Characterization of the NarS/NarL and KdpD/KdpE Two-Component Signal Transduction Systems in *Mycobacterium tuberculosis*: Recombinant Protein Expression, Polyclonal Antibody Development, and Construction of Gene Knockout Vectors

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Abstract:
Mycobacterium tuberculosis survives hostile host environments through two-component regulatory systems (TCSs), including the NarS/NarL and KdpD/KdpE pathways, which play important roles in environmental sensing and bacterial adaptation. This study aimed to characterize these signaling systems by expressing recombinant NarS, NarL, KdpD, and KdpE proteins in *Escherichia coli*. The proteins were purified using Ni-NTA affinity chromatography, and polyclonal antibodies against NarL and KdpE were generated in rabbits. Antibody specificity and titre were evaluated using ELISA and Western blotting. A suicide delivery vector was also constructed by cloning the downstream homologous region of the NarS/NarL genes into the p2NIL vector for targeted gene knockout in *Mycobacterium bovis* BCG. The recombinant proteins and antibodies demonstrated high specificity and are suitable for functional studies. The constructed knockout vector provides a valuable tool for investigating gene function through homologous recombination. Overall, this study establishes important molecular resources for understanding bacterial stress adaptation, signal transduction, and virulence regulation in *M. tuberculosis*, thereby supporting future tuberculosis research and therapeutic target identification.

Keywords: *Mycobacterium tuberculosis*; Two-component system; NarS/NarL; KdpD/KdpE; Signal transduction; Recombinant protein; ELISA; Western blot; Gene knockout; p2NIL vector.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains one of the leading infectious diseases worldwide and continues to be a major public health concern. According to the World Health Organization (WHO), tuberculosis (TB) remained a major global health challenge in 2023, with an estimated **10.8 million incident cases** and **1.25 million deaths** worldwide despite the availability of effective treatment(1) The increasing prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis underscores the need to better understand the molecular mechanisms that enable bacterial survival and persistence (2). One of the key adaptive mechanisms in *M. tuberculosis* is the two-component signal transduction system (TCS), which enables the bacterium to detect and respond to environmental stresses such as hypoxia, nutrient deprivation, oxidative stress, and osmotic changes (3,4). Among these systems, NarS/NarL regulates nitrate metabolism and adaptation to low-oxygen conditions, whereas KdpD/KdpE controls potassium homeostasis and contributes to stress adaptation and intracellular survival (5,6). Although these regulatory pathways are considered potential therapeutic targets, their molecular functions remain incompletely understood. Therefore, the present study aimed to characterize the NarS/NarL and KdpD/KdpE signaling systems through recombinant protein expression, purification, polyclonal antibody production, and construction of a suicide delivery vector for targeted gene knockout. These molecular tools provide a valuable platform for investigating bacterial signal transduction, virulence regulation, and the development of novel anti-tuberculosis strategies (7–10).

Materials and Methods

Overexpression and Purification of Recombinant Proteins

Recombinant NarS, NarL, KdpD, and KdpE proteins were overexpressed in *Escherichia coli*, a widely used host for recombinant protein production because of its rapid growth, ease of genetic manipulation, and high expression efficiency (11,12). Recombinant plasmids were isolated by the PEG–NaCl method and confirmed by agarose gel electrophoresis. Competent *E. coli* cells were prepared using the calcium chloride ($CaCl_2$) method and transformed with the recombinant plasmids (13). Positive transformants were cultured in Luria–Bertani (LB) medium containing the appropriate antibiotic, and protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cell pellets were harvested by centrifugation, lysed by sonication, and separated into soluble and insoluble fractions. Soluble recombinant proteins were purified using Ni-NTA affinity chromatography, whereas proteins present in inclusion bodies were solubilized, refolded under optimized conditions, and purified (14,15). Protein purity was assessed by SDS–PAGE, and protein concentrations were determined using the Bradford protein assay (16,17).

Production and Purification of Polyclonal Antibodies

Purified recombinant NarL and KdpE proteins were used as antigens for raising polyclonal antibodies in New Zealand White rabbits. Primary immunization was performed with Complete Freund's Adjuvant (CFA), followed by booster immunizations with Incomplete Freund's Adjuvant (IFA) at regular intervals (18). Serum samples were collected after each booster, and antibody titres were determined by indirect enzyme-linked immunosorbent assay (ELISA). The specificity of the antibodies was further confirmed by Western blot analysis using purified recombinant proteins (19,20).

Construction of the Suicide Delivery Vector

A suicide delivery vector was constructed for targeted disruption of the NarS/NarL genes in *Mycobacterium bovis* BCG. Genomic DNA of *Mycobacterium tuberculosis* was isolated, and the downstream homologous region of the target genes was amplified by PCR. The amplified fragment was cloned into the p2NIL suicide vector using standard molecular cloning procedures. Recombinant plasmids were confirmed by restriction digestion and sequencing before being used for homologous recombination-mediated gene knockout studies (21).

Results

Expression and Purification of Recombinant NarS, NarL, KdpD, and KdpE Proteins

The recombinant plasmids carrying the NarS, NarL, KdpD, and KdpE genes were successfully transformed into *Escherichia coli* expression strains. SDS–PAGE analysis demonstrated successful expression of all four recombinant proteins following IPTG induction. Among the expressed proteins, NarS, NarL, and KdpD were predominantly detected in the soluble protein fraction, whereas KdpE was largely recovered as inclusion bodies within the insoluble fraction. The insoluble KdpE protein was subsequently solubilized, refolded, and purified.

Purification of the His-tagged recombinant proteins was achieved using Ni-NTA affinity chromatography. The purified proteins appeared as distinct bands corresponding to their expected molecular weights with minimal contaminating proteins, indicating efficient purification. Dialysis removed excess salts and imidazole, resulting in stable protein preparations suitable for downstream immunological and molecular analyses.

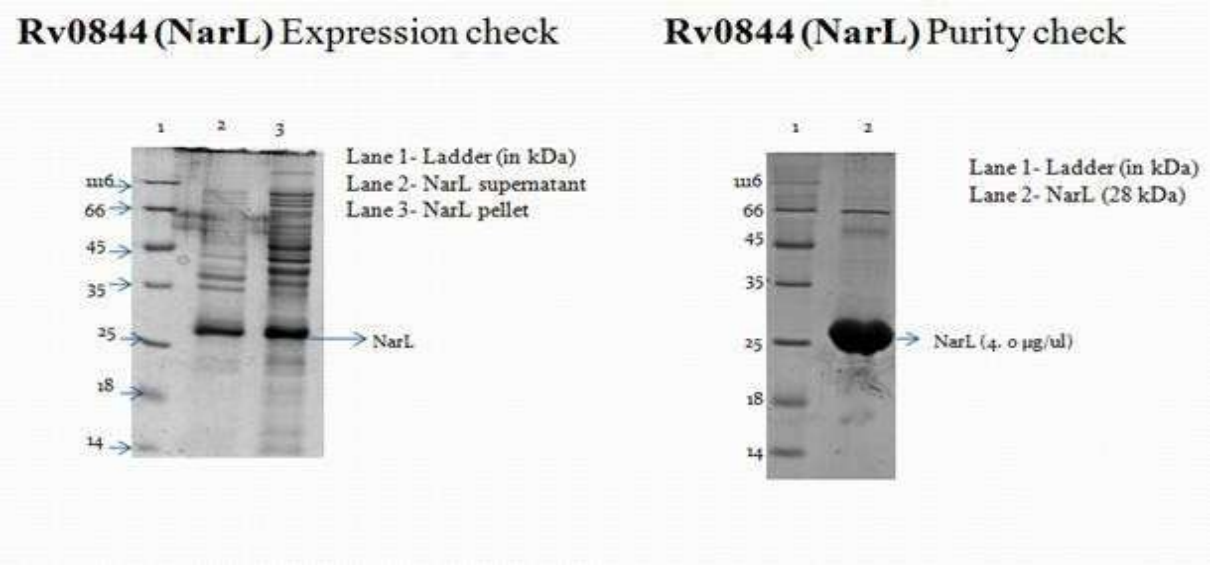
Protein concentrations determined by Bradford assay showed differences in the yield of the purified proteins. NarL exhibited the highest protein concentration (4.0 µg/µL), followed by KdpE (1.8 µg/µL), KdpD (0.4 µg/µL), and NarS (0.3 µg/µL), demonstrating variable expression efficiencies among the recombinant proteins.

Table 1. Quantification of purified recombinant proteins

Protein	Absorbance (595 nm)	Protein concentration (µg/µL)
NarS	0.0153	0.3
NarL	0.2040	4.0
KdpD	0.0204	0.4
KdpE	0.0918	1.8

The purified protein was analyzed on SDS gel (Figure.1.a-c) and the protein was quantified using Bradford assay (Figure.2 and Table.2)

Figure. 1. Protein purification. a) Soluble fraction protein purification of NarL, b) Soluble fraction purification of NarS and KdpD, c) Insoluble fraction protein purification of KdpE



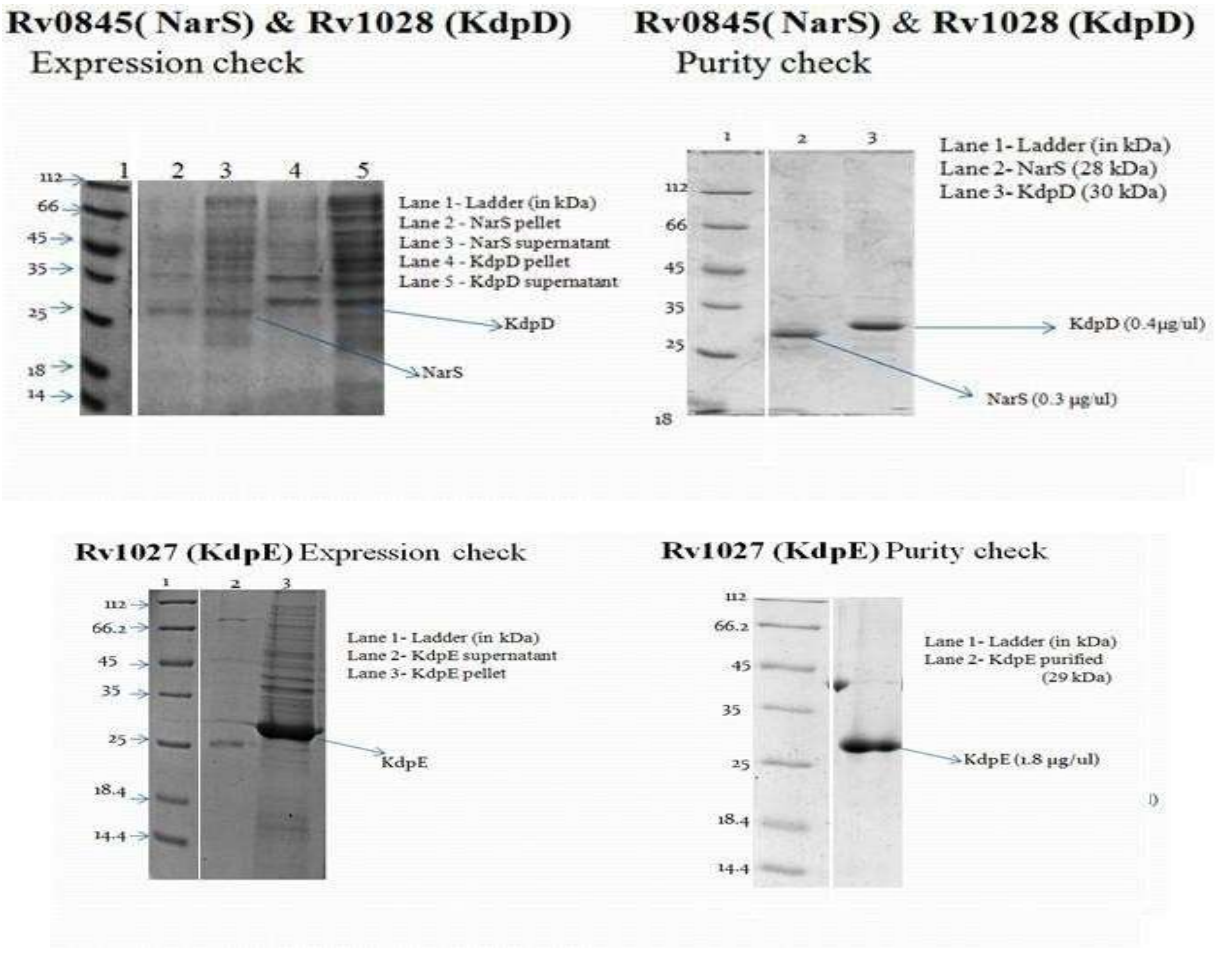
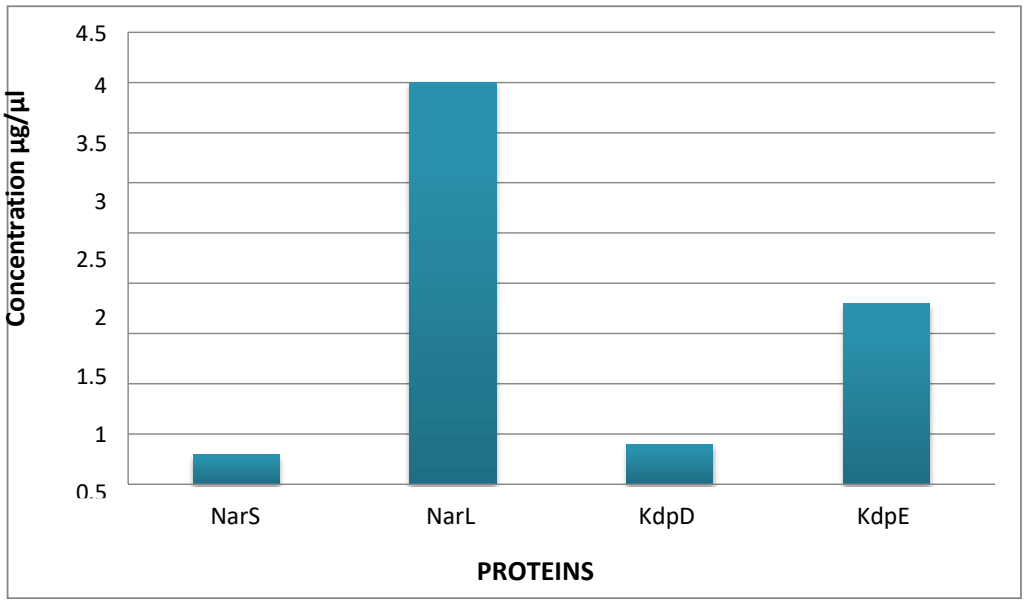


Figure.2. Bradford's assay read out of purified NarS, NarL, KdpD and KdpE proteins



Anti- Rv0844 titer determination

Table.2 Titer of anti-Rv0844 using ELISA

S. No.	Day	Bleed	Titer
1.	29	Bleed 1	10000
2.	58	Bleed 2 part 1	20000

3.	60	Bleed 2 part 2	30000
4.	100	Bleed 3	40000
5.	104	Bleed 4	150000
6.	106	Bleed 5	200000
7.	108	Bleed 6	250000

Anti- Rv1027 titer determination

Table.3 Titer read out of anti-Rv1027 using ELISA

S. No.	Day	Bleed	Titer
1.	29	Bleed 1	100000
2.	58	Bleed 2 part 1	150000
3.	60	Bleed 2 part 2	150000
4.	100	Bleed 3	200000
5.	104	Bleed 4	200000
6.	106	Bleed 5	250000
7.	108	Bleed 6	300000

Production and Characterization of Polyclonal Antibodies

Purified NarL and KdpE proteins were used as immunogens for raising polyclonal antibodies in rabbits. Sequential booster immunizations resulted in a progressive increase in antibody titre throughout the immunization schedule.

Indirect ELISA demonstrated a gradual enhancement of antibody response after each booster injection. The highest antibody titres were observed in the final bleeds, indicating successful immunization and strong antigen-specific immune responses. Purified antibodies retained high antigen-binding activity following affinity purification.

Western blot analysis further confirmed the specificity of the generated antibodies. Anti-NarL antibodies recognized the recombinant NarL protein with a clear immunoreactive band, while anti-KdpE antibodies specifically detected recombinant KdpE protein. Limited cross-reactivity with other recombinant proteins was observed, confirming the suitability of these antibodies for immunological applications including Western blotting, ELISA, and chromatin immunoprecipitation (ChIP).

ELISA Antibody Titre Analysis

Serial dilution ELISA demonstrated a consistent increase in antibody production following repeated immunization. The anti-NarL antibody reached a maximum titre of approximately 2.5×10^5, whereas the anti-KdpE antibody attained approximately 3.0×10^5 during the final immunization cycle. These findings indicate efficient stimulation of the rabbit immune system and successful production of high-titre polyclonal antibodies.

The purified antibodies maintained strong antigen recognition even after purification, demonstrating their suitability for subsequent functional studies involving signal transduction proteins.

Construction of the NarS/NarL Suicide Delivery Vector

To investigate the biological role of the NarS/NarL signaling pathway, a suicide delivery vector was constructed using the p2NIL plasmid backbone. The downstream homologous region of the target genes was successfully amplified by PCR and cloned into the vector using restriction enzyme-mediated ligation.

Restriction digestion analysis confirmed successful insertion of the amplified DNA fragment into the cloning vector. The recombinant plasmid generated in this study provides an efficient platform for homologous recombination-mediated targeted gene deletion in *Mycobacterium bovis* BCG.

The validated construct represents an important molecular tool for generating knockout mutants that will facilitate future investigations into the physiological functions of NarS/NarL under environmental stress conditions such as hypoxia, nutrient deprivation, acidic pH, and nitrogen limitation.

Discussion

The present study successfully established molecular tools for investigating the NarS/NarL and KdpD/KdpE two-component regulatory systems of *Mycobacterium tuberculosis*. Recombinant expression in *Escherichia coli* enabled efficient production and purification of the target proteins, although KdpE was predominantly recovered as inclusion bodies, a common feature of heterologous protein expression **(11,22)**. The purified proteins generated high-titre polyclonal antibodies with excellent specificity, as confirmed by ELISA and Western blotting, demonstrating their suitability for immunological and functional studies **(23,24)**. Construction of the **p2NIL** suicide delivery vector provides an effective platform for homologous recombination-based gene knockout and functional analysis of the NarS/NarL signaling pathway

(25). Since NarS/NarL and KdpD/KdpE regulate nitrate metabolism, potassium homeostasis, stress adaptation, and intracellular persistence, these molecular tools will facilitate future investigations into bacterial survival mechanisms and virulence regulation **(26,27)**. Further validation through phosphorylation assays, transcriptomic analysis, and infection models will improve understanding of these regulatory networks and may contribute to the identification of novel therapeutic targets for tuberculosis **(28,29)**. This study establishes an important experimental framework for investigating two-component signaling systems in *Mycobacterium tuberculosis*. The successful production of recombinant proteins, high-titre polyclonal antibodies, and a validated suicide delivery vector significantly expands the molecular toolkit available for tuberculosis research. These resources will facilitate future investigations into bacterial stress adaptation, persistence, and virulence and may ultimately contribute to the identification of novel therapeutic targets for combating tuberculosis

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