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Narrative Review: Use of Competent Stimulating Peptide in Gene Transfer Via Suicide Plasmid in *Streptococcus pneumoniae*

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Abstract

Natural competency for genetic transformation of *Streptococcus pneumoniae* causes the emergence of novel or non-vaccine preventable pneumococcal serotypes. This phenomenon has become a global concern as it can spread quickly in the population through inhalation and close contact. The colonisation of *S. pneumoniae* at the upper respiratory tract can either become commensal or pathogenic. Once the bacterium invades into the body system, it will secrete its toxin and virulence protein to facilitate the invasion. Besides, *S. pneumoniae* can undergo natural biological transformation via uptake of exogenous DNA by horizontal gene transfer for integration and recombination of the genome. *S. pneumoniae* natural transformation is aided by competence-stimulating peptide (CSP) that induces the competence of bacteria. Natural transformation cascade of *S. pneumoniae* via CSP is triggered in the presence of conserved 17-amino acids peptide which is regulated and encoded by *comC*, *comD* and *comE* operon, where *comC* is responsible in secreting precursor CSP. Nowadays, researchers transforming *S. pneumoniae* by inserting the mutated *S. pneumoniae* gene through a vector, suicide plasmid. Suicide plasmids such as pID701, pAUL-A and pVA891 can be transferred but cannot replicate in the bacteria. Homologous recombination process occurs once the mutated gene of suicide plasmid is integrated with wild-type *S. pneumoniae*. Previous studies had used the transformation of suicide plasmid into S. pneumoniae as it can integrate with host DNA at specific insert for gene transfer. But there is no evidence on the role of CSP in horizontal/gene replacement via suicide plasmid in *Streptococcus pneumoniae*. This narrative review's scope as per defined purpose statement is to relate and recommend the use of competent stimulating peptide in efficient horizontal gene transfer via suicide plasmids in *Streptococcus pneumoniae*.

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Introduction

Streptococcus pneumoniae (*S. pneumoniae*) comprises an extensive capsule and underneath of capsule is a cell wall that comprises of polysaccharides, choline-binding protein, teichoic acid and sortase processed protein [1,2]. The variability in the capsular region is the result of serotypes differentiation and identification of *S. pneumoniae* [3]. *S. pneumoniae* is a gram-positive diplococcus that grows in chains [2] and grouped as α-hemolytic viridans streptococci [1]. Pneumococcal identification can be done through Gram staining, optochin susceptibility test, catalase test and followed by bile solubility test for further confirmation [4,5]. Based on phylogenetic reconstruction of 16S-rRNA-sequence, *S. pneumoniae* is one of the streptococci Mitis group that has different structure of cell wall carbohydrate as well as sequence of competence pheromone [6]. Several members of Mitis group naturally produce specialized competence-stimulating peptide (CSP) and its cognate receptors, which are responsible for genetic transformation at population level to accomplish goals which are impossible by individual cells [7]. Competence was an early example of bacterial response organized by scattered peptide signals, termed quorum-sensing (QS) and found in both gram-positive and gram-negative bacteria [8,9]. The virulence of bacterium is associated with genetic competence, biofilm production and acid tolerance which are controlled by CSP based QS system [10]. Quorum sensing in *S. pneumoniae* is caused by reserved 17-aminoacid peptide pheromone, which is the product of *comC* gene and later processed by ABC transporter (*ComAB*). Regulatory system depends on two components, histidine kinase receptor (*ComD*) and its associated response regulator (*ComE*), responsible to recognize the concentration of CSP in medium and initiate the response [1,2].

Among strains of *S. pneumoniae*, six well-defined CSPs have been recognized due to variation in their sequence, but majority of strains secrete either one of the two pheromones: CSP1 and CSP2 [10]. Each distinct CSP variant or group of strains recognized as a pherotype and pherotype genetically carrying one of two variants are not able to respond in the presence of other pheromones because of its receptor specificity, i.e. strain carrying *comC1* (CSP1) is unable to respond to extracellular peptide (CSP2) product of *comC2* [13]. Competent bacteria bind double-stranded DNA available in environment and transfer these fragments within cytoplasm in single-stranded form. A foreign DNA fragment can integrate within bacterial chromosome and change genotype and phenotype of the cell [12]. Isolates sharing similar antibiotic resistance have similar pherotypes, e.g. penicillin-resistant isolates have CSP-1. In pneumococcal population, pherotype vary independently of serotype and has clonal property. More divergence in genetic material have more probability to show different pherotype [3]. Competent cells can lyse the non-competent pneumococci, through fratricide mechanism. In pneumococci, fratricide plays a key role in lateral transformation by lysing non-competent cells and providing naked DNA in environment [14,15]. In natural population, genetic exchange between Intra pherotype prevails than inter pherotype [3]. This review study aims to discuss the comparative efficiency of gene transformation in the presence and absence of CSP.

Methods

Data mining concerned to the literature of the present study was sorted out by using key words including suicide plasmid in *Streptococcus pneumoniae*, homologous recombination in *Streptococcus pneumoniae*, Competence-stimulating peptide in *Streptococcus pneumoniae*, pneumococcal disease in google scholar and PubMed database. Articles retrieved under key words limitation were narrow down based on the study's content. Where feasible, literature was limited to those articles which were recently issued in last decade, unless the essence was pivotal to flourish the article. We elected a total of 59 peer-reviewed articles, 55 original research articles and 4 review articles, and summarized into this conceptual and comprehensive review.

Discussion

S. pneumoniae invades the body system via inhalation of small water droplet and colonize at the upper respiratory tract [1]. The bacterium can be either non-invasive or invasive [16]. Non-invasive bacteria reside at non-sterile parts inclusive of ears, otitis media, bronchitis and sinusitis. However, once be invasive and pathogenic, it can invade sterile parts including blood, cerebrospinal fluid, synovial fluids, pleural fluid and pericardial fluid causing invasive pneumococcal disease (IPD) [16,17].

Based on phylogenetic reconstruction of 16S-rRNA-sequence, *S. pneumoniae* is one of the streptococci Mitis group that has different structure of cell wall carbohydrate as well as sequence of competence pheromone [6]. *S. pneumoniae* had undergone many evolutionary lineages resulting in cluster separation with *S. oralis* and *S. infantis* [6]. Specifically, this bacterium consists of many serotypes under few serogroups [18]. The previous study stated the isolation and identification of 93 pneumococcal serotypes among 46 serogroups. However, only 10 serogroups were only related to pneumococcal disease [18].

Pneumococcal disease, public concern has been classified to be an airborne disease and to be spread by direct contact. Children and elderly are more prone to get infected by this bacterium, since, they are immunocompromised. In immunocompetent person, *S. pneumoniae* colonise asymptomatically at upper respiratory tract [17]. However, the inflammatory response is activated when the virulent genes of cell wall such as hyaluronidase [1], pneumococcal surface protein A [19], pneumococcal surface protein C [20] and α-enolase adhere to the epithelial cells of the upper respiratory tract for progression of disease [21]. Eventually, the inflammatory response becomes intense leading to the development of pneumococcal pathogenesis [1].

Antibiotics are used as a treatment of ailments to treat pneumococcal infection. In 1970s all pneumococcal isolates were susceptible towards antibiotics prescribed such as penicillin, clindamycin, rifampin, trimethoprim-sulfamethoxazole, macrolides, cephalosporin and vancomycin [22]. However, in 1990s most of pneumococcal clinical isolate in the United States showed resistance towards the antibiotics. *S. pneumoniae* had to go through genetic diversity by altering penicillin-binding protein receptor on its cell wall [6]. Due to inefficiency of treatment, another strategy had been done to improve public health status and prevention of *S. pneumoniae* is by implementing vaccination.

Vaccination is a preventive measure to counter pneumococcal disease among population. This prevention strategy was considered successful as the vaccination can target 75% of pneumococcal disease in children and after one year, the rate of infected children is reduced to more than half. However, in years later, the vaccination fails to overcome the disease due to emergence of non-vaccine type pneumococcal strains [18,23].

S. pneumoniae can evolve into different types of strains by genetic recombination. This highly recombinogenic bacterium will possess natural transformation mediated by horizontal gene transfer [24], resulting in high genetic diversity of *S. pneumoniae* that is difficult to be covered by monovalent vaccine [25]. Nowadays, scientists aim to construct new vaccines such as life attenuated vaccine that can yield wide coverage of *S. pneumoniae* serotypes.

S. pneumoniae natural transformation discovered by Griffth in 1928 proved to be a crucial finding in research area [26]. This transformable bacterium can take up naked extracellular DNA from the environment and integrating it with its own genome [26]. *S. pneumoniae* takes up double-stranded linear DNA by using different methods, including direct transformation of a DNA based product [27], direct transformation of plasmid DNA [28] and conjugation [29]. These modes of transfers can be with or without exogenous CSP. Natural transformation is one of quorum sensing system of *S. pneumoniae* that plays a role in communication and interaction of bacteria to manage and regulate gene expression as per environmental changes and signals [30].

The transformation cascade is triggered by the presence of CSP which constitutes of 17-amino acids to induce the competency of bacteria [31]. Competence regulation of *S. pneumoniae* (figure 1) is regulated by its core protein encoded by *comC*, *comD* and *comE* operon, where *comC* is responsible in secreting precursor CSP [32,33]. The maturation and exportation of CSP outside cell are in charge by ATP-binding cassette (ABC) transporter, membrane-associated peptide permease, *comA* and accessory gene; *comB* [34-36]. CSP binding with histidine kinase receptor, *comD* autophosphorylation [37] which concurrently phosphorylates *comE* [38]. Transcriptional regulator, *comE* transcribed 24 early phase genes as well as *comX* and the activated ComX factor regulates about 81 late genes which consist of coding effector for DNA uptake and recombination [39]. Competence induction for genetic transformation of *S. pneumoniae* consists of pherotypes which are CSP1 and CSP2 [41]. These two variants have similar primary structures but they are highly specific to their receptors, *comD1* and *comD2*. Therefore the specificity of phenotype depends on the type of pneumococcal strain [3]. In contrast with natural regulation of CSP, extracellular CSP is exogenously targets membrane-embedded histidine kinase receptor *comD* before initiates the downstream cascade events to achieve competence state [41]. However, when *S. pneumoniae* is induced to competent, fratricide factor encoded late genes; CbpD and CibAB are transcribed to upregulate LytA synthesis in the non-competent cell [26]. The stimulation of LytA, a murein hydrolase triggers pneumococcal autolysin that releases intracellular materials including chromosomal DNA [42]. The DNA uptake and release are in maximum level showing that the processes are simultaneously occurring [42,43]. The presence of exogenous CSP indicates that competent cell takes up extracellular DNA by killing neighbouring non-competent cell results in cell population reduction [43].

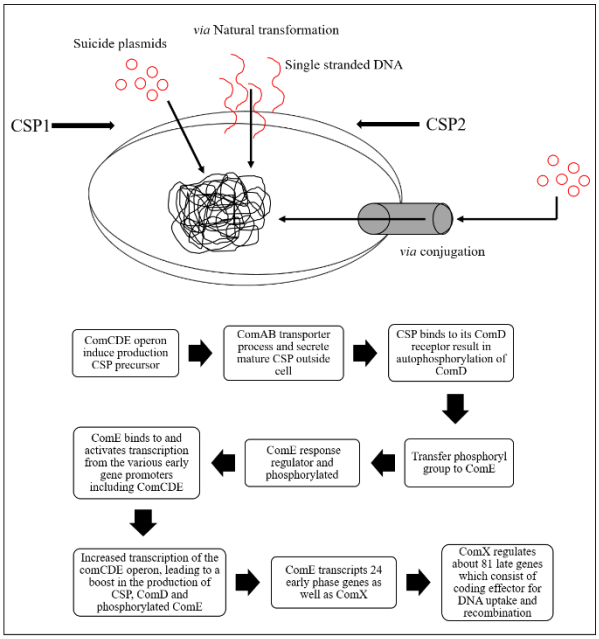

Figure 1: Competency and regulation of DNA uptake in *Streptococcus pneumoniae*. Conceptually driven and adapted from [8,12,39,40].

Process of DNA uptake occurs in two parts which are at outer membrane binding site and admission of DNA into the cell without being affected by DNase [44]. The binding of donor DNA to outer membrane of *S. pneumoniae* result in single-strand breaks before moving into the cell. Upon entry, one strand of DNA is degraded to oligonucleotides and another strand is introduced into the cell in single-stranded form [44]. The previous study stated that the incoming donor DNA strands move into

No	Title of Citation	*S. pneumoniae* strain	Cloning Vector	Suicide plasmid	*Ori*	Methodology Used	Reference
1	*phgABC*, a Three-Gene Operon Required for Growth of *Streptococcus pneumoniae* in Hyperosmotic Medium and In Vivo	Capsular serotype 3, a clinical isolate	Plasmid DNA from *E. coli*	pID701-Disruption vector for *S. pneumoniae* derived from pEVP3; Cm^r	ColE1	a. RNA samples b. RT-PCR (Amplify internal portion of genes) c. Transform into E. coli strain DH5α d. Subcloned into suicide plasmid pID701 e. Plasmid insert identities were confirmed by DNA sequencing f. Transformation of S. pneumonia g. Confirm mutants by PCR. h. Tested retain Cmr in Todd Hewit broth with 5% yeast extract (THY) without antibiotic selection, with 100% of the 100 colonies	[28]
2	A functional Genomic analysis of type 3 *Streptococcus Pneumoniae* virulence	Serotype 3	Plasmid DNA from *E. coli*	pID701	ColE1	a. Ligation of chromosomal into SmaI (S) site of pID70, tagged with variable nucleotide sequence tags into KpnI (K) site b. Transform into E. Coli DH5α c. Amplified by growing bacteria in Luria Bertani d. Purify plasmid e. Transform into S. pneumoniae f. Transformants stored into Todd-Hewitt broth contain 10% glycerol	[53]
3	Characterization of Pit, a *Streptococcus pneumoniae* Iron Uptake ABC Transporter	Capsular serotype 3, a clinical isolate	Plasmid DNA from *E. coli*	pID701-Disruption vector for *S. pneumoniae* derived from pEVP3; Amp^r Cm^r	ColE1	a. RNA samples b. RT-PCR (Amplify internal portion of genes) c. Transform in the *E. coli* strain DH5α d. Subcloned into pID701 e. Plasmid insert identities were confirmed by DNA sequencing f. Transform into *S. pneumonia* g. Mutant confirmed by PCR. h. Tested retain Cm^r in Todd Hewit broth with 5% yeast extract (THY) without antibiotic selection, with 100% of the 100 colonies	[54]
4	Visualizing Pneumococcal Infections in the Lungs of Live Mice Using Bioluminescent *Streptococcus pneumoniae* Transformed with a Novel Gram-Positive *lux* Transposon	*S. pneumoniae* strain D39 and clinical isolates of *S. pneumoniae* A66.1	Plasmid from *E. coli* DH5α	pAUL-A	ColE1	a. Cut Tn*4001 luxABCDE* Kmr cassette from pMGC57 using the enzymes *Eco*RI and *Xho*I and ligated with the corresponding *Eco*RI and *Sal*I sites of pAUL-A. b. Ligation and electroporated into *E. coli* DH5a and spread onto LB agar containing erythromycin c. Screened for bioluminescence using an ICCD camera d. Isolate plasmid DNAs e. Transform into *S. pneumoniae* strain D39	[55]
5	A Novel Metallo-β-Lactamase Involved in the Ampicillin Resistance of *Streptococcus pneumoniae* ATCC 49136 Strain	*S. pneumoniae* ATCC 49136	pUC18 vector	pVA891	p15A	a. Insertion mutagenesis of MBL gene b. Coding region of the MBL gene inserted at the EcoRI restriction site of pVA891 c. Transformed into *S. pneumoniae*. d. Screened mutants from the erythromycin-containing agar plate	[56]
6	Streptococcal integration vector for gene inactivation and cloning	*S. pneumoniae* *S. mutans* *S. gordonii* *S. pyogenes*	Plasmid from *E. coli*	pEVP3 pSF151 pSF152 pSF143	ColE1 CMV CMV CMV	a. Isolate genomic DNA from streptococci b. Use insertion and duplication method in the plasmid to inactivate the gene c. Transform into *E. coli* d. Purify plasmid e. Transform into streptococcal strain f. Incubate in Todd Hewit broth agar plate contain antibiotic g. Confirm plasmid insertion by Southern hybridization	[57]
7	Serotype competence and penicillin resistance in *Streptococcus pneumoniae*	Clinical isolate (6B, 14, 19F, 9V, 23F, 3, 18C)	Plasmid from *E. coli* DH5α	pDL278	pBR322	a. Culture early log phase in Todd Hewit Yeast, pH6.8, 1mmol/L $Cacl_2$, and 0.2% bovine serum albumin b. Add 100ng/ml CSP-1 or CSP-2 in 10mmol/L glucose and 10% horse serum c. Incubate for 15minutes in 37°C d. Add pDL278 e. Incubate samples one hour at 35°C in 5% CO_2 f. Spread on blood agar plate containing 500 µg/ml spectinomycin	[40]

Table 1: List of suicide plasmids

the cells in a 3'to 5' direction [45]. Throughout the entry, the single-stranded DNA is coated with protein secreted by competent cells [46] and concurrently integrate with recipient chromosome complementary strand [47]. However, the transforming action cannot be performed due to lacking DNA processing factors such as *comC* encoded in production of competence stimulating peptide (CSP) and *comD* encoded for CSP receptor to phosphorylate *comE* [32,48].

Therefore, another approach had been used by cloning the gene of *S. pneumoniae* is into plasmid before introduced into the bacteria for gene recombination via transformation [29]. Homologous recombination will take place when the nucleotide sequences of two similar molecules of DNA are exchanged [49]. Throughout this process, Holliday junction takes place in which two double-stranded of DNA molecules are separated into four strands and covalently linked to exchange part of genetic material and to make the genetic crossing over more specific, researchers are using suicide plasmid or shuttle plasmid [50,51]. Besides able to execute multiple mutations, the suicide plasmid can integrate with DNA by homologous recombination inside the chromosome without damages [51]. Suicide plasmid moves into the competent cell and integrates with target sequence by homologous recombination before eliminating itself through second cross-over event result in allelic exchange [51,52].

Transformation of suicide plasmid into *S. pneumoniae* through exogenous CSP had been reported [51] but the comparative transformant efficiency between with and without CSP induction have not been done yet.

Therefore, the transformant efficiency of *S. pneumoniae* is low with positive selection of using competence-stimulating peptide due to autolysis as compare to the use of suicide plasmid for gene replacement in *S. pneumonia* [26,51]. Besides, there is a study that previously had used conjugation method by transferring plasmid pIP501 consist of DNAase resistant into *S. pneumoniae* through nitrocellulose filter [29]. The process is also known as filter mating that resembles conjugation mechanism. Along the process, pIP501 showed capability to mobilize a non-transmittable plasmid, pMV158 into clinical isolate group B streptococcus [58]. As stated (Table 1), some researchers used suicide plasmids that contain mutated gene of interest for replacement, to be transformed into *S. pneumoniae* such as pID701 [28,54], pAUL-A [55], pVA891 [56], pEVP3 [57], pSF151, pSF152, pSF143, pSF151 [57], pSF152 [57], pSF143 [57] and pDL278 [40].

Conclusion and Recommendations

1. There is a need to study the comparative transformant efficiency between; with 2nd method. CSP indication in different serotypes of S. pneumonia.
2. Transformation of *S. pneumoniae* by using suicide plasmid as a vector is an alternative to genetic recombination. Besides using the proper protocol, choosing suitable types of plasmid for *S. pneumoniae* transformation is necessary as it is a key for successful experiment.
3. Transforming bacteria as mentioned in this study needs an alternative and more efficient method.

Furthermore, the mechanism of biological properties of S. pneumoniae competence membrane needs to be studied so that the suicide plasmid used for transformation is compatible with the bacteria used. It is undeniable that with the combination of comprehensive theory and protocol, genetic recombination of *S. pneumoniae* will be succeeded and be chosen as one of an alternative way to improve the prevention strategy against pneumococcal disease under the umbrella of CSP.

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Author Contributions:

N.A.S., M.H.: Data mining and write manuscript; A.A.B., M.A.K., M.K.Z: Study design; A.Z.L., M.U.K., N.B.S, A.A.B: review manuscript and supervised

Conflict of interest:

The authors declare that they have no conflict of interest.

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