains can also be affected by catalytic activity of foldases. PPIases are a type of foldases that catalyze the isomerization between the *cis* and *trans* forms of peptide bonds, by the 180° rotation about the prolyl bond (Fig. 3) (Lin et al. 2019) and expedite the folding of nascent polypeptides...
as well as the refolding of unfolded and misfolded proteins (Compton, et al. 1992; Schmid et al. 1993). Due to steric hindrance exerted by side chains of amino acids, almost all peptide bonds exist as trans-conformers. However, in the case of proline, due to the formation of the pyrrolidine ring, the peptide bond can be present either in normal trans- or in cis-configuration. The presence of the abnormal proline-cis bond spontaneously affects folding, refolding, and protease-mediated degradation of the polypeptide chain and consequently influences protein function(s) (Brandts et al. 1975; Cook et al. 1979). PPIases are mostly found to be localized in the bacterial periplasm, inner membrane, and cytoplasm and are sometimes present in the supernatant (secreted) (Hayano et al. 1991; Kim et al. 2002; Söderberg and Cianciotto. 2008; Delpino et al. 2009). Localization in different compartments suggests a variety of differentially distributed targets for PPIases.

The PPIases can be grouped under one superfamily comprising three families of proteins, namely cyclophilins (Cyps)—PpiA, FK506-binding proteins (FKBPs)—FkpA, SylD, and parvulins like SurA (Ünal and Steinert, 2014). PpiA is localized in the periplasm. FKBP-type peptidyl-prolyl cis-trans isomerase (FkpA), a product of the fkpA gene, and cyclophilin PpiA catalyze the same isomerization reaction. Chaperone SurA encoded by the surA gene helps in the correct folding and assembly of outer membrane proteins. Structurally, SurA has an N-terminal region, two parvulin-like domains, and a C-terminal tail. The PPIase activity resides in one of the parvulin domain. The N-terminal region and the C-terminal tail are necessary and sufficient for the chaperone activity of SurA. This was demonstrated by a study in which a variant of SurA composed of only N-terminal region and the C-terminal tail (lacking the parvulin domains) exhibited chaperone activity in spite of lacking the PPIase parvulin domain (Behrens et al. 2001).

**PPIase in bacterial virulence**

PPIases are shown to be induced during the accumulation of misfolded proteins, heat and cold stresses, (Kandror and Goldberg, 1997; Söderberg and Cianciotto, 2008; Fasseas et al. 2012) and infection processes (Port and Freitag, 2007). The role of PPlase in bacterial virulence is mostly explained by its ability to facilitate proper folding of secreted proteins, adhesins, and other virulence factors (Hermans et al. 2006; Purdy et al. 2007; Alonzo and Freitag, 2010; Behrens-Kneip, 2010; Forster et al. 2011). In *Streptococcus suis*, *Listeria monocytogenes*, and *Clostridioidei difficile*, PPIases are required for resistance against several stresses including thermal, oxidative, and acid stresses, thereby contributing to virulence in mice (Bigot et al. 2006; Wu et al. 2011; Ünal et al. 2018). PPlase gene deletion strains of *E. coli* and *Yersinia pestis* showed defective adherence to and invasion of host cells (Justice et al. 2006; Obi and Francis, 2013) and virulence in mice (Hermans et al. 2006; Cron et al. 2009). A recent study demonstrated the role of *Legionella pneumophila* PPlase in the infection of both *Acanthamoeba castellanii* and human macrophages (Rasch et al. 2018).

**PPIase in Salmonella**

The *fkpA* and *surA* genes are required for *Salmonella* survival during long-term carbon starvation and the cross-resistance of carbon-starved cells to acidic pH, high temperature, and antimicrobials (Kenyon et al. 2010). Deletion mutant strains of *fkpA* and *surA* genes were found to be defective in survival in epithelial cells and macrophages and showed attenuated virulence in mice (Horne et al. 1997; Sydenham et al. 2000; Humphreys et al. 2003). Cyclophilin A (CypA) is a eukaryotic protein belonging to the PPlase family. A recent study suggested the role of CypA in the membrane ruffling

![Fig. 3 Conversion of cis- and trans- forms of proline by PPlase](image)
and internalization of S. Typhimurium into HeLa cells (Dhanda et al. 2018).

**Methionine sulfoxide reductases**

The sulfur-containing amino acids (Met and Cys) are highly prone to oxidation (Hawkins et al. 2003). Upon oxidation, Met residues convert into methionine sulfoxides (Met-SO) and further oxidation leads to the formation of methionine sulfones. MSRs reductively repair Met-SO to Met; however, sulfone repair enzymes are not yet known (Fig. 4). MSR-mediated repair of Met-SO plays two important roles in the cell. First, the MSR-mediated repair restores the functions of Met-rich oxidized proteins (Mahawar et al. 2011; Kuhns et al. 2013). Second, surface-exposed Met residues in proteins act as oxidant sinks. These surface-exposed Met residues get oxidized during the respiratory burst of host immune response and sop up excess oxidants, thus limiting damage to the cell until oxidized Met-SO gets repaired by MSRs (Abulimiti et al. 2003; Luo et al. 2009; Benoit and Maier, 2016; and Schmalstig et al. 2018).

**Mechanism of MSR-mediated repair**

Oxidation of sulfur in methionine forms either S or R epimers. According to localization, there are two types of MSRs, cytoplasmic and periplasmic (Boschi-Muller et al. 2008). MsrA and MsrB, which are present in cytoplasmic compartment repair S and R epimers of Met-SO, respectively. A third MSR was later discovered in the cytoplasm and named as MsrC. It is specific for free Met-R-SO and had been first described in E. coli (Lin et al. 2007) and then in S. Typhimurium (Denkel et al. 2011). MsrA reduces both free and protein-bound Met-S-SO whereas, MsrB reduces mainly protein-bound Met-R-SO with limited action on free Met-R-SO. All these MSRs repair Met-SO in thioredoxin-thioredoxin reductase manner where NADPH serves as an electron donor for the reduction process. The catalytic mechanism of MSR is a three step process involving three cysteine residues. In the first step, a nucleophilic Cys residue (CysA) attacks a Met-SO substrate, which leads to the formation of a sulfinic acid (–SOH) group on CysA and the release of reduced Met. In the second step, a nucleophilic Cys residue (CysB) attacks CysA–SOH, which leads to the formation of an intramolecular disulfide intermediate and the release of a water molecule. In the third step, the intramolecular disulfide intermediate is reduced by a Trx protein, and a catalytically active MSR enzyme is regenerated (Ezraty et al. 2017).

Periplasmic MSR (MsrP) has been discovered very recently in Gram negative bacteria. This new methionine sulfoxide reductase system, named MsrPQ, involves two proteins encoded in the same operon. MsrP, which carries out the reductase activity, is a periplasmic, soluble protein with a molybdenum atom in its active site. It was previously named YedY until its MSR activity was discovered (Loschi et al. 2004). To be functional in vivo, MsrP has to be specifically associated with MsrQ, an integral B-type heme-containing membrane-spanning protein, previously named YedZ (Drew et al. 2002). For the reduction reaction, MsrP receives electrons from MsrQ which in turn acquires electrons from quinones (Juillan-Binard et al. 2017).

**Role of MSRs in bacterial virulence**

The role of bacterial msr gene in combating various stresses within the host system has been well established. Further, its role in virulence also has been shown in many studies. An msrA gene deletion mutant of Mycoplasma genitalium exhibited hypersusceptibility to H₂O₂.
in comparison to wild-type strain and showed decreased ability to colonize in hamster lungs (Dhandayuthapani et al. 2001). In certain bacteria, MsRA and MsRB activities are carried out by a single fused protein. In *Neisseria gonorrhoeae*, one such protein called PilB, which was earlier supposed to have a role in pilin gene expression, was found homologous to both MsRA and MsRB of *E. coli* (Skaar et al. 2002). PilB is found in both secreted and cytoplasmic form, but only the secreted form of PilB was involved in the oxidative stress survival of the bacteria which was evident from the increased sensitivity of ΔpilB null mutant and mutant overexpressing truncated form of PilB to hydrogen peroxide and superoxide compared to the wild-type strain (Skaar et al. 2002). ΔmsrAB double deletion mutant strain of *Helicobacter pylori* was shown to be defective in host colonization in vivo suggesting the role of MSR in pathogenesis and virulence of *H. pylori* (Alamuri and Maier, 2004). In *Campylobacter jejuni*, MsRA and MsRB proteins were found to protect against oxidative and nitrosative stress. Moreover, the ΔmsrAB double mutant strain of this bacteria showed a severe growth defect in in vitro media due to accumulation of Met-SO in proteins (Atack and Kelly, 2008). msrA and msrB genes were very essential for the protection of *Enterococcus faecalis* against H2O2 stress, and the deletion of these genes lead to attenuated virulence in mice model studies (Zhao et al. 2010). The msrA and B gene deletion mutant strain of *E. coli* was hypersusceptible to HOCl-mediated killing (Rosen et al. 2009). *Mycobacterium tuberculosis* lacking both MsRA and B were shown to be hypersusceptible to nitrite and HOCl stress (Lee et al. 2009). The msrA mutant strain of *Staphylococcus aureus* were sensitive to H2O2 stress in vitro and phagocytic cells. Further, this mutant was less adherent to human lung epithelial cells and showed reduced survival in mouse model (Singh et al. 2015, Singh et al. 2018). In *Francisella tularensis*, the msrB deletion mutant was shown to be defective in growth significantly in comparison to wild type in in vitro media. Further, this strain was hypersusceptible to H2O2 stress and showed decreased growth in macrophages and defective in colonization in mice (Saha et al. 2017) indicating that MsRB contributes to virulence.

**MSRs in Salmonella**

In *S. Typhimurium*, three cytoplasmic MSRs namely, MsRA, MsRB, and MsRC, have been reported. All the three are present in cytoplasmic compartment. MsRA repairs protein-bound as well as free Met-SO and is essential for the survival of this bacterium under oxidative stress (Denkel et al. 2011; Trivedi et al. 2015). MsRB repairs protein-bound Met-SO though it is dispensable for stress survival of this bacterium (Denkel et al. 2011). The msrA and msrC gene deletion strains are hypersusceptible to nitrite and HOCl stress (Lee et al. 2009). The msrA and msrB gene deletion mutant strain of *E. coli* was hypersusceptible to HOCl-mediated killing (Rosen et al. 2009). *Mycobacterium tuberculosis* lacking both MsRA and B were shown to be hypersusceptible to nitrite and HOCl stress (Lee et al. 2009). The msrA mutant strain of *Staphylococcus aureus* were sensitive to H2O2 stress in vitro and phagocytic cells. Further, this mutant was less adherent to human lung epithelial cells and showed reduced survival in mouse model (Singh et al. 2015, Singh et al. 2018). In *Francisella tularensis*, the msrB deletion mutant was shown to be defective in growth significantly in comparison to wild type in in vitro media. Further, this strain was hypersusceptible to H2O2 stress and showed decreased growth in macrophages and defective in colonization in mice (Saha et al. 2017) indicating that MsRB contributes to virulence.

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hypersensitive to phagocytic cells and showed attenuated virulence in mice and poultry (Denkel et al. 2011; Sarkhel et al. 2017). *Salmonella* also harbors MsrP, though the role of this protein in stress survival of most bacterial pathogens including *Salmonella* is not known.

**Conclusions**

Different stresses induce unfolding and covalent modifications in bacterial proteins that result in protein inactivation and consequently affecting cellular survival. Chaperones can refold unfolded proteins. Covalently modified amino acids are repaired by special enzymatic systems (PIMT, MSR, and PPlase). The overall goal of protein repair is to reactivate the damaged proteins without their de novo synthesis, and this process helps bacterial pathogens to overcome stress conditions, as those encountered during the infection (Fig. 5). The ways that can inhibit this process can pave the way to develop novel prophylactic and therapeutic agents against bacterial diseases.

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**Consent for publication**

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**Competing interests**

The authors declare that they have no conflict of interest.

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