In vivo characterization of *Streptococcus pneumoniae* genes involved in the pathogenesis of meningitis by differential fluorescence induction

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ABSTRACT

الأهداف: فهم أمراض التهاب السحايا للمكورات الرئوية، و إعطاء عناصر مرشحة لتطوير الأدوية.

الطريقة: أجريت الدراسة في مختبر المفتاح لتشخيص الطب – وزارة التعليم – جامعة شونجكين الطبية – شونجكين – الصين خلال الفترة من مارس 2006م حتى ديسمبر 2007م. تم نقل فخ مجموعة العقدية الرئوية TIGR4 بواسطة البروتين الأخضر المتألق الذي تم إنشائه و استخدامه لنقل العدوى لغاز BALB/c عدد=15 و وضع نموذج التهاب السحايا. لقحت مجموعة التحكم عدد=5 بمحلول الفوسفات المعقم. تم فحص البكتيريا التي تحتوي على معزز مندمج ناتج فقط في التهاب السحايا بأنسجة الدماغ و ليس في المختبر وذلك بواسطة تحريض البروتين المتألق المختلف. تم تجهيز البكتيريا المعطاة لإعادة نقل العدوى لما هو مشار أعلاه. انتشرت البكتيريا الموجودة في أغار صويا الترابيكاز، 5% صفائح دم أغار للجسم التي تحتوي على مضاد حيوي بمقدار (2.5µg/mL)، و يستخدم في استنتاخ DNA، و التسلسل، و تحليل شبكة المعلومات البيولوجية المتعلقة بالتكنولوجيا البيولوجية و التنوع البيولوجي.

النتائج: أخذ مقدار 52 جين. أظهرت تحليل شبكة المعلومات البيولوجية المتعلقة بالتكنولوجيا البيولوجية و التنوع البيولوجي أن الجينات الناتجة في المختبر تشترك في الوظائف مثل الالتصاق، استقلاب الطاقة، و نقل المادة الغذائية، انتظام النسخ، و استقلاب DNA، و تركيب جدار الخلية. بجانب ذلك، هناك جينات تماثل البروتينات الافتراضية بعوامل وهمية، أو غير معروفة.

خاتمة: تشترك بعض جينات المكورات الرئوية في التهاب السحايا الذي تم تعريفه في هذه الدراسة. هناك عناصر محتملة لفهم أمراض التهاب السحايا للمكورات الرئوية.

Objectives: To further understand the pathogenesis of pneumococcal meningitis, and provide some target candidates for the development of drugs.

Methods: This study was performed at the Department of Laboratory Medicine, Key Laboratory of Diagnostic Medicine (Ministry of Education), Chongqing Medical University, Chongqing, China from March 2006 to December 2007. A promoter-trap library of *Streptococcus pneumoniae* TIGR4, reported by green fluorescent protein was constructed, and used to infect BALB/c mice (n=15) intranasally, to set up a meningitis model. The control group (n=5) were inoculated with sterile phosphate buffered saline. The bacteria containing the promoter fusions induced only in meningitis brain tissue, not *in vitro* were screened by differential fluorescence induction. The obtained bacteria were prepared to re-infect the mice and re-screened, as above. The sorted bacteria were spread on trypticase soy agar with 5% sheep blood agar plates containing chloramphenicol (2.5 µg/mL), and were used for DNA cloning, sequencing, and bioinformatics analysis.

Results: A total of 52 genes were obtained. Bioinformatics analysis revealed that these *in vivo* induced genes were involved in functions such as, adherence, energy metabolism, nutrient substance transport, transcription regulation, DNA metabolism, as well as, cell wall synthesis. In addition, there were some genes encoding for some hypothetical proteins with unknown, or putative functions.

Conclusion: Pneumococcal genes involved in meningitis identified in this study are potential targets to understand the pathogenesis of pneumococcal meningitis.

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Streptococcus pneumoniae (*S. pneumoniae*) is still one of the most important human pathogens, which causes approximately 11% of all deaths in children younger than 5 years.[1] It can cause a number of invasive diseases including sepsis, meningitis, and otitis media. Among them, pneumococcal meningitis has relatively higher mortality and morbidity. It is generally recognized that pneumococcal meningitis progresses after bacterial colonization in the upper respiratory tract mucosa, pneumococci enter the bloodstream, and subsequently invade the central nervous system via the blood-brain barrier (BBB).[2] However, little is known on how many, or which factors are involved in the invasion of pneumococci to the brain. Choline-binding protein (Cbp) A and pneumolysin (Ply) are so far the 2 most impressive virulence factors contributing to the development of pneumococcal meningitis.[3,4] Hence, the main focus of the present study was to reveal more pneumococcal factors involved in the pathogenesis of pneumococcal meningitis, which may help to understand the pathogenic mechanism of pneumococcus, and provide some target candidates for the development of drugs and vaccines. Several strategies have been employed to screen *in vivo* induced genes including *in vivo* expression technology (IVET),[5] signature-tagged mutagenesis (STM),[6] differential fluorescence induction (DFI),[7,8] and DNA microarray.[9] Apart from DNA microarray, DFI is the most widely used screening technology, which has been successfully performed in a number of pathogens.[10,11] Additionally, the best advantage for DFI is its ability to allow temporal and spatial monitoring, even for a single cell, of pathogen gene expression in infected animals.[8] Pneumococcal genes involved in sepsis, pneumonia, and otitis media was identified by several methods mentioned above. Nevertheless, pneumococcal genes involved in meningitis have been identified only by DNA microarray, in which a pneumococcal meningitis model was induced by direct intracisternal injection with *S. pneumoniae* D39.[9] Its main drawbacks would be: 1) Intracisternal infection is different from the natural infection; 2) *S. pneumoniae* D39 is not a common strain causing meningitis because of its thick capsule.[12] In the present study, we used pneumococcal strain TIGR4[13] to set up a pneumococcal meningitis model via intranasal inoculation, which mimics the natural route of infection in humans, and then identify the virulence factors that contribute to pneumococcal meningitis by DFI.

Methods. This study was performed at the Department of Laboratory Medicine, Key Laboratory of Diagnostic Medicine (Ministry of Education), Chongqing Medical University, Chongqing, China, from March 2006 to December 2007.

First, a promoter trap plasmid vector, designated herein pEVP3-SDGFP, was constructed by inserting a fragment with *BamH* I ends. A translation initiation region (TIR) includes a translational enhancer (ENH) and a Shine-Dalgarno (SD) sequence. The fragment consisting of TIR and a promoterless gfpmut2 from plasmid pGreenTIR that was kindly provided by Lindow[14] was inserted into the *BamH* I site of the *Escherichia coli (E. coli)/S. pneumoniae* suicide vector

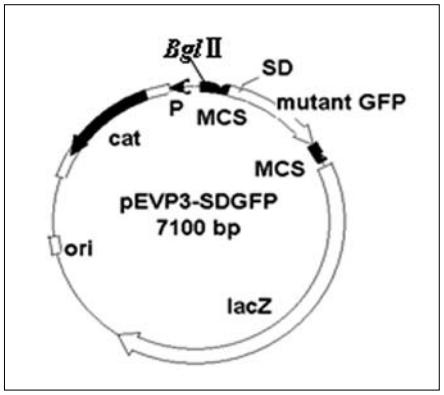

Figure 1 - Diagram of *Streptococcus pneumoniae* promoter trap plasmid vector pEVP3-SDGFP showing the locations of Shine-Dalgarno (SD), and promoterless gfp. *Bgl II* site in the mutiple cloning site is unique in the vector. GPF - green fluorescent protein, MCS - multiple cloning site, cat - chloramphenicol acetyl transferase gene, lacZ - beta-galactosidase gene, ori - origin of gene replication, P - promoter

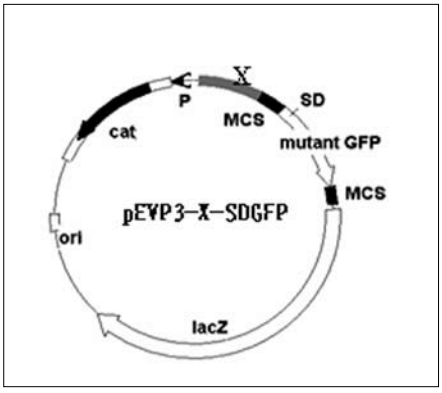

Figure 2 - Plasmid library including DNA fragments of *Streptococcus pneumoniae* (pEVP3-X-SDGFP). SD - Shine-Dalgarno, GPF - green fluorescent protein, MCS - multiple cloning site, cat - chloramphenicol acetyl transferase gene, lacZ - beta-galactosidase gene, ori - origin of gene replication, P - promoter

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pEVP3 to ensure the upstream promoters can be reported efficiently (**Figure 1**). Secondly, a promoter trap library was constructed. The chromosomal DNAs of *S. pneumoniae* TIGR4 (ATCC BAA-334) were partially digested with *Sau3A* I. Fragments varying in size from 200-800 bp were purified from agarose gels, and inserted upstream of the promoterless gfpmut2 gene in pEVP3-SDGFP at *Bgl* II site (*Sau3A* I and *Bgl* II have the same digesting ends). The ligation mixture was transformed into *E. coli* DH5α, and selected on Luria-Bertani broth (LB) agar supplemented with chloramphenicol (25 μ/mL). Colonies were collected manually, and plasmid DNAs were prepared, the resultant DNAs was termed the plasmid library (**Figure 2**). Plasmid DNAs were transformed into *S. pneumoniae* TIGR4 as described by Sung et al.[15] After 24 hours incubation at 37°C, the transformants were counted and collected.

Three-week-old female BALB/c mice, weighing 12-14 g were purchased from the Laboratory Animal Center of the Chongqing Medical University Chongqing, China. All experimental procedures were approved by the Chongqing Medical University Institutional Animal Ethics Committee. The mice were lightly anesthetized by 2% chloral hydrate (0.01ml/g mice) via intraperitoneal injection. The treatment group contains 15 mice. Each mouse was intranasally inoculated with 50 μl bacterial suspension of TIGR4 (approximately $3x10^7$ colony forming units [CFU]). The control group (5 mice) were inoculated with sterile phosphate buffer saline (PBS). The mice were then recovered from anesthesia, and were given *ad libitum* access to food and water. Generally, symptoms such as somnolence and arched back occurred 48 hours post infection, and mice were then sacrificed. The brains were removed, half of which were fixed in 10% buffered formalin for histopathologic analysis. The other half was washed with sterile PBS until achromic, homogenized in 5 ml PBS, and centrifuged at 1000 g for 5 minutes at 4°C. The supernatants were used for fluorescence activated cell sorting (FACS) by flow cytometry. The FACS was performed under sterilized conditions according to the instructions of FACS Calibur machine (Becton Dickinson ImmunoCytometry Systems, San Jose, CA, USA). The gate was adjusted to sort bacteria with fluorescence above the background, which was determined on the basis of wild *S. pneumoniae* TIGR4 (without insert sequence). The *S. pneumoniae* TIGR4 with recombinant pEVP3-ply-SDGFP plasmid served as positive control. At least 10,000 fluorescent events were collected from the supernatants of homogenized brain tissues. The sorted cells, which contain promoter fusions induced in brain tissues were cultured in casamino acids + yeast extract (C+Y) medium containing chloramphenicol (2.5 μg/mL) overnight, and cells with lower fluorescence intensity, which contain promoters repressed *in vitro* but active in brain tissues were collected using FACS. The obtained cells were prepared to re-infect the mice, and re-screened as described above. The sorted cells were then spread on trypticase soy agar (TSA) with 5% sheep blood agar plates containing chloramphenicol (2.5 μg/mL), and were used for sequencing. The fragments integrated into the pEVP3-SDGFP were herein termed as the X fragments, which were cloned as described by Meng et al.[16] Briefly, *BamH* I was used to digest chromosomal DNA, and the ensuing self-ligation was performed. The resulting product was transformed into *E. coli* DH5α. The recombinant suicide plasmids incorporating X fragments were selected from LB plates containing 25 μg/ml chloramphenicol, and further sequenced with primer P1 (Shanghai Sangon Biological Engineering Technology and Service Company Ltd., Shanghai, China).

The basic local alignment search tool (BLAST) programs[17] were used for homology analysis. The sequences were aligned with the complete genome of *S. pneumoniae* TIGR4 in the GeneBank (AE005672). Open reading frames (ORFs) were identified using the National Center for Biotechnology Information (NCBI) ORF finder,[18] and the promoter regions were analyzed by online promoter analysis software.[19]

Results. Most fragments resulted from the digestion of *S. pneumoniae* TIGR4 chromosomal DNA with *Sau3A* I varying from 200-800 bp in size (**Figure 3**). Approximately 58,000 colonies were generated, and plasmid DNAs were prepared. Considering the size (sequencing results shown the average size was 400 bp), and inserted orientation of DNA fragments, this library

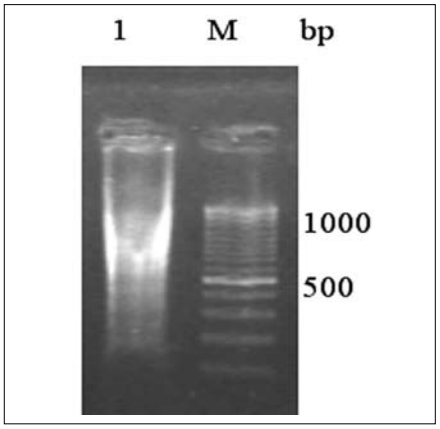

Figure 3 - *Sau3A*I digestion of TIGR4 genomic DNA. Lanes: 1 - TIGR4 genomic DNA digested by *Sau3A*I; M - molecular size marker, bp - base pair

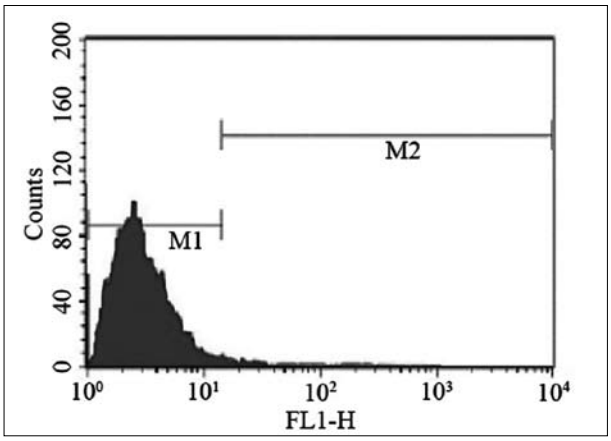

Figure 4 - Flow cytometry analysis of *Streptococcus pneumoniae* promoter-trap library in mice brain tissue. M1 - low fluorescence intensity bacterial population (according to the value of the control); M2 - high fluorescence intensity bacterial population *in vivo.* FL1-H - fluorescence intensity of channel 1-height

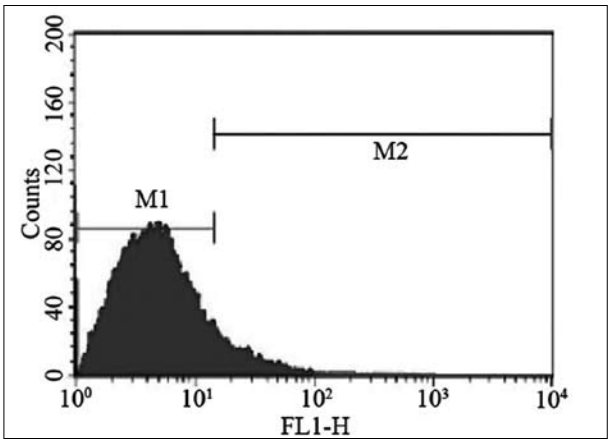

Figure 5 - Flow cytometry analysis of high fluorescence intensity bacterial after culturing in medium C+Y. M1 - low fluorescence intensity bacterial population (according to value of the control); M2 - high fluorescence intensity bacterial population *in vitro.* FL1-H - fluorescence intensity of channel 1-height

was estimated to represent 5.3-fold coverage of the 2.2 Mb *S. pneumoniae* genome (58,000 x 400 bp/2.2 Mb /2). The polymerase chain reaction (PCR) sequencing revealed that more than 90% (73 clones out of 80 random clones) had the insert segments of *S. pneumoniae*, and the library preserved suitable complexity. After the plasmid DNAs were transformed into *S. pneumoniae* TIGR4, a total of 500,000 recombinants (promoter trap library) were obtained from TSA (5% defibrinated sheep blood) containing 2.5 μg/ml chloramphenicol. Such a big trap library was estimated to ensure the diversity of the fragments. Bacteria were harvested from the dying mice, and subjected to FACS sorting. The cell portion with higher fluorescence intensity (**Figure** 4 M2) was collected, and cultured *in vitro*. The cultured cells were subjected to FACS sorting again, while at this time the part with lower fluorescence intensity (**Figure** 5 M1) was collected. Therefore, the obtained bacteria contained promoters induced *in vivo,* but not *in vitro*. A total of 220 colonies were collected, and genome DNAs were isolated and further sequenced. Twenty-four isolated DNA fragments were obtained by searching the GenBank,[17] and a total of 52 ORFs were found (**Table** 1).

Discussion. *S. pneumoniae* has more than 90 serotypes varied in pathogenesis, and has been shown to cause organ-specific infections on a serotype bias. Mice infected intranasally with A66.1 (serotype 3) developed only pneumonia, those challenged with D39 (serotype 2) experienced high-grade sepsis, while TIGR4 (serotype 4) infection resulted in low-grade pneumonia and bacteremia, ultimately progressing to meningitis.[12] The strain TIGR4 was used in this study, and a meningitis model via intranasal inoculation was set up successfully. We identified the virulence factors of *S. pneumoniae* that contribute to pneumococcal meningitis by using a model, which mimics the natural route of infection in humans.

In the present study, 52 genes were obtained, and most of them were different from those induced in brain tissues of a meningitis model via intracisternal infection.[9] This implies that the pathogenesis of *Pneumococcal meningitis* caused by different infection route is different. Some known virulence genes that relate to pneumococcal meningitis, such as Ply and CbpA were not identified. We may attribute this failure to the drawbacks of the method itself, which has to exclude genes expressed *in vitro*, as we know, Ply are commonly expressed in *in vitro* culture.[20] There may be some unknown virulence genes relating to pneumococcal meningitis expressed both *in vivo* and *in vitro*, but cannot be identified by this method.

Of these 52 genes we identified, as many as 18 genes were involved in the transport and metabolism of amino acid and carbohydrates, which implies the importance of more efficient energy used in bacterial survival at, or shortly thereafter invasion. It is commonly believed that bacterial density is also an important factor for virulence. In order to survive the host, bacteria themselves should accelerate DNA replication and repair. In this study, 4 genes responsible for DNA replication, recombination, and repair were obtained, including genes pheS, pheT, pnp, and gene rp1I. These genes were upregulated in pneumococcal meningitis, which is reminiscent of enhanced pneumococcal virulence of bacterium in the pathogenesis of meningitis. Operon psaBCA encodes ATP-binding protein, putative manganese ABC transporter permease, and manganese-binding adhesion

Table 1 - Open reading frames (ORF) analysis of *Streptococcus pneumoniae* (*S. pneumoniae)* genes involved in meningitis identified by differential fluorescence induction (DFI).

Strain	Gene in TIGR4	Gene name	ORF description
DFI009	*SP*_0293		Hypothetical protein (AAK74471)
DFI010	*SP*_0447	*ilvC*	Ketol-acid reductoisomerase (AAK74608)
DFI015	*SP*_0151		ABC transporter, ATP-binding protein (AAK74333)
DFI033	*SP*_0204		ORF1: acetyltransferase, GNAT family (AAK74384)
	*SP*_0205[a]	*nrdG*	ORF2: anaerobic ribonucleoside-triphosphate (AAK74385)
DFI039	*SP*_0750[a]	*livH*	ORF1: branched-chain amino acid ABC transporter (AAK74890)
	*SP*_0751[a]	*livM*	ORF2: branched-chain amino acid ABC transporter (AAK74890)
	*SP*_0752[b]	*livG*	ORF3: branched-chain amino acid ABC transporter (AAK74891)
	*SP*_0753[b]	*livF*	ORF4: branched-chain amino acid ABC transporter (AAK74892)
DFI062	*SP*_2176	*dltA*	ORF1: D-alanine-activating enzyme (AAK76230)
	*SP*_2175	*dltB*	ORF2: *dltB* protein (AAK76229)
	*SP*_2174	*dltC*	D-alanyl carrier protein (AAK76228)
	*SP*_2173	*dltD*	*dltD* protein (AAK76227)
DFI097	*SP*_2205		ORF1: DHH subfamily 1 protein (AAK76256)
	*SP*_2204	*rplI*	ORF2: 50S ribosomal protein L9 (AAK76255)
	*SP*_2203	*dnaC*	ORF3: replicative DNA helicase (AAK76254)
	*SP*_2202		ORF4: hypothetical protein (AAK76253)
	*SP*_2201[a]	*cbpD*	ORF5: choline-binding protein D (AAK76252)
DFI099	*SP*_0588	*pnp*	Polyribonucleotide nucleotidyltransferase (AAK74741)
DFI103	*SP*_0579	*pheS*	ORF1: phenylalanyl-tRNA synthetase alpha subunit (NP_345093)
	*SP*_0580		ORF2: acetyltransferase, GNAT family (AAK74734)
	*SP*_0581	*pheT*	ORF3: phenylalanyl-tRNA synthetase, beta subunit (AAK74735)
DFI106	*SP*_0287		ORF1: xanthine/uracil permease family protein (AAK74465)
	*SP*_0288		ORF2: hypothetical protein (AAK74466)
DFI121	*SP*_0979		ORF1: oligoendopeptidase F (AAK75100)
	*SP*_0980		ORF2: O-methyltransferase (AAK75101)
DFI122	*SP*_0321		ORF1: PTS system, sugar-specific IIA component (AAK74497)
	*SP*_0322		ORF2: glucuronyl hydrolase, putative (AAK74498)
	*SP*_0323		ORF3: PTS system, IIB component (AAK74499)
	*SP*_0324		ORF4: PTS system, IIC component (AAK74500)
	*SP*_0325		ORF5: PTS system, IID component (AAK74501)
	*SP*_0326	*yajC-1*	ORF6: preprotein translocase, YajC subunit (AAK74502)
	*SP*_0327		ORF7: hypothetical protein (AAK74503)
DFI125	*SP*_0641		Serine protease, subtilase family (AAK74791)
DFI127	*SP*_1151	*rexB*	ORF1: exonuclease RexB (AAK75260)
	*SP*_1152	*rexA*	ORF2: exonuclease RexA (NP_345621)
DFI135	*SP*_2109	*malC*	ORF1: maltodextrin ABC transporter, permease protein (AAK76168)
	*SP*_2110	*malD*	ORF2: maltodextrin ABC transporter, permease protein (AAK76169)
DFI151	*SP*_2146		Conserved hypothetical protein (AAK76203)
DFI160	*SP*_1341		ORF1: prolyl oligopeptidase family protein (AAK75441)
	*SP*_1342		ORF2: conserved hypothetical protein (AAK75442)
DFI165	*SP*_0915		Putative IS1239, transposase (AAK75039)
DFI167	*SP*_0117[a]	*pspA*	Pneumococcal surface protein A(AAK74303)
DFI178	*SP*_0330	*regR*	Sugar binding transcriptional regulator RegR (AAK74506)
DFI191	*SP*_1784		ORF1: hypothetical protein (AAK75857)
	*SP*_1783		ORF2: MutT/nudix family protein (AAK75856)
DFI195	*SP*_1175		Conserved domain protein (AAK75284)
DFI199	*SP*_0663		ORF1: conserved hypothetical protein (AK74808)
	*SP*_0664	*zmpB*	ORF2: zinc metalloprotease ZmpB (AAK74809)
DFI205	*SP*_1648	*psaB*	ORF1: ATP-binding protein (AAK75728)
	*SP*_1649	*psaC*	ORF2: putative manganese ABC transporter permease (ABC75780)
	*SP*_1650	*psaA*	ORF3: manganese ABC transporter (AAK75729)

[a]Genes also expressed in cerebrospinal fluid identified by DNA microarray.[9] *SP* - *Streptococcus pneumoniae* (in the gene bank), AAK - amino acid kinase, *ilvC* - isoleucine-valine operon C, ATP - adenosine triphosphate, ABC - ATP-binding cassette, GNAT - GCN5-related N-acetyltransferase, *nrdG* - anerobic ribonucleotide reductase, *liv* - leucine/isoleucine/valine transporter, H - subunit H, DDH - desert hedgehog homolog, PTS - phosphotransferase system, *yajC* - SecYEG protein translocase auxillary subunit C, *rex* - rII exclusion, *mal* - maltose transport system permease, *zmp* - *psa* - pneumococcal surface adhesin,

liprotein. Increased operon psaBCA in pneumococcal meningitis model further supported previous observations that operon psaBCA was necessary for bacterial growth and adherence.[21] Teichoic acid (TA) is virtually shared by all gram-positive bacteria, which is covalently attached to peptidoglycan, or anchored to the cell membrane. As a surface component, TA has important roles in a variety of biological functions as bacterial autolysis, biofilm formation, adherence, and virulence. These activities were believed to correlate with the D-alanine abundance. The D-alanine is conjugated with TA to form D-alanyl-lipoteichoic acid, which was catalyzed by dltABCD encoded products. Previous studies have shown that bacteria were easier to be killed by immune cells in a status of dlt operon deletion.[22,23] However, the mechanism of bacteria crossing BBB helped by D-alanine is unclear, and should be further investigated.

The putative ZmpB protein, encoded by zmpB gene, is anchored on the pneumococcal surface via LPxTG motif in the N terminal domain. Evidence has shown that ZmpB might act as a sortase, functioning in the attachment of surface proteins to the pneumococcal cell wall.[24] Mutation in zmpB resulted in reduced localization of CbpA, CbpE, CbpF, CbpJ, and LytA on the cell wall.[24,25] In addition, the mutation in CbpA could lead to the absence of pneumococcal meningitis.[3] Collectively, upregulated ZmpB may function in the development of meningitis by elevating CbpA level. The identified gene products also include Cbp's, such as PspA and CbpD. Such Cbps were commonly attached to the cell surface. The PspA is a well-known virulence factor, whose primary function would be its ability to combine lactoferrin and decrease C3 deposit on pneumococcal surface, and hence, avoid killing by host immune cells via antibody-mediated opsonophagocytosis.[26,27] Previous microarray results have also shown that PspA overexpressed in rabbit meningitis were raised by intracisternal injection.[9] Though with different infection route, our results confirmed their observation that PspA was induced in pneumococcal meningitis, strengthening the key role of PspA in the pathogenesis of pneumococcal meningitis.

From the results shown in **Table 1**, *SP*_0293, *SP*_0327, *SP*_1784, *SP*_2202, *SP*_0663 were encoded for the putative or unknown functional proteins. The functions of these proteins could accordingly be predicted, and function investigations are needed to further confirm their roles in pneumococcal meningitis. The identified genes in this study were induced in the brain during the progress of meningitis caused by the natural route of infection instead of by intracisternal injection, and they are potential targets to understand the pathogenesis of pneumococcal meningitis.

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