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Synthesis of Purified Pneumolysin and Autolysin Proteins of *S. pneumoniae* in Prokaryotic System by Modified Recombinant DNA Technology

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Abstract: ***This study aimed to produce purified recombinant proteins encoded by ply and lytA genes of S. pneumoniae in prokaryotic system. Gene specific primers were used to amplify ply and lytA genes from* S. pneumoniae *ATCC 49619 strain. The ply and lytA PCR products and pUC29 cloning vector were digested, ligated and transformed into E.coli (DH5α). Sequence confirmed ply and lytA genes were sub cloned in pET28a expression vector. Proteins were over expressed by IPTG induction and purified using Ni^{2+}-NTA based affinity column purification with imidazole gradient. PCR amplicons of size ~1413 bp for ply and ~954 bp for lytA were obtained. Sequencing analysis of clone's revealed 100% match with the ply and lytA genes of S. pneumoniae. Expression of proteins was maximum at 16 hours of IPTG induction. Higher quantity of ply and lytA proteins were found in 500mM imidazole elution with >90% purity. Methods employed in prior studies for the production of ply and lytA proteins were laborious, time consuming and contributes poor yield and purity. Our strategy offers efficient and economical approach for the production of pure recombinant ply & lytA proteins.***

Keywords: *Streptococcus pneumoniae*, Pneumolysin, Autolysin, Recombinant proteins, Ni^{2+}-NTA affinity chromatography

1. Introduction

Streptococcus pneumoniae is one of the most extensively studied microorganism. Mortality due to pneumococcal pneumonia is high, regardless of appropriate antibiotic therapy. The rates of complication may intensify with increasing resistance of *S.pneumoniae* to commonly used antibiotics. This aspect has led to rekindling of interest in establishment of etiology of the disease in clinical settings [1]. *S. pneumoniae* expresses a wide variety of surface proteins to interact with host cell components during the colonization or dissemination stages of the bacterium. They are also involved in the pathogenesis of the disease both as mediators of inflammation and as a direct part on host tissue attack [2]. Several pneumococcal proteins like pneumococcal toxin pneumolysin (*ply*), autolysin (*lytA*/CBP), pneumococcal surface adhesin A (*PsaA*) and pneumococcal surface protein A (*PspA*) have been investigated in the past two decades as an alternative to the expensive conjugate vaccines, as an antigen in potential protein-based vaccines or as carriers for polysaccharides aiming to broaden serotype coverage [3].

Ply is a 471 amino acids monomeric protein with a molecular mass of 53 KDa, folded in four globular domains. This protein shows a highly conserved tryptophan rich 11 residues sequence in one of the domains. As suggested by earlier studies [4] this region is responsible for anchoring at host cell membrane and forming a transmembrane pore complex. The pores upset the delicate osmotic balance between the cell and its environment, allowing material to leak in and out freely, quickly leading to lysis of the target cell [5]. In high concentrations, this enzyme is toxic to ciliated bronchial epithelial cells, reducing the ciliary movement, destroying the integrity of joints and cellular bronchial epithelial monolayer, facilitating the spread of pneumococcal infection.

S. pneumoniae has a characteristic autolytic response that is induced during the stationary growth phase and leads to the excessive lysis of cultures *invitro*. The major autolysin of *S. pneumoniae* is *N*-acetylmuramoyl-L-alanine amidase (*lytA*) and is considered as a main virulence factor [6]. The translated product of the *lytA* gene is a low-activity form (E-form) that is converted to the fully active form (C-form) with choline at low temperature [7]. The N-terminal moiety of *lytA* contains the active center of the enzyme, whereas the C-terminal part is composed of seven choline-binding repeats, responsible for recognition of and attachment to the choline residues of the pneumococcal cell wall teichoic acid. The *lytA* amidase is also responsible for the characteristic, clinically relevant lysis-prone phenotype exhibited by pneumococcal isolates in the presence of deoxycholate [8]. However, several authors have reported the isolation of pneumococcal strains that does not lyse with deoxycholate but still harbor a *lytA* gene [9].

The detection of pneumolysin, C-polysaccharide or mixed capsular polysaccharide antibodies trapped in immune complexes has been evaluated as a diagnostic tool for pneumococcal pneumonia in Kenyan adults, but its sensitivity and specificity were insufficient [10]. Although serology is not affected by prior antibiotic therapy, its use is limited to epidemiological studies, and has drawbacks with the detection of antibodies induced by carriage and the difficulty in obtaining acute and convalescent sera from the patient.

The methodologies employed in the previous studies for the production of *ply* and *lytA* were laborious, time consuming and involved various techniques such as ultracentrifugation, construction of cosmid gene bank and cell fractionation with poor yield. To further investigate the properties of the *ply* and *lytA* antigens and use them as diagnostic markers in

point of care test (POCT) while utilizing lateral immunochromatographic platform, we cloned and expressed *ply* and *lytA* genes in *Escherichia coli (E.coli).* The recombinant *ply* and *lytA* proteins were purified as a Histidine tagged fusion protein by a simple and efficient affinity chromatography method.

2. Materials and Methods

Bacterial strains and plasmids
Standard strain of *S. pneumoniae* (ATCC 49619), obtained from American type culture collection; Rockville, Md.pUC29 and pET-29a (Novagen, Cambridge) were used as cloning and expression vectors respectively. *E.coli* DH5α (Life Technologies, Waltham, MA USA) and BL21.de3 (Invitrogen, Waltham, MA USA) were used as hosts for recombinant plasmids.

DNA extraction
DNA extraction from *S. pneumoniae* ATCC 49619 strain was done using QIAamp DNA Mini Kit (*Qiagen, Hilden, Germany*). *S. pneumoniae* was grown on 5% sheep blood agar and a loopful of the overnight growth was resuspended in 180µl of Qiagen's suspension buffer followed by lysis buffer, the remaining procedure followed the manufacturer's protocol.

PCR amplification of *ply* and *lytA* genes
Gene specific primers with sites for restriction enzymes *XhoI* and *NdeI* were designed using primer-3 software for the amplification of *ply* and *lytA* genes from *S. pneumoniae* (Table. 1). Primers were synthesized from BioServe India (Hyderabad, India). Thermocycler from Applied Biosystems was used to perform PCR. PCR reaction mix contained 50ng of template, 100ng/µl of each forward and reverse primers, 10mM dNTPs, 10X ChromTaq assay buffer, 3U/µl of ChromTaq enzyme (Chromous Biotech Pvt. Ltd, India) and 36.5 µl of PCR-grade H_2O was added to adjust the final reaction volume to 50µl.Thermocycling conditions were optimized to one cycle of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, extension at 72°C for 1.30 min and one cycle of final extension at 72°C for 15 min. PCRamplicons were analyzed on 1% agarose gel.

Table 1: Primer Sequences for PCR amplification of ply and lytA genes

Primer	Sequence	Product size (bp)	GenBank accession no
ply Forward *ply* Reverse	5′ –GACTATCATATGGCAAATAAAGCAGTAAATGACTTTATAC– 3′ (NdeI) 5′–GTACAACTCGAGGTCATTTTCTACCTTATCTTCTACCTGAGG– 3′ (XhoI)	1413	AE005672.3
lytA Forward *lytA* Reverse	5′ –GACTATCATATGGAAATTAATGTGAGTAAATTAAGAACAG– 3′ (NdeI) 5′ –GTACAACTCGAGTTTTACTGTAATCAAGCCATCTGGCTCTA– 3′ (XhoI)	954	AE005672.3

Molecular cloning of the *ply and lytA* genes.
PCR products were gel purified by using gel purification kit (Chromous Biotech Pvt. Ltd, Bangalore) as per the manufacturer's instructions. Gel purified *ply*, *lytA* PCR products were cloned into pUC29 cloning vector at *NdeI* and *XhoI* restriction sites and transformed into *E.coli* DH5α as per the standard protocol [11].

Sequence analysis
Positive *ply* and *lytA* clones were sequence confirmed by the dideoxy chain termination method with an automated ABI Prism 3500XL DNA sequencer (Applied Biosystems, California, US). Sequence analysis was performed by NCBI nucleotide blast software and Clustal W.

Sub-Cloning of Sequence Confirmed *lytA* & *ply* genes in pET28a
The *lytA* and *ply* genes were released from sequence confirmed pUC29 clones and sub cloned into pET28a expression vector at *NdeI/ XhoI* restriction sites and transformed into DH5α competent cells [11].

Expression and purification of *rply* and *rlytA* genes
Positive pET28a/*ply* and pET28a/*lytA* clones were then transformed in BL21.de3 expression host and screened for protein expression. For small batch expression testing, single colonies were screened for checking gene expression in a small batch (5ml). The culture was grown to an OD of 0.6 at 600nm and induced with 1mM IPTG. Before induction, 4 h after induction & 16 h (overnight) induction samples were processed for protein extraction and loaded on 12% SDS-PAGE.

Big batch protein expression and purification was performed on the basis of small batch culture results. Single colony was inoculated for big batch expression & protein purification. The culture was grown till 0.6 OD and induced with 1mM IPTG at 37°C for overnight. Before and after induction samples were analyzed on SDS-PAGE (12%). The culture was pelleted down and suspended in 1X PBS. The suspended pellet was subjected to sonication and centrifuged at 10,000 rpm for 15 min at 4°C. Sonicated supernatant and pellet were loaded on 12% SDS-PAGE for testing. The supernatant containing protein of interest was further processed for protein purification using Ni^{2+}-NTA (nickel nitrile tri-acetic acid) based affinity column purification (Qiagen, Hilden, Germany) with Imidazole gradient elution (250mM, 500mM, 500mM, 750mM and 1M Imidazole). Purified fractions from the Ni^{2+}-NTA column were loaded on 12% SDS-PAGE and analyzed.

3. Results

In this study, PCR using *ply* and *lytA* gene specific primers resulted in amplicons of size ~1413 bp and ~954 bp respectively, which were analyzed on 1% agarose gel (Fig. 1). Cloning and ligation of the gel purified *ply* and *lytA* gene inserts into the cloning vector (pUC29) was successfully achieved by digesting both the PCR products of *ply* and *lytA* gene and the pUC29 vector with *NdeI* and *XhoI* restriction enzymes. The transformation of the DH5α competent cells with clones/ligation mix consisting of *ply* and *lytA* gene inserts resulted in characteristic white colonies on AXI plates during blue and white screening.

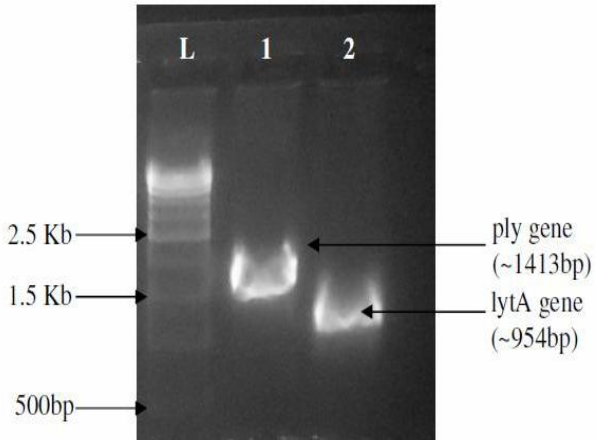

Figure 1: PCR amplified *ply* and *lytA* amplicons loaded on 1% agarose gel
Lane description
L – 500bp DNA Ladder
1 – PCR amplicon of *ply* gene (1413bp)
2 – PCR amplicon of *lytA* gene (954bp)

The plasmids isolated from the white colonies on AXI plates analogous to the pUC29-*ply* & pUC29-*lytA* clones, released bands of 1413bp, 954bp and 2.5Kb corresponding to *ply*, *lytA* gene inserts and vector backbone respectively upon digestion with *NdeI* and *XhoI* restriction enzymes (Fig. 2). The sequencing data analysis of the positive pUC29-*ply* & pUC29-*lytA* clones revealed 100% match with the *ply* and *lytA* genes of *S. pneumoniae.*

Sequence confirmed pUC29-*ply*, pUC29-*lytA* clones and expression vector pET28a which were digested at *NdeI*/*XhoI* restriction sites resulted in the release of the *ply* & *lytA* genes and the digestion of the pET28a for cloning. The released genes were then sub cloned into pET28a expression vector at *NdeI*/*XhoI* sites and the ligation mixture was inoculated onto the kanamycin containing media. Screening of twenty-four colonies from each kanamycin plate for probable pET28a-*ply* & pET28a-*lytA* clones by isolating the plasmids followed by digestion resultedin *ply*, *lytA* gene specific bands and a vector backbone of 2kb in size (Fig. 3).

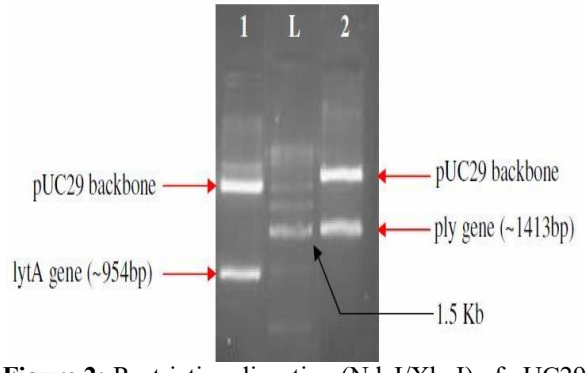

Figure 2: Restriction digestion (NdeI/XhoI) of pUC29 clones.
Lane description
1 – Digested clone (pUC29-*lytA*)
2 – Digested clone (pUC29-*ply*)
L – 500bp DNA Ladder

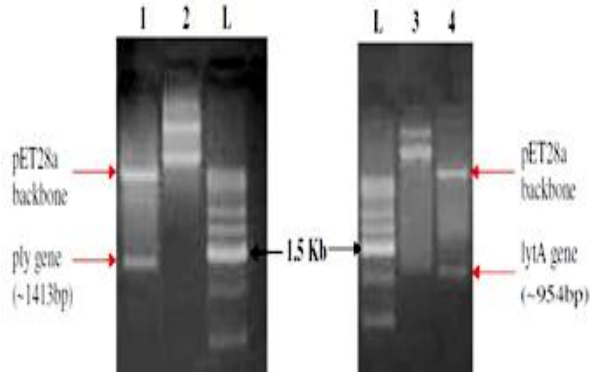

Figure 3: Restriction digestion (NdeI/XhoI) of pET28a clones.
Lane description
1 – Digested clone (pET28a -*ply*)
2 – Undigested clone (pET28a -*ply*)
3 – Digested clone (pET28a -*lytA*)
4 – Undigested clone (pET28a -*lytA*)
L – 500bp DNA Ladder

Positive pET28a clones were transformed into BL21.de3 expression host and screened for protein expression before induction, 4 h after induction & 16 h (Overnight) induction of the whole cell extract. Maximum expression for both *ply* and *lytA* genes was seen in 16h induced sample followed by 4h.

From the results of small batch culture, further protein purification experiments were performed from 16 h (overnight) induced culture. SDS-PAGE analysis of sonicated supernatant and sonicated pellet of both *ply* and *lytA* induced cultures showed the presence of the protein of interest in supernatant (Fig. 4).

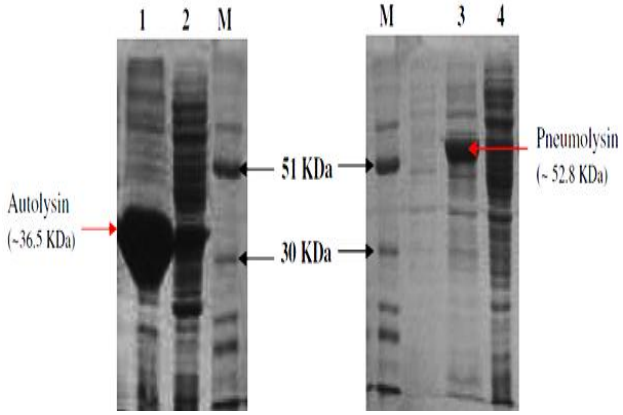

Figure 4: 1X PBS sonicated supernatant and pellet loaded on 12% SDS PAGE
Lane description
1 – Sonicated supernatant of *lytA* induced culture
2 – Sonicated pellet of *lytA* induced culture
3 – Sonicated supernatant of *ply* induced culture
4 – Sonicated pellet of *ply* induced culture
M – Protein Marker

5mg of highly purified *rply* and *rlytA* proteins were obtained using Ni^{2+}-NTA based affinity column purification with Imidazole gradient elution. Analysis of 12% SDS-PAGE revealed that maximum quantity of *rply* and *rlytA* were found in 500mM Imidazole elution (Fig.5).

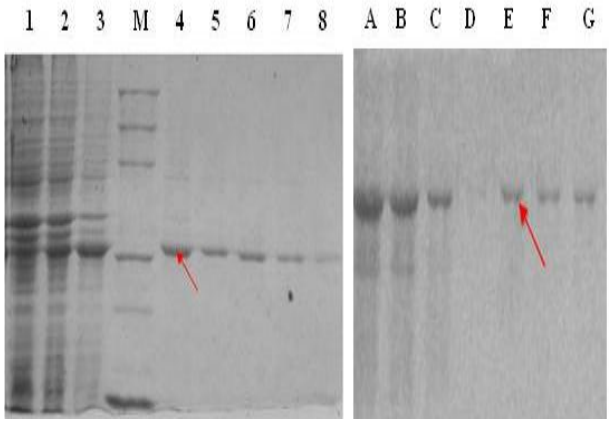

Figure 5: Ni^{2+}-NTA purified 1X PBS sonicated supernatant and pellet loaded on 12% SDS PAGE.

Lane description

1 – Sonicated supernatant of lytA
2 – Flow through
3 – Wash
4 – Elution – *lytA* (500mM Imidazole)
5 – Elution – *lytA* (500mM Imidazole)
6 – Elution – *lytA* (750mM Imidazole)
7 – Elution – *lytA* (750mM Imidazole)
8 – Elution – *lytA* (1M Imidazole)
M – Protein Marker

A – Sonicated supernatant of ply
B – Flow through
C – Wash
D – Elution – *ply* (250mM Imidazole)
E – Elution – *ply* (500mM Imidazole)
F – Elution – *ply* (750mM Imidazole)
G – Elution – *ply* (1M Imidazole)

4. Discussion

Streptococcus pneumoniae is an opportunistic pathogen and is accountable for a wide array of diseases together with pneumonia, septicemia and meningitis. Precise and prompt diagnosis of pneumococcal infections is very critical to initiate appropriate treatment [6]. Pneumococcus divides rapidly, but its culture is far too difficult and slow in growth, to be of any clinical use [12]. Hence, research for appropriate antigens for protein based vaccines and diagnostic markers that will allow accurate and rapid identification of *S. pneumoniae* is obligatory [10]. Hence in this study we made an attempt to synthesize r*ply* and r*lytA* proteins in prokaryotic system which were easily purified using 6x*Histidine*-tag/Ni^{2+}-NTA system.

Gene cloning has enabled numerous insights into gene structure, function and regulation utilizing large number of host organisms and molecular cloning vectors [13]. In this study gene recombination experiments were performed in *E. coli (DH5α)* host cells using pUC29 plasmid vector as these are technically sophisticated, versatile, widely available, and offer rapid growth of recombinant organisms with minimal equipment making the whole process economical [14].

All the plasmids transformed into host cells may not contain the desired gene insert hence; we used conventional Blue-white screening method for screening and selection of the positive clones. This method allowed identification of successful products of cloning reactions through the color of the bacterialcolony. The plasmid pUC29 carries multiple cloning sites (MCS) within the *lacZα* sequence which can be cut by restriction enzymes so that the foreign DNA (in this study *ply* and *lytA* gene inserts) is inserted within the *lacZ*α gene, thereby disrupting the gene and inhibiting α complementation resulting in nonfunctional β-galactosidase. The presence of an inactive β-galactosidase cannot be detected by X-galand results in white colonies which otherwise should produce characteristic blue color colonies [14,15].

We utilized pET system (pET28a vector) for *rply* and *rlytA* protein expression. pET28a vector is an optimum choice as it offers highest expression levels, tightest control over basal and induced expression. Choice of N-terminal (6x *Histidine*-tag) and C-terminal fusion tags for detection, purification and localization makes the system versatile. The added advantage is the presence of expanded multiple cloning sites in all three reading frame [16]. This vector also has kanamycin resistance gene for the selection of the positive clones. Hence, screening was performed by allowing the transformed cells to grow on kanamycin plates.

E.coli competent cells (BL21.de3 expression host) transformed with positive pET28a clones were screened for protein expression [17]. BL21.de3 cells are one of the most commonly used systems for producing recombinant proteins [18]. We utilized IPTG as an inducer of Lac operon in BL21.de3 expression host instead of lactose. IPTG is a structural mimic of lactose that binds to the Lac repressor and induces a conformational change that greatly reduces its affinity. Unlike lactose, IPTG is not part of any metabolic pathways, so it is not broken down or used by the cell. This ensures that the concentration of IPTG added remains constant, making it a more useful inducer of the Lac operon than lactose [19]. Our findings showed maximum protein expression for both *ply* and *lytA* genes in 16 h after induction samples and was in contrary with the previous studies that reported lower productivity with induction times longer than 6 h [17].

When the recombinant protein is expressed intracellularly in host cells, it is necessary to achieve high cell growth in order to yield high amounts of that protein. Thus, higher the cell

growth, the more the recombinant protein is synthesized [20]. As a consequence, different operational strategies are developed to increase cell growth. The characteristics of recombinant protein production, the molecule location, and protein destination determine its purification process. However, it is also known that protein may be expressed in a soluble or insoluble form into inclusion bodies. Several works in the literature consider the formation of these inclusion bodies as a drawback of the expression system in *E. coli*, since they require further addition of a complex process of isolation and purification, in which proteins are denatured and refolded in vitro. Therefore, the recovery of protein may be very low, which adds to the losses in all subsequent stages of purification [21]. Thus, a major challenge in recombinant expression is to design strategies for soluble production.

In the present study, purification of r*ply* and r*lytA* proteins were achieved using Ni^{2+}-NTA based affinity column purification with Imidazole gradient elution, where 6xHis tag in the recombinant proteins was obtained from the pET28a expression vector. Ni^{2+}-NTA chromatography is based on the interaction between a transition Ni^{2+} ion immobilized on a matrix and the histidine side chains [22,23]. The extra chelating site prevents nickel-ion leaching, thus providing greater binding capacity and high-purity protein preparations. Under denaturing conditions, this system is the ideal choice since the 6x*Histidine*-tag/Ni^{2+}-NTA interaction tolerates high concentrations of urea and guanidine. Under physiological conditions, host proteins with histidine stretches or host proteins containing metal ions may contaminate the protein preparation. Working with appropriate concentration of Imidazole can reduce this problem [23].

In the past, purification methods for *ply* and *lytA* have used preparative electrophoresis as last step [24,25]. We did not use this method for protein purification, as it results in poor yield. The molecular weight of *rply* (53 KDa) and *rlytA* (36.5 KDa) observed in our study was similar to that observed by Paton and coworkers [25]. Other studies using gel filtration and sucrose density gradient centrifugation have reported these proteins with different molecular weights [26].

As per our knowledge this is the first attempt to produce *rply* and *rlytA* proteins with a 6x *Histidine*-tag using Ni^{2+}-NTA purification system. Hence, our preliminary results could be considered prominent. This method is dependable and economical which can be used to synthesize pure recombinant *ply* & *lytA* proteins with higher recovery. Availability of these proteins will permit a number of investigations and the use of these purified proteins as carrier in conjugate vaccines and as targets in point of care test.

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Author Profile

Feroze Ganaie is a Research Scholar in KIMS H & RC affiliated to RGUHS, Bangalore. Since 2012, he is working in the field of pneumococcal research. This article represents related work he accomplished during his Ph.D.