Design, Expression, and Oral Delivery of a Recombinant Fusion Protein (Tp0751–CtB–TprK) in Yeast as a Candidate Syphilis Vaccine

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Abstract

Syphilis, caused by *Treponema pallidum*, has resurged globally in recent years, particularly in East Asia, highlighting the urgent need for preventive vaccines. Despite being a well-characterized pathogen, traditional vaccine development for *T. pallidum* faces substantial challenges due to its inability to be cultured in artificial media. This study presents the development of a novel oral vaccine candidate utilizing recombinant *T. pallidum* outer membrane proteins TprK and Tp0751 expressed in yeast (*Saccharomyces Boulardii*). Our approach incorporates a gene cassette containing the conserved N-terminal region of TprK, cholera toxin B subunit (CtB) as an immune adjuvant, and Tp0751, connected by glycine-serine flexible linkers under the control of the ADH promoter. This strategy aims to overcome the cultivation limitations of *T. pallidum* while potentially inducing protective mucosal immunity through oral administration. Structural analysis using AlphaFold predictions confirms the stability of the recombinant construct. This research represents a significant advancement toward developing an accessible and effective vaccine against syphilis, with important implications for global public health.

Introduction

Syphilis, caused by the spirochete bacterium *Treponema pallidum*, continues to be a significant public health challenge globally despite being an ancient disease. Recent epidemiological data demonstrates alarming increases in syphilis cases, particularly in East Asia. Japan reported a record 13,228 cases in 2022, showing a dramatic increase since 2013 when cases first exceeded 1,000. Similarly, South Korea has experienced a substantial surge, with 1,881 cases

reported between January and August 2024, approximately 4.5 times the total number (416) reported in the previous year.

This trend is not limited to Asia. According to the U.S. Centers for Disease Control and Prevention (CDC), syphilis cases in the United States increased by 26% in 2021 compared to the previous year, reaching the highest levels since 1948. The European Centre for Disease Prevention and Control (ECDC) has also reported a consistent increase in syphilis infection rates across the European Union and European Economic Area from 2010 to 2019.

Despite the availability of effective antibiotic treatments, primarily penicillin, several factors make vaccine development a valuable pursuit. First, the disease progression includes latent periods during which patients may remain infectious despite symptom resolution. Second, the characteristic ulcerative lesions cause significant physical discomfort and psychological stigma. Third, reinfection remains possible despite prior exposure and treatment.

Conventional vaccine development approaches face substantial challenges with *T. pallidum*. The bacterium cannot be cultured on artificial media, requiring rabbit testes for propagation, which severely limits the viability of traditional live or killed vaccine strategies.

This study explores an innovative approach utilizing recombinant protein technology to express key *T. pallidum* outer membrane proteins in yeast, specifically targeting TprK and Tp0751, which have shown promising immunogenic potential. By incorporating these proteins with the cholera toxin B subunit (CtB) as an immune adjuvant, we aim to develop an oral vaccine candidate that could elicit mucosal immunity against syphilis infection, potentially providing a practical solution to this resurging public health threat.

Outer Membrane Proteins as Vaccine Targets

Given these limitations, subunit vaccines targeting key outer membrane proteins represent a promising alternative. This study focuses on two specific proteins:

> **TprK (Treponema pallidum repeat protein K)**: TprK is a surface-exposed lipoprotein comprising a conserved N-terminal region and seven variable regions (V1-V7). These variable regions undergo sequence variation through gene conversion, allowing the bacterium to evade host immune responses. While this variability poses a challenge, the

conserved N-terminal region represents a potential target for vaccine development that could elicit cross-reactive immunity.

Tp0751 (Pallilysin): Tp0751 is an atypical lipocalin with an eight-stranded beta-barrel structure that plays a crucial role in host-pathogen interactions. It binds to extracellular matrix (ECM) components such as fibrinogen, fibronectin, and collagen I and IV, facilitating *T. pallidum*'s attachment to host tissues and dissemination. Previous research has shown that immunization with recombinant Tp0751 reduces bacterial burden in infected organs in animal models, highlighting its potential as a vaccine candidate.

Oral Vaccine Delivery and Mucosal Immunity

Oral vaccine delivery offers several advantages for addressing sexually transmitted infections, including:

Ease of Administration: Simplified delivery without needles increases compliance and reduces the need for trained healthcare providers.

Cost-Effectiveness: Lower production costs and elimination of cold chain requirements improve accessibility.

Mucosal Immunity: Oral vaccines can induce robust mucosal immune responses, which are particularly relevant for pathogens that initially encounter mucosal surfaces, such as sexually transmitted infections.

Mucosal immunity, particularly the production of secretory IgA antibodies at mucosal surfaces, may be crucial for preventing syphilis infection at the point of entry. By targeting the production of these antibodies through oral vaccination, we aim to establish a first line of defense against *T. pallidum* invasion.

Materials and Methods

Gene Cassette Design

We designed a gene cassette containing the following elements:

Promoter: ADH (alcohol dehydrogenase) promoter for high-level constitutive expression in yeast.

Target Gene 1: TprK N-terminal conserved region 87-272 AA (UniPort: A0A089FK68, https://www.uniprot.org/uniprotkb/A0A089FK68/entry).

Linker 1: (GGS)×3 flexible glycine-serine linker to ensure independent folding of protein domains.

Adjuvant: Cholera Toxin Subunit B (CtB) for enhanced immunogenicity and mucosal targeting.(UniPort: Q7X2D2, https://www.uniprot.org/uniprotkb/Q7X2D2/entry)

Linker 2: (GGS)×4 flexible glycine-serine linker.

Target Gene 2: Tp075 focusing on the beta-sheet regions important for immunogenicity. 99-237 AA (UniPort: O83732, https://www.uniprot.org/uniprotkb/O83732/entry)

Terminator: ADH terminator for efficient transcription termination.

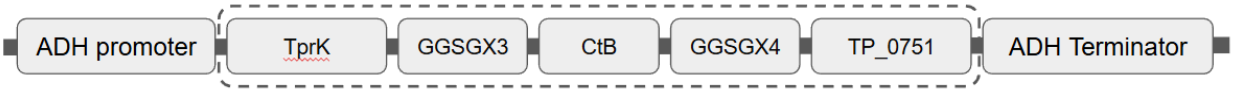

Figure 1. Schematic Diagram of the Gene Cassette Construct (ADH promoter - TprK - Linker - CtB - Linker - Tp0751 - ADH terminator)

The amino acid sequences incorporated in our construct were as follows:

TprK (N-terminal conserved region):

VYAEINVKALKLSLESNGGAKFDTKGSAKTIEATLHCYGAYLTIGKNPDFKSTFAALWEP
WTANGDYKSKGDKPVYEPGFEGAGGKLGYKQTDIAGTGLTFDIAFKFASNTDWEGKPN

GNVPAGVTPSKYGLGGDILFGWERTREDGVQEYIKVELTGNSTLSSGYATARAGADILW
DVGAKVSMK

CtB (Cholera toxin B subunit):

MTPQNITDLCAEYHNTQIYTLNDKIFSYTESLAGKREMAIITFKNGAIFQVEVPGSQHIDS
QKKAIERMKDTLRIAYLTEAKVEKLCVWNNKTPHAIAAISMANGT

Tp0751 (Pallilysin, key antigenic region):

VQTAMRIALWNRATHGEQGALQHLLAGLWIQTEISPNSGDIHPLLFFDREHAEITFSRAS
VQEIFLVDSAHTHRKTVSFLTRNTAISSIRRRLEVTFESHEVIHVRAVEDVARLKIGSTSM
WDGQYTRYHAGPASAPSP

The gene sequences incorporated in our construct were as follows (The yellow part is the linker):

ATGgtctatgcggaaattaatgttaaagcgttgaagttgagtttagagtcaaatggtggagcaaagtttgacacgaagggtt
ctgcaaagacgatagaggcaaccctgcactgttatggggcctacctgaccattgggaagaatcctgattttaagtcaacgttt
gctgctttgtgggagccgtggaccgcgaatggggattataagtctaagggagataagccggtgtatgagccggggtttgag
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acacacaccgcaagacggtgtcatttctcacgcgcaacaccgcaatttccagcatccgccgccgccttgaggtaacatttg
aatcccacgaggtgatacacgtaagggcggttgaagacgtagcacggctcaaaattggcagcacgtcgatgtgggacg
gtcaatacaccagatatcacgccggtccggctagtgctccttcgcccTGA

Structural Analysis

The three-dimensional structure of the fusion protein was predicted using AlphaFold to evaluate proper folding and accessibility of key antigenic domains. (https://alphafoldserver.com/fold/2141425e7cdc8352) Particular attention was paid to:

1. The preservation of the beta-sheet regions in Tp0751, which are crucial for its immunogenicity
2. The pentameric structure of CtB, important for its adjuvant function
3. The accessibility of the conserved epitopes in the TprK N-terminal region

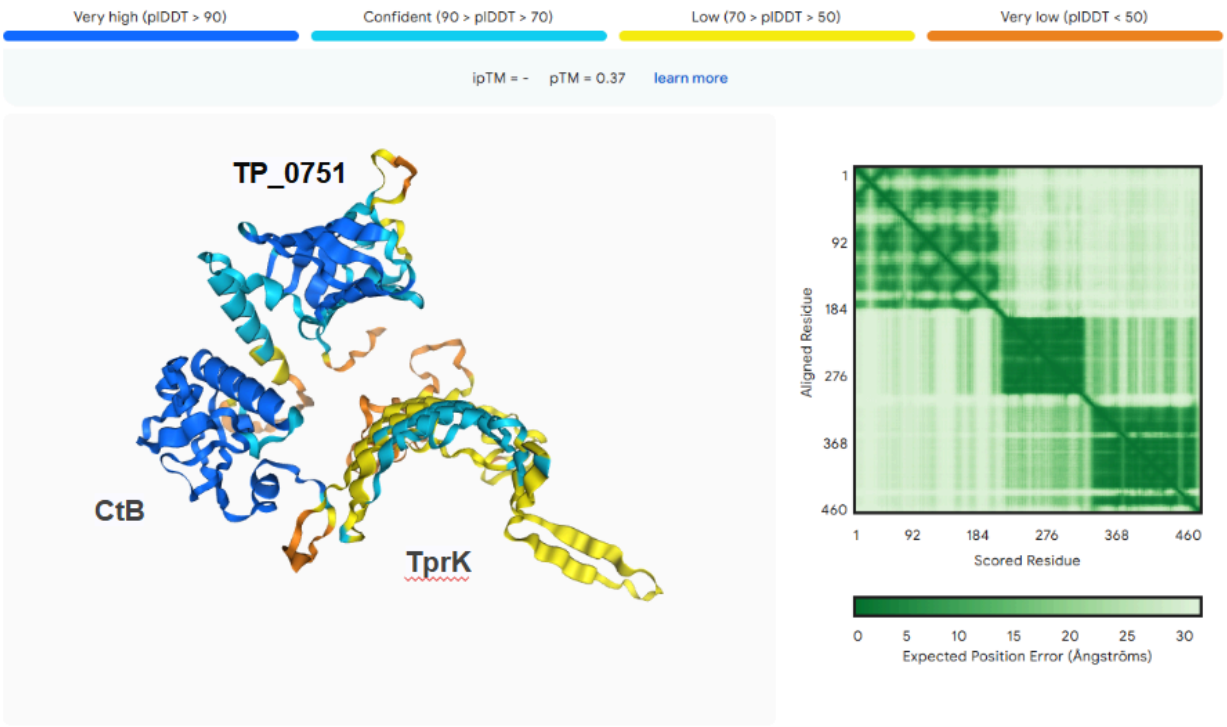

Figure 2. AlphaFold-Predicted Structure of the Fusion Protein Demonstrating Key Immunogenic Domains (TprK N-terminal Epitopes-CtB Pentamer-Tp0751 β-sheet)

Yeast Strain and Transformation

Saccharomyces Boulardii was used as the expression host due to its well-characterized genetics and strong protein expression capabilities. The designed gene cassette was synthesized and cloned into the yeast expression vector pICE ADE2-HphM(Addgene) under the control of the ADH promoter/terminator. Yeast cells were transformed with the expression vector using the lithium acetate method.

Recombinant Protein Expression and Verification

Transformed yeast cells were cultured in YPD medium at 30°C with shaking (200 rpm) for 48 hours to induce protein expression. Cells were harvested by centrifugation (3,000 × g for 10 minutes), and protein expression was analyzed by SDS-PAGE on 12% polyacrylamide gels stained with Coomassie Blue.

Yeast Processing for Oral Delivery

Successfully transformed yeast cells expressing the recombinant protein were processed for oral delivery using the following protocol:

1. **Harvest**: Cells were harvested at the optimal growth phase (late log phase) by centrifugation.
2. **Yeast Processing:** The harvested yeast culture was subjected to heat drying to inactivate the yeast cells, rendering them non-viable while maintaining antigen stability.
3. **Formulation**: The heat-dried yeast cells were processed into powder form and formulated into enteric-coated capsules to protect against gastric degradation.

Discussion

The increasing incidence of syphilis globally, particularly in East Asia, necessitates innovative approaches to prevention. Our study presents a novel strategy for syphilis vaccine development using yeast-expressed recombinant outer membrane proteins of *T. pallidum*.

1. **Cultivation Challenges**: By expressing key *T. pallidum* antigens in yeast, we bypass the need for cultivation of the pathogen itself.
2. **Antigenic Variation**: By focusing on the conserved N-terminal region of TprK, we target epitopes that are less subject to variation while still inducing potentially protective immune responses.
3. **Delivery and Compliance**: The oral delivery system offers advantages in terms of ease of administration and potential compliance compared to injectable vaccines.
4. **Mucosal Immunity**: By incorporating CtB as an adjuvant and utilizing oral delivery, our approach aims to induce mucosal immunity at potential sites of infection, which may be crucial for preventing initial invasion by *T. pallidum*.

The selection of TprK and Tp0751 as vaccine targets is based on their important roles in pathogenesis and previous evidence of their immunogenic potential. The conserved N-terminal region of TprK represents a stable target despite the variability in other regions of the protein. Tp0751's role in host-pathogen interactions, particularly its interaction with extracellular matrix components, makes it an attractive target for neutralizing antibodies that could prevent bacterial dissemination.

The yeast expression system offers several advantages for vaccine production, including:

1. **Safety**: *S. Boulardii* has GRAS (Generally Recognized As Safe) status
2. **Scalability**: Yeast cultivation is well-established and can be scaled up efficiently
3. **Cost-effectiveness**: Production costs are potentially lower than for other recombinant systems
4. **Post-translational modifications**: Yeast can perform many of the post-translational modifications required for proper protein folding

The oral delivery approach offers unique advantages for a syphilis vaccine. By targeting mucosal immunity, we aim to establish a first line of defense at potential sites of infection. The

use of CtB as an adjuvant enhances this approach, as it has been shown to be effective in stimulating mucosal immune responses when administered orally.

While our results are promising, several limitations and challenges remain. The actual immunogenicity and protective efficacy of the vaccine candidate need to be evaluated in animal models. The complex immune evasion mechanisms of *T. pallidum* may still pose challenges even with a carefully designed vaccine. Further optimization of antigen selection, dosage, and formulation may be required to achieve optimal protection.

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