Mechanism of Tol/Pal System in Antibacterial Therapies

Ciamak Ghazaei

Abstract

The Tol/Pal system is a group of complex proteins found in Gram-negative bacteria that has a crucial function in bacterial outer membrane development and safety. It is a promising target for potential antibacterial therapy. Initially, this Tol/Pal system was thought to be associated with the uptake of toxins such as colicins by Escherichia coli. However, the latest research has revealed that this system has much broader features beyond that. The system has been extensively studied, and this article discusses some of the conclusions drawn from those studies. Most significantly, the Tol/Pal system is a prerequisite for the pathogenicity of many Gram-negative bacteria, indicating that it has a remarkable role in how these bacteria cause diseases. Moreover, this system plays a vital role in the growth and overall fitness of specific pathogens. This indicates that it may be a promising target for growing antimicrobial therapies. Significantly, one of the proteins in this system, called Pal, is highly recognizable through the immune system and may trigger each of the adaptive and innate immune responses. A lot of these features make the Tol/Pal system an exciting area of research for the development of antibacterial therapies, and may trigger each of the adaptive and innate immune responses. A lot of these features make the Tol/Pal system within the context of bacterial disease development and its potential utilization as a vaccine to counter respective bacterial infections.

Keywords: Gram-negative bacteria, Virulence, Drug resistance, Antimicrobial chemotherapy, Vaccine, Bacterial pathogenesis

Introduction

Antibiotics are frequently employed to prevent and treat bacterial infections, but an expanding portion of bacteria has developed resistance to traditional medications. Consequently, there is a pressing requirement to explore alternative approaches to treatment, including the development of vaccines and novel drug therapies. Gram-negative microorganisms possess an outer membrane (OM) outside their cell wall and an inner membrane, which acts as a protective barrier. This OM restricts the penetration of drugs into bacterial cells. Consequently, many Gram-negative bacteria have a natural resistance to certain types of antimicrobial agents. These bacteria become even more challenging to treat when they acquire additional resistance. Different categories of antimicrobial agents are nowadays being employed in medical and industrial settings to combat infections caused by these pathogens. However, the extensive use of such agents has led to the emergence of drug-resistant bacteria. The Tol/Pal system comprises a group of proteins that form two complexes spanning the OM, inner membrane, and middle periplasmic space. These proteins include TolA, TolR, TolQ, TolB, and Pal. While initially discovered in Escherichia coli, similar genes, known as orthologous genes, are present in numerous Gram-negative bacteria. These genes are organized into the ybgC-tolQ-tolR-tolA and tolB-pal-cpoB operons. A single, larger transcript encompassing ybgC-tolQ-tolR-tolA-tolB-pal-cpoB is observed as well. The TolA-TolQ-TolR complex found in the inner membrane plays a crucial role in how these bacteria cause diseases and potentially serve as a vaccine to counter respective bacterial infections.
genes, ybgC, and cpoB (formerly known as ybgF), which encode a cytoplasmic lipid thiosterase and a regulator involved in peptidoglycan (PG) peptide crosslinking in the periplasmic space, respectively.\textsuperscript{19, 20} It is important to note that either of these genes, YbgC or CpoB, is not involved in this Tol/Pal system. Mutations in these genes do not produce similar observable characteristics as mutations in the Tol/Pal genes.\textsuperscript{21} Mutations related to these genes have a wide range of impacts on various aspects of bacterial behavior, which are referred to as pleiotropic effects. Initially, Tol/Pal genes were identified for conferring tolerance capability to \textit{E. coli} against colicins, which are a type of bacteriocin produced by this bacterium.\textsuperscript{22} Research has demonstrated that mutants with mutations in the Tol/Pal genes exhibit resistance to colicins and have a reduced capability to take up colicins, allowing these cells to avoid cell death. Furthermore, subsequent studies have revealed that the mutations of these genes disrupt the integrity of the OM, leading to the development of mucoid bacterial colonies characterized by enhanced colonic acid production. Further, these mutations reduce the transfer of filamentous bacteriophages into the bacterial cytoplasm.\textsuperscript{23-26} Interestingly, when the integrity of the OM is disrupted due to these mutations, it makes the bacteria more vulnerable to certain antimicrobial agents. This increased susceptibility includes antibiotics such as colistin, novobiocin, vancomycin, and \(\beta\)-lactams.\textsuperscript{27-30} Increasing evidence points out the crucial role of the Tol/Pal system in the virulence of various Gram-negative bacterial species, and it is essentially needed for cell growth in certain cases. Consequently, the Tol/Pal system holds promise as a target for drug development.\textsuperscript{31-33} Moreover, mutants with Tol/Pal gene mutations might serve as viable candidates for live attenuated types of vaccines, which can stimulate a significant immune response. Notably, the Pal protein has been identified as an important bacterial surface antigen and is capable of eliciting an immune response; however, clinical evidence to support this claim is currently lacking.\textsuperscript{34, 35} The subsequent sections of this article delve into various studies dedicated to understanding the mechanism and function of the Tol/Pal system in the pathogenicity of bacteria. Additionally, the study aims to explore the potential applications of proteins in the Tol/Pal system in the realms of drug development and vaccine-related research.\textsuperscript{36, 37} In antibacterial therapy, this system operates in several ways, as follows:

**Outer Membrane Vesicles**

Many Gram-negative bacterial species release tiny structures called outer membrane vesicles (OMVs), which comprise various immunogenic components from the bacterial surfaces, such as proteins of the OM, phospholipids, and lipopolysaccharides.\textsuperscript{38-40} Interestingly, some pathogens with Tol/Pal mutants, including \textit{Shigella}, \textit{Helicobacter pylori}, \textit{Salmonella}, and \textit{Buttiauxella agrestis}, were found to release such a large number of OMVs, which can trigger immune responses.\textsuperscript{37, 41-46} For instance, in \textit{Salmonella} research, OMVs from the wild-type \textit{S. choleraesuis}, as well as tolB, tolA, and tolR mutants, were used to immunize mice, leading to immunity.\textsuperscript{43, 47} When challenged with a lethal dose, mice immunized with OMVs obtained from tolA and tolR gene mutants showed longer survival than those with OMVs obtained from wild-type strains. Intriguingly, OMVs obtained from the tolB mutants provided 40% protection to mice, indicating that they conferred stronger immunity. In another \textit{Salmonella}-related research study, OMVs obtained from the mutants of the non-typhoidal \textit{Salmonella tolR} gene stimulated the production of immunoglobulin G (IgG) antibodies in mice, reducing the colonization of bacteria later infected with the wild-type \textit{Salmonella} strains.\textsuperscript{43} Similarly, OMVs produced from the mutants of the \textit{Shigella flexneri tolR} gene elevated the levels of MHC II and CD40 molecules and induced the production of IgG antibodies. OMVs obtained from the mutants of the \textit{Shigella boydii tolA} gene raised the production of mucosal IgA and IgG antibodies in mice, along with certain pro-inflammatory cytokines, such as interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ).\textsuperscript{37, 41} In the case of \textit{Helicobacter pylori}, OMVs produced from \textit{Pal} and \textit{tolB} gene mutants induced significantly higher levels of IL-8 expression in human gastric adenocarcinoma cells compared to the wild-type strain.\textsuperscript{45}

### Sensitizing Antibiotic-resistant Bacteria

In cases where bacteria have developed resistance to antibiotics due to impermeable OMs, targeting the Tol/Pal system could sensitize these bacteria to existing antibiotics. This makes them once again susceptible to treatment, sensitizing antibiotic-resistant bacteria through the Tol-Pal system, and disrupting the stability of the OM of Gram-negative bacteria. This approach enhances antibiotic entry, allows for combination therapies, and can overcome bacterial resistance mechanisms. Research in this field holds the potential to develop novel treatments for infections that are otherwise challenging to treat due to antibiotic resistance.\textsuperscript{46}

### Outer Membrane Permeability

The Tol/Pal system has a vital role in maintaining OM integrity in Gram-negative bacterial species. The disruption of this system can lead to increased permeability of the OM, causing bacteria to become more susceptible to certain antimicrobial agents.\textsuperscript{4} The Gram-negative bacterial OM acts as a selective barrier, controlling the entry of molecules, including antibiotics, into the cell.\textsuperscript{6} Tol/Pal protein complexes can be visualized in Figure 1.

OM permeability is a significant challenge in antibiotic
therapy, and addressing this issue is crucial for developing effective treatments for infections caused by antibiotic-resistant bacteria. Researchers are actively exploring various strategies to enhance antibiotic penetration and overcome this barrier.

**Target for Antibiotics**

As a consequence of its role in OM integrity, mutations or disruptions in the Tol/Pal system can render bacteria more susceptible to antibiotics, including colistin, vancomycin, β-lactams, and novobiocin. These antibiotics, which may have had limited success against antibiotic-resistant strains, can exploit the changes in OM permeability to exert their bactericidal effects. The Tol/Pal system plays a crucial role in maintaining OM integrity in Gram-negative bacteria. When this system is compromised through mutations or disruptions, the OM’s properties change, rendering bacteria more susceptible to antibiotics. This presents a promising avenue for improving antibiotic therapy and addressing antibiotic resistance, as it allows existing antibiotics to be more effective against resistant strains.

**Role of the Tol/Pal System in Bacterial Pathogenesis**

Research into the Tol/Pal system’s role in the bacterial pathogenesis mechanism has revealed intriguing findings across various bacterial species (Table 1). These studies consistently indicate that mutants lacking functional Tol/Pal components exhibit distinctive characteristics that significantly affect their ability to infect hosts and cause diseases. Research on Tol/Pal has demonstrated its significant contribution to PG synthesis in bacteria. TolB is required for the localization of the functional Pal protein to support PG binding and OM constriction, where energy-driven recruitment of the OM TolB-Pal complex by the inner membrane TolA-TolQ-TolR complex occurs during the cell division process. The Tol/Pal system is also actively involved in PG remodelling through the splitting of cell wall glycans to aid the separation of daughter cells during cell division. Hence, the Tol-Pal complex plays a crucial role in lipid homeostasis through retrograde phospholipid trafficking to maintain OM stability in bacteria, which works as an effective barrier against toxic agents such as antibiotics. Host responses to Tol/Pal mutants are notable, as infected hosts tend to exhibit a decreased bacterial burden. Additionally, these mutants often display reduced fitness, slower growth rates, and heightened sensitivity to environmental stressors. This reduction in fitness has profound implications for the overall virulence and persistence of these bacteria within host settings. Another crucial aspect influenced by this system is the production of essential bacterial proteins linked to virulence, including toxins and flagellar proteins. Mutations in these Tol/Pal genes can disrupt the synthesis of these virulence factors, directly impacting the bacterium’s ability to cause...
disease. Specific bacterial species interact with the Tol/Pal system in distinct ways (Table 1).

*Shigella flexneri* relies on the Tol/Pal system to perform the invasion process and growth within host cells. Mutations in the Tol/Pal genes lead to reduced invasiveness, decreased virulence in mice, and heightened sensitivity to various compounds.37 *Burkholderia cenocepacia*, responsible for chronic respiratory infections, depends on the *Pal* gene for attachment to host cells. Mutants exhibit reduced attachment capability and lower lethality in certain host organisms.39 *Haemophilus ducreyi*, a pathogen of genital ulcer disease, produces the *Pal* protein abundantly during infection. Mutants lacking *Pal* exhibit reduced lesion formation and compromised survival, highlighting *Pal’s* importance.40,45 *Salmonella enterica* relies on the Tol/Pal system for survival within host cells and resistance to environmental stressors. Mutations impact OM’s composition and sensitivity to serum and bile acids.53,56,57 *Klebsiella pneumoniae*, typically part of the normal bacterial community, demonstrates increased sensitivity to host defenses and reduced virulence in the presence of *Pal* mutations.67,68 *Dickeya daffaditii*, a plant-pathogenic bacterium, depends on the Tol/Pal system for maintaining pectinolytic enzymatic activity and optimal virulence. Mutants show reduced enzyme activity and mobility.54,77,78 *Vibrio cholerae*, responsible for cholera, requires the Tol/Pal system for growth and the acquisition of a critical phase. Mutations affect growth and the ability to take up the phase.75 *Pseudomonas aeruginosa*, known for its antimicrobial resistance and biofilm formation, represents an essential role for TolB in growth, making it a potential therapeutic target.69,79 *Edwardsiella ictaluri*, a pathogenic bacterium affecting catfish, relies on the TolQ and TolR genes for virulence. Mutants lacking these genes exhibit reduced mortality rates in infected catfish.64 *E. coli* pathogenic subgroups such as enterohemorrhagic *E. coli* (EHEC) and uropathogenic *E. coli* (UPEC) have distinct interactions with the Tol/Pal system. EHEC depends on TolB for effector protein secretion, while UPEC relies on TolB/Pal for flagella-dependent colonization and survival in the urinary tract.41,42,62,67 In summary, the Tol/Pal system plays a pivotal role in the pathogenicity of bacteria from diverse species. Its impact on bacterial fitness, virulence factors, and interactions with host cells underscores its significance in bacterial infections. Understanding these interactions provides valuable insights into potential therapeutic targets for combating bacterial diseases, with the collective knowledge gained from studying Tol/Pal mutants contributing to a broader understanding of their central role in bacterial pathogenesis.

### Table 1. Roles of the Top/Pal System in the Pathogenesis of Gram-negative Pathogens

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Pathogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) <em>Acinetobacter baumannii</em></td>
<td>It provides intrinsic resistance to antibiotics and detergents (sodium dodecyl sulfate) by reducing membrane permeability and maintaining membrane integrity.</td>
</tr>
<tr>
<td>(2) <em>Burkholderia cenocepacia</em></td>
<td>It causes bacterial adaptation and, in turn, its survival in the lungs by promoting attachment to lung epithelial cells and inflammation during cystic fibrosis infections.</td>
</tr>
<tr>
<td>(3) <em>Citrobacter rodentium</em></td>
<td>Enteritis and lethality in mice are associated with tolB, contributing to cell motility, adherence to host cells, and secretion of the proteins EspA/B from the Type III secretion system.</td>
</tr>
<tr>
<td>(4) <em>Dickeya daffaditii</em> (<em>Erwinia chrysanthemi</em>)</td>
<td>It shows pectate lyase activity (a pectinase enzyme) and promotes bacterial growth by increasing cell integrity and motility.</td>
</tr>
<tr>
<td>(5) <em>Enterohemorrhagic Escherichia coli</em></td>
<td>It promotes cell envelope integrity, confers virulence through the type III secretion system, promotes bacterial adherence through attaching and effacing (A/E) lesions in the host cells, and aids in flagellar biosynthesis and activity for infections in the enteric area.</td>
</tr>
<tr>
<td>(6) <em>Uropathogenic Escherichia coli</em></td>
<td>It promotes bacterial internalization and aggregation in bladder epithelial cells and improves bacterial colonization and motility. It also leads to capsule formation and resistance to serum.</td>
</tr>
<tr>
<td>(7) <em>Edwardsiella ictaluri</em></td>
<td>It maintains outer membrane (OM) integrity and causes chronic meningonephritis and acute septicemia in catfish.</td>
</tr>
<tr>
<td>(8) <em>Haemophilus ducreyi</em></td>
<td>As a causative agent of genital ulcer disease, <em>Pal</em> expression confers antibiotic resistance and causes the pustular stage of the disease.</td>
</tr>
<tr>
<td>(9) <em>Klebsiella pneumoniae</em></td>
<td>It causes OM integrity and bacterial cell shrinkage, along with the production of OM vesicles to confer resistance toward antibiotics.</td>
</tr>
<tr>
<td>(10) <em>Pseudomonas aeruginosa</em></td>
<td>It leads to biofilm formation and bacterial growth.</td>
</tr>
<tr>
<td>(11) <em>Salmonella enterica</em> (<em>Typhimurium</em>)</td>
<td>It promotes OM glycerophospholipid homeostasis and is the regulator of capsule synthesis (Rcs) signaling inactivity, which aids in antibiotic (rifampicin) resistance, survival in macrophages and mice, and innate tolerance toward bile acids and the serum, leading to bacteremia.</td>
</tr>
<tr>
<td>(12) <em>Salmonella enterica</em> (<em>Choleraesuis</em>)</td>
<td>It promotes bacterial growth and motility, confers resistance to bile salts and antibiotics (vancomycin), and causes diseases such as meningitis, pneumonia, and hepatitis in swine.</td>
</tr>
<tr>
<td>(13) <em>Vibrio cholerae</em></td>
<td>It facilitates CTXphi temperate bacteriophage infection in bacteria, which confers the ability of cholera toxin production to bacteria. In addition, it confers bile resistance and increases bacterial colonization.</td>
</tr>
<tr>
<td>(14) <em>Xylella fastidiosa</em></td>
<td>It is a plant pathogen in which the Tol/Pal complex plays a role in biofilm development during bacterial growth in the xylem vessels of cultivable plants, leading to the progression of disease and damage to such plants.</td>
</tr>
</tbody>
</table>
Virulence Attenuation

Interestingly, even though some pathogens’ tol/pal mutants are less virulent or even avirulent, they can still trigger immune responses. This makes these mutant strains of the Tol/Pal genes potential candidates for attenuated live vaccines. For example, the tolB mutants of *Citrobacter rodentium*, which is used as an alternative model for evaluating EHEC virulence in vivo, can stimulate IgG production and various cytokines such as IL-17 and IFN-γ.\(^{52,60}\) This immunization protects from the pathogenicity of the lethal parental wild-type strain. Similarly, inoculation with the less-virulent mutants of the tolA gene protects mice from subsequent infection with *Salmonella typhimurium*.\(^{80}\) When compared to naïve control mice, those immunized with the mutant strains of the tolA gene had significantly reduced bacterial loads than those immunized with wild-type strains later on. Furthermore, immunizing mice with the Pal gene mutants of *K. pneumoniae* allows them to survive infections caused by highly virulent wild-type strains, demonstrating the potential of these mutants as vaccines.\(^{81}\) Additionally, the *Edwardsiella ictaluri* mutants of the tolQ and tolR genes were found to confer resistance against infections from the highly virulent wild-type *E. ictaluri*.\(^{84}\)

Vaccine Development

Some surface molecules of bacteria, such as lipoproteins, capsular components, lipopolysaccharides, flagellin, and pilin, serve as antigens that trigger defensive immune responses. Within the Tol/Pal system, the protein Pal belongs to the group of bacterial lipoproteins.\(^{21}\) Furthermore, Pal can stimulate the production of proinflammatory cytokines by activating Toll-like receptor 2.\(^{84}\) Therefore, Pal has the potential to act as an immunogenic antigen, prompting both innate and adaptive immune responses. Various vaccine approaches have been proposed utilizing whole recombinant forms of Pal proteins or synthetically made peptide fragments.\(^{82,83}\) For example, immunizing mice with the recombinant form of the Pal protein from the bacteria *Legionella pneumophila* led to the production of specific IgG antibodies against the Pal protein and proinflammatory cytokines (e.g., IL-6 and TNF-α), conferring immune protection against *L. pneumophila*-related infections. Researchers identified an epitope within the Pal protein of *L. pneumophila* that is responsible for immune system activation, and by using a synthetic peptide of this epitope, along with a TLR9 agonist called CpG-oligodeoxynucleotides, as an adjuvant, vaccination could effectively boost cytotoxic T cell responses, lower bacterial burden, and protect mice by providing immunity against *L. pneumophila* infections.\(^{84,85}\) DNA encoding the Pal protein has also been explored as a vaccine candidate. One study showed that using Pal DNA of *L. pneumophila* induced Pal-specific IgG antibody production and cytotoxic T-lymphocyte-based responses in mice, with stronger CD8+ T cell responses, as compared to mice immunized with the Pal protein alone.\(^{84}\) Another study utilized a hybrid DNA construct with the Pal gene, along with the pilE and flaA genes, which encode flagellin and type IV pilin, respectively, in *L. pneumophila*.\(^{85}\) A vaccine comprising this plasmid DNA stimulated a range of Th1 and Th2 cytokines and IgG antibody production and protected vaccinated mice from lethal pulmonary infections.\(^{86}\) Similarly, in the case of Tol genes, deletion of the tolR gene in *S. flexneri* demonstrated enhanced sensitivity of tolR mutants to detergents due to changes in bacterial cell envelope properties. A subunit vaccine showed good stability against temperature and other external factors, along with the safe and effective capability of modulating the immune system in mice, providing a platform for OMV-based vaccine production against *Shigella* spp.-related infections.\(^{87}\) Hence, the Tol/Pal system can be used in immunoprophylaxis to develop effective immunity against related bacterial infections.

Conclusion

The Tol/Pal system plays a crucial role in enhancing the virulence of many Gram-negative pathogens while also being essential for the survival and growth of certain bacterial species. This system offers a promising and novel target for antibacterial therapy, setting it apart from conventional target molecules such as ribosomes, primary metabolic enzymes, cell wall synthases, and RNA polymerase. The disruption of the Tol/Pal system presents a potential strategy for combating drug-resistant bacteria that have become impervious to traditional drugs. One promising avenue is the development of molecules that can interfere with protein-protein interactions or bind to proteins of the Tol/Pal system, such as TolA, TolR, TolQ, TolB, and Pal, which could effectively disrupt this system. Additionally, this Tol/Pal system possesses significant immunogenic properties, making it a valuable resource for vaccine development. Mutants lacking components of the Tol/Pal system have shown the ability to trigger protective immune responses against wild-type bacteria. Notably, these mutants can lead to the production of OMVs in some species, which contribute to immune responses. Pal proteins, a part of the Tol/Pal system, can stimulate both the innate and adaptive types of the immune systems. It acts as a ligand for Toll-like receptor 2, inducing a proinflammatory cytokine response, and serves as an antigen recognized by specific antibodies in infected individuals. Consequently, this remarkable Tol/Pal system holds potential as a target for vaccine development. In the coming years, further research on the Tol/Pal system is expected to address these important considerations, paving the way for potential therapeutic and vaccine applications of this system.
References


10. Liberati NT, Urbach JM, Miyata S, et al. An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. Proc Natl Acad Sci U S A. 2006;103(8):2833-2838. doi:10.1073/pnas.0511001010


29. Onodera K, Rolfe B, Bernstein A. Demonstration of missing


