Bacterial factors required for *Streptococcus pneumoniae* coinfection with influenza A virus

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**Abstract**

**Background:** *Streptococcus pneumoniae* is a common cause of post-influenza secondary bacterial infection, which results in excessive morbidity and mortality. Although 13-valent pneumococcal conjugate vaccine (PCV13) vaccination programs have decreased the incidence of pneumococcal pneumonia, PCV13 failed to prevent serotype 3 pneumococcal disease as effectively as other vaccine serotypes. We aimed to investigate the mechanisms underlying the co-pathogenesis of influenza virus and serotype 3 pneumococci.

**Methods:** We carried out a genome-wide screening of a serotype 3 *S. pneumoniae* transposon insertion mutant library in a mouse model of coinfection with influenza A virus (IAV) to identify the bacterial factors required for this synergism.

**Results:** Direct, high-throughput sequencing of transposon insertion sites identified 24 genes required for both coinfection and bacterial infection alone. Targeted deletion of the putative aminotransferase (*PA*) gene decreased bacterial growth, which was restored by supplementation with methionine. The bacterial burden in a coinfection with the *PA* gene deletion mutant and IAV in the lung was lower than that in a coinfection with wild-type pneumococcus and IAV, but was significantly higher than that in an infection with the *PA* gene deletion mutant alone. These data suggest that IAV infection alters host metabolism to benefit pneumococcal fitness and confer higher susceptibility to pneumococcal infection. We further demonstrated that bacterial growth was increased by supplementation with methionine or IAV-infected mouse lung homogenates.

**Conclusions:** The data indicates that modulation of host metabolism during IAV infection may serve as a potential therapeutic intervention against secondary bacterial infections caused by serotype 3 pneumococci during IAV outbreaks in the future.

**Keywords:** Influenza A, *S. pneumoniae*, Transposon mutant library, Coinfection, Metabolome

**Introduction**

Influenza A virus (IAV) infection dramatically increases the susceptibility to secondary *Streptococcus pneumoniae* infections, resulting in significantly greater morbidity and mortality during IAV outbreaks [1]. In the most recent influenza pandemic caused by a triple-reassortment IAV subtype H1N1 occurred in 2009, bacterial pneumonia complicated between 25 and 50% of the severe infections, in both children and adults [2–4]. *S. pneumoniae* remained one of the important complicating organisms [5]. *S. pneumoniae* is a common human nasopharyngeal commensal that colonizes 10–40% of children under the age of 5 years [6] and 8–15% of adults [7]. For progression from colonization to lower respiratory tract infection, pneumococci must overcome physical barriers and escape immune defense mechanisms. Influenza virus disrupts...
lungs physiology to promote pneumococcal adherence and interferes with host immune responses to facilitate secondary pneumococcal invasion [8, 9]. Furthermore, influenza neuraminidases remove terminal sialic acid residues from host glycoconjugates to promote pneumococcal adherence and growth during coinfection [10, 11]. On the pneumococcal side, the bacterial mechanism underlying this process remain largely unknown. It has been reported that virulence factors, such as pneumococcal surface protein A (PsaP), choline-binding protein A (CbpA), and pneumococcal serine-rich repeat protein ( PsrP), can be used for increased adherence to the basement membrane or elements of the extracellular matrix, such as fibrin, fibrinogen, and collagen, in the presence of influenza [12].

Until now, there have been no systemic studies to identify genes of S. pneumoniae involved in IAV coinfection. Development of a 13-valent pneumococcal conjugate vaccine (PCV13) has substantially reduced the global burden of pneumococcal disease [13]. Nevertheless, PCV13 failed to effectively prevent serotype 3 mucosal disease as other vaccine serotypes, which is associated with a higher risk of death [14, 15]. In the present study, a clinical isolate of serotype 3 (Taian-S3), recovered from a child with severe pneumonia [16], was used to construct a mini-mariner transposon library [17]. Using high-throughput sequencing to sequence the transposon insertion site can determine the relative fitness of bacterial mutants in the presence and absence of prior IAV infection for comprehensive understanding the interaction between IAV and pneumococcus [17, 18]. Here, we report a genome-wide profiling of the S. pneumoniae genes contributing to strong fitness benefits during coinfection with IAV in comparison with bacterial infection alone in mice.

Materials and methods

S. pneumoniae and influenza A virus culture conditions

Pneumococcal isolates Taian-S3 were grown at 37 °C in Todd–Hewitt broth supplemented with 0.5% yeast extract (THY), chemically defined medium (CDM) [19] or cultured on blood agar plate (supplemented with 5% defibrinated sheep blood) in the presence of 5% CO₂. Escherichia coli (E. coli) was grown in Luria broth (LB). Antibiotics were added at the following concentrations, when required: spectinomycin at 250 mg/L for S. pneumoniae and at 100 mg/L for E. coli; ampicillin at 100 mg/L for E. coli; chloramphenicol at 10 mg/L for S. pneumoniae and at 30 mg/L for E. coli. The A/PR/8/34 (PR8) strain of influenza virus were grown in Madin-Darby Canine Kidney (MDCK) cells.

Mutant library construction

A mutant library of the Taian-S3 strain was constructed using mini-transposon magellan6 random insertional mutagenesis as previously described [17]. Please see Additional file 1 for more detail methods.

S. pneumoniae coinfection post influenza A virus infection in a mouse model

Eight to ten-week-old female BALB/c mice were intranasally inoculated with 250 plaque forming unit (PFU) of PR8 in 50 μL PBS, under light isoflurane anesthesia. On day 4 post influenza IAV infection, bacterial mutant library pool or mutants of S. pneumoniae were intranasally inoculated with 2.5 × 10⁴−1× 10⁶ colony forming unit (CFU) in 50 μL PBS, under light isoflurane anesthesia (Fig. 1A). One or two days post bacterial infection, the mice were euthanized via decapitation after administering anesthesia using isoflurane as per guidelines issued by the Institutional Animal Care and Use Committee (IACUC) of Chang Gung Memorial Hospital. The lungs were removed, rinsed with PBS, and homogenized. Lung homogenates were serially diluted and plated on blood agar, to determine the number of viable pneumococci. The mouse lung with or without IAV infection was homogenized in PBS and centrifuged using benchtop centrifuges, following which the supernatant of the mouse lung was collected and stored at −80 °C until use. The mice were monitored daily for disease manifestation. Mice with severe sickness were euthanized humanely, in accordance with the guidelines provided by the institutional ethical committee and protocols approved by the IACUC of the Chang Gung Memorial Hospital. All experiments were approved by the local ethical committee for animal research (approval number: 2017072001). All animals were housed in an animal facility at 22 °C, with a relative humidity of 55%, in a 12 h light/12 h dark cycle, with sterile tap water and food available ad libitum.

Fitness calculation of the S. pneumoniae mutant library in a mouse model

Sample preparation for Illumina sequencing and single gene fitness calculation of transposon mutant library of S. pneumoniae were performed as previously described [17]. After sequencing, 20-bp reads were mapped to S. pneumoniae genomes (accession number: JAFNBN000000000, Taian-S3) using the program Bowtie v1.2 [20]. Only reads mapping to single sites and insertions with two or more reads at one time point were included in the analysis. For the input (before mice challenge) and output (after mice challenge) treatments, the
Fig. 1  IAV co-infection increased the total bacterial burdens of the mice. **A** Schematic S. pneumoniae serotype 3 mutant library construction and experimental design of mouse infection model. Intranasal challenge of 8- to 10-week-old female BALA/c mice, with or without inoculation of 250 PFUs RP8 influenza virus for 4 days. Fifty microliters of S. pneumoniae serotype 3 mutant (2.5 x 10^4 CFU) library pools were used for intranasal inoculation. **B** The mice were sacrificed 2 days post bacterial infection and the bacterial burdens of the mouse lung was calculated via plate counting (n=5 in each group). Each symbol represents the bacterial burdens value for an individual animal. Mice pre-challenged with influenza viruses RP8 displayed an increase in the total bacterial burdens in the lung (*, p < 0.01, Mann–Whitney U test). IAV influenza A/PR8 virus, PFU plaque-forming units, CFU colony-forming units, Mock without pre-challenged with IAV.
number of reads at each transposon insertion location was recorded and analyzed.

**Construction of deletion mutants and complementation strain of S. pneumoniae**

Target gene deletion mutants were constructed as previously described [21]. The ribA, zmpA, prtA, and putative aminotransferase gene (PA) deletion mutants (S3-ΔribA, S3-ΔzmpA, S3-ΔprtA, and S3-ΔPA, respectively) were generated by replacing the gene with a spectinomycin resistance gene cassette, as described below, using primers listed in Table 1. Briefly, coding regions and flanking fragments for the four genes of interest (goi) from the clinical isolate serotype 3 strain, Taian-S3, were amplified using PCR with the following primer pairs: goi-up-F/goi-dn-R for goi (as mentioned above: ribA, zmpA, prtA, and PA). The resulting PCR products were cloned into the pJET2.1 plasmid (Thermo Fisher Scientific). The coding regions of the goi were removed using inverse PCR (iPCR) with the primer pairs: goi-inverse-F/goi-inverse-R and ligated to a PCR-amplified spectinomycin antibiotic cassette (spec-F/spec-R) from the pDL278 plasmid, to create a deletion construct vector. This vector was transformed into S. pneumoniae Taian-S3 using competence stimulating peptide-1 (CSP-1), and the transformants were selected using spectinomycin. To construct a PA gene complementation strain, a single copy of the PA gene was inserted into the non-coding

| Table 1 | Primers and plasmids used in this study |
|---------|-----------------------------------------|
| Primer name | Sequence (5’ to 3’) | Purpose |
| ribA-flank-F | GTA TCA TAT GCG ATA CAC ΔribA | ΔribA (PCR) |
| ribA-flank-R | GAG GTG GCTAT CAT GAT G | |
| ribA-inverse-F | TGACTGATTATCCCTTTCG | ΔribA (iPCR) |
| ribA-inverse-R | ATGACACTTTAGAAGTTAATTAG | |
| zmpA-flank-FF | CTC AGA AAA AGG AAA AAA TC ΔzmpA | ΔzmpA (PCR) |
| zmpA-flank-FR | TCC TAA TAG ATAC ATATCCCTCCTTG | ΔzmpA (PCR) |
| zmpA-flank-RR | CAC GAC TGA GAG ATA ATT CTAA | |
| zmpA-inverse-F | TATTCCCTTTGAATAATATATATA TATAG | ΔzmpA (PCR) |
| zmpA-inverse-R | GTTTTACTATTAGAATAAAG | |
| Putative aminotransferase-flank-F | TGACACGCGCTTCACTTTTCA | ΔPA (PCR) |
| Putative aminotransferase-flank-R | CTTTCCTGTTGGAGCCACAG | |
| Putative aminotransferase-inverse-F | CTTTTGTCTTATCCCTCTAAG | ΔPA (PCR) |
| Putative aminotransferase-inverse-R | AAATCCAGCTTCTAGGAG | ΔPA (PCR) |
|prtA-flank-FF | GAA GGA ACG CCC GAC GAC GCTG | ΔprtA (PCR) |
|prtA-flank-RR | ATGAA AGA AT AAG AAGAGCAAGCTATAGA | |
|prtA-flank-R | CATCAACTGAGCCAGAAATTTTG | ΔprtA (PCR) |
|prtA-inverse-F | TTTAACCTTTACATATTTATTTAAC | ΔprtA (PCR) |
|prtA-inverse-R | GACAAAGCTATAGAATAATAT | |
| Psepc-F_EcoRIa | CTGAATTCAAGATCGATTTCGTTG | Promoter region sequence of spectinomycin |
| Psepc_R_PLP_F_Ol | ATCATATTTTCATATTGTAGTACC | |
| PA-F_Psepc-R_Ol | GTGACTAATCAAATTTTGGAAATATGATT | Putative aminotransferase gene |
| PA-R_CAT-F_Ol | TTTCAATTATCTACCTAAAGGTTG | Chloramphenicol |
| CAT-F_PLP-R_Ol | TGGCCAAAGCTTAATGATAGATTGA | ΔPA:PA (PCR) |
| CAT-R_EcoRIa | GTGAATTCATTATTCGAGAAGTC | |
| 12133-F | TGGCAAGATAAGAGATTAC | |
| 12136-R | TTAACCGATATTCTTATAG | |

| Plasmid | Features | Reference |
|---------|----------|-----------|
| pDL278 | spec⁺, E. coli-Streptococcus shuttle vector | [47] |
| pJET1.2/blunt | Cloning vector | Thermo Fisher |

* Boldface letter indicates EcoRI cutting site.
region between SPNOXC16740 and SPNOXC16750 (homologs to OXC141; accession number: FQ312027.1) as described below. First, a 2055-bp fragment of the insertion site was PCR-amplified using the primer pair 12133-F/12136-R and cloned into pJET2.1, to create p12133-12136::pJET. Next, the promoter sequence of the spectinomycin (Pspec) gene was PCR-amplified from the pDL278 plasmid, the PA gene was PCR-amplified from Taian-S3, and the chloramphenicol (cat) antibiotic cassette sequence was PCR-amplified from the pKO3-Km plasmid. These three PCR-amplified products were fused together using an overlapping PCR method, with the primers listed in Table 1. The PCR products were digested using EcoRI and sub-cloned into the EcoRI site of the p12133-12136::pJET plasmid, to generate the complementation vector p12133-Pspec-PA-cat-12136::pJET. The complementation strain (S3-ΔPA::PA) was created by transforming the p12133-Pspec-PA-cat-12136::pJET plasmid into the S3-ΔPA strain using CSP-1 and selecting for chloramphenicol.

Metabolomics analysis
Bacteria were grown until OD₆₀₀ = 0.5–0.8, following which the number of bacteria was adjusted to 10⁹ CFU, and extracted using extract solution (methanol: acetonitrile: ddH₂O = 2:2:1 with 2 mg/L 2-chloro-l-phenylalanine), then homogenized using 5 μm glass beads. The sample was centrifuged and vacuum dried then reconstituted with 50% acetonitrile and storage at −80 °C until for analysis. Each sample (10 μL) was injected into a vanquish-focused ultra-high-performance liquid chromatography (UHPLC) system (Thermo Orbitrap Elite) coupled with an Orbitrap Elite Mass Spectrometer (Thermo Fisher Scientific) using electrospray ionization. Please see Additional file 1 for more detail methods.

Statistical analysis
Prism 5.0 (GraphPad) was used for statistical analysis. Comparisons of CFU recovered from murine lung were made using Mann–Whitney U test. Data have been presented as mean ± standard deviation (SD) of more than three independent experiments. Differences with p ≤ 0.05 were considered statistically significant.

Results

Genome-wide fitness screening of S. pneumoniae mutants using a murine IAV coinfection model
At 2 days post-infection with S. pneumoniae, the mice were sacrificed, and the total bacterial burden in the mouse lung was calculated. The results showed that pre-challenge with IAV significantly increased the total bacterial burden in the mouse lung (Fig. 1B, p = 0.0079). To identify the bacterial genes that contribute to IAV and S. pneumoniae coinfection, we generated a transposon insertion mutant library comprising approximately 2000 individual mutants and inoculated the mutant library pool into a mouse model with or without IAV infection. After calculating fitness, genes (size > 1 kb and a read count ≥ 8) with a fitness score of 0 were designated as required under a given in vivo condition. We identified 24 genes that were required for both bacterial infection and IAV coinfection, and six that were not required for both bacterial infection and IAV coinfection. No gene was solely essential for single or coinfection (Additional file 2). Among the 24 genes that were required for both bacterial infection and IAV coinfection, eight genes encoded hydrolases, five genes encoded transferases, three genes were related to transport, and two genes encoded hypothetical proteins. The remaining genes were related to carbohydrate metabolic processes, lyases, helicases, oxidoreductases, protein kinases, and transcription regulators. One of the identified genes, nanA, was previously reported to have a synergistic effect on S. pneumoniae coinfection with IAV [11, 22]. Four of the 24 genes [ribA (SPNOXC02060 homolog), zmpA (SPNOXC10390 homolog), prtA (SPNOXC05890 homolog), and PA (SPNOXC13360 homolog)] were selected, and deletion mutants of these genes were created for further studies (Table 2 and Additional file 2).

| Number | Gene/product name | Putative function | Relative referencea |
|--------|-------------------|------------------|---------------------|
| 1      | ribA              | 3,4-Dihydroxy-2-butanone 4-phosphate synthase / GTP cyclohydrolase II | [48, 49] |
| 2      | prtA              | CSa peptidase precursor | [23, 50] |
| 3      | zmpA              | IgA-specific metalloendopeptidase | [51, 52] |
| 4      | Putative aminotransferase | PLP-dependent beta-cystathionase / Maltose regulon repressor | |

a Previously studies to be associated with the virulence of S. pneumoniae
Validation of the roles of the selected bacterial genes in a coinfection mouse model

The four selected genes were individually deleted in the clinical serotype 3 strain Taian-S3, which is the parental strain used for mutant library construction, by precise replacement of each coding region with the spectinomycin antibiotic resistance sequence. The four deletion mutants, S3-ΔribA, S3-ΔzmpA, S3-ΔprtA, and S3-ΔPA, were tested in a mouse model with or without IAV infection. The ribA gene was only required for colonization of mouse lung in the bacterial infection alone (Fig. 2A, B). Two genes (prtA and PA) were required for colonization of the mouse lung in the single and coinfection models (Fig. 2A, B) as predicted from the genome-wide fitness analysis. PrtA was previously reportedly associated with S. pneumoniae virulence [23], which is consistent with the results of our study. Thus, this association was not investigated further.

Putative aminotransferase is involved in the cystine and methionine metabolic pathway and regulates the growth of S. pneumoniae

Analysis of the growth kinetics of the four deletion mutants showed that the growth of S3-ΔPA was significantly slower than the kinetics of the other mutants, which grew similar to the wild-type Taian-S3 strain (Additional file 1: Fig. S1A). A PA gene-complemented strain (S3-ΔPA::PA) showed restored growth kinetics similar to that of the wild-type strain (Additional file 1: Fig. S1B). PA from Taian-S3 shares 39% amino acid identity with the Bacillus subtilis PatB polypeptide. PatB is a class I pyridoxal phosphate (PLP)-dependent aminotransferase [24] that participates in the synthesis of methionine and cystine [24, 25]. To investigate the effect of methionine supplementation on growth kinetics, the growth of the S3-wild-type strain and the S3-ΔPA mutant were compared in both THY complete medium and CDM medium with restricted methionine content. In THY, there was no growth difference between the S3-wild-type strain and the S3-ΔPA mutant with or without methionine (Fig. 3A). However, in CDM, the growth of both the S3-wild-type strain and S3-ΔPA mutant were increased by the addition of methionine (Fig. 3B). We speculated that, in a nutrient-restricted environment, the addition of methionine can improve growth. We then comprehensively investigated the differences in the metabolite composition of the S3-wild-type strain and S3-ΔPA mutant using an untargeted mass spectrometry (MS)-based metabolomics analysis. In the negative charge metabolite analysis, 26 metabolites were found to be significantly different between the S3-wild-type strain and S3-ΔPA mutant. In the positive charge metabolite analysis, 51 metabolites were found to be significantly different between the S3-wild-type strain and the S3-ΔPA mutant (Additional file 3). Most of the metabolites could not be annotated (MS1 and MS2), and only a few could be identified by their metabolite IDs in the KEGG database. Among those that were identified,
such as L-aspartic acid (KEGG: C00049) and glutathione (KEGG: C00051), which are involved in the cystine and methionine metabolic pathways, were higher in the S3-ΔPA mutant than in the S3-wild-type strain.

**Lung colonization is impaired in putative aminotransferase gene-deficient pneumococci and is partially restored upon IAV coinfection**

To assess the role of the PA gene in lung colonization by *S. pneumoniae* during coinfection with influenza, mice were infected intranasally with *S. pneumoniae* Taian-S3 wild type or its isogenic PA deletion mutant (S3-ΔPA) at 4-day post IAV infection. Since the growth kinetics of S3-ΔPA were slower, to better discriminate the difference in the bacterial burden in the mouse lung, we increased the bacterial infection dosage from 2.5 × 10⁴ CFU/mouse to 1 × 10⁶ CFU/mouse. The mice were sacrificed at one or two days post bacterial infection. The results showed that the bacterial burden in the lung of S3-ΔPA-infected mice was significantly lower than that of S3-wild-type
strain-infected mice under single infection conditions (Fig. 4). Coinfection with IAV significantly increased the lung colonization density of both the S3-wild-type strain and S3-ΔPA mutant when compared to that with bacterial infection alone (Fig. 4). Based on this finding together with increased bacterial growth upon methionine addition in vitro, we speculated that the improved growth of S3-ΔPA mutant and S3-wild-type strain after IAV infection might be due to changes in the nutrients/metabolites present in the mouse lung upon IAV infection.

**IAV-infected murine lung homogenate increases S. pneumoniae growth**

Supplementation with methionine promoted bacterial growth in vitro, and coinfection with IAV increased the bacterial burden in mouse lung. Next, we sought to examine whether the mouse lung (post-IAV infection) could directly increase pneumococci, to rule out the host immune system. We added a crude lung homogenate extracted from mice with or without IAV infection to a *S. pneumoniae* culture in CDM medium (with restricted nutrient content) and assessed the fold change in bacterial growth. As expected, the fold change in bacterial growth of the S3-wild-type strain was higher in cultures with homogenate from virus-infected mice than in cultures with homogenate from mock-treated (without IAV infection) mice (Fig. 5). In summary, we demonstrated that a crude extract from mouse lung post-IAV infection promoted bacterial growth, which is coherence with the observed increase in bacterial burden in mouse lung after IAV infection.

**Discussion**

Serious secondary bacterial pneumonia frequently occurs after influenza infection. *S. pneumoniae* is the major pathogen causing secondary bacterial pneumonia post-influenza infection [1, 26]. Primary influenza infection can modulate the host immune response by increasing either the anti-inflammatory or pro-inflammatory response, reducing bacteria clearance and leading to poor outcomes from secondary bacterial pneumonia [27, 28]. In addition, virus-induced cytotoxicity leads to lung epithelial cell damage resulting in cell attachment side exposure, thus increasing bacterial adhesion and physical barrier dysfunction in the airway [1, 26]. These changes lead to an increase in host susceptibility to secondary infections. Viral-bacterial synergism also contributes to serious secondary bacterial infections [11, 18]. Neuraminidase, which is expressed in both influenza and pneumococcus, possesses sialidase activity and contribute to release of sialic acid from host airway mucins [10, 11]. Therefore, pneumococcus can utilize more host sialylated substrates as a nutrient source to promote bacterial growth [11] and biofilm formation [11, 22] after influenza infection. However, the detailed mechanisms and bacterial virulence factors that contribute to the synergistic effect between influenza and pneumococcus in coinfections are not completely understood. To determine if there are other genes or synergism mechanisms that are
involved in this viral-bacterial interaction, a systematic genome-wide bacterial fitness screening of a *S. pneumoniae* mutant library pool was used. The screen identified the putative aminotransferase gene, which is involved in the cystine and methionine biosynthetic pathway. Based on our analyses performed in vitro and in vivo for the PA gene-deficient mutant of a serotype 3 strain, we hypothesized that the increase in *S. pneumoniae* growth after influenza infection may result due to changes in the host metabolome, and the utilization of these metabolites may promote bacterial fitness after influenza infection.

Studies have shown that influenza infection dynamically changes the metabolome in serum, the lungs, and BALF of mice [29]. In previous studies in animals, the common trends in the metabolome post-influenza infection include alterations in amino acids and related molecules, in select lipids, and in some nucleosides, nucleotides, and analogs; increases in carbohydrates and related molecules; and decreases in mannitol, myo-inositol, and glyceric acid [29–32]. Metabolite changes in the host post virus infection might reflect the consequences of viral manipulation of the host metabolism to favor the production of new viral particles and modulation of pro-inflammatory and anti-inflammatory metabolites that contribute to disease pathogenesis. Moreover, infection with other viruses, such as respiratory syncytial virus (RSV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), can also alter the host metabolome [33–35]. Infections with these respiratory viruses are also often followed by severe secondary bacterial pneumonia [36, 37]. The metabolome changes accompanying influenza infection that affect metabolic pathways are closely related to pro- and anti-inflammatory cytokine expression levels. For example, amino acid metabolites are correlated with IFN-γ and IL-6 expression [30]. In addition, the metabolic reprogramming associated with influenza infection in adipose tissue and preadipocytes promotes influenza virus replication [38]. Therefore, obesity, which changes the T-cell metabolome, is a risk factor for increased mortality from influenza infection [39]. Accordingly, the effects of the metabolome changes that occur with influenza infection, which may increase the incidence of secondary bacterial infection, are worthy of further study. One study revealed that capillary leakage, with the efflux of nutrients, especially glucose, into the alveolar space after influenza infection promotes pneumococcal growth [40]. The results of the current study were consistent with previous research showing that the metabolites of the host lung were changed after influenza infection. In addition, our findings showed that supplementation of a bacterial culture with a crude homogenate from influenza-infected lungs increased *S. pneumoniae* growth also revealed that the metabolome changes in the mouse lung could directly promote bacterial growth.

Bacteria can increase their fitness in the host niche by producing metabolites, such as essential amino acids, to promote colonization [19], virulence [18, 41], biofilm expansion [42], and rapid growth [40]. Methionine is one of the scarce amino acid in physiological fluids but its importance cannot be underestimated. Methionine biosynthetic genes are essential for the full virulence of many bacteria, including *Haemophilus parasuis* [43], *Brucella melitensis* [44], *Salmonella enterica* [45], and *S. pneumoniae* [41]. Consistent with previous findings, the results of this study showed that the poor growth rate of a PA gene-deficient mutant could be restored by supplementation with methionine, and pre-infection with influenza A virus could also increase the bacterial growth of the PA mutant.

In this study, only 30 genes were identified in the genome-wide screening as being related to infection. One identified gene, *nanA*, was previously reported to have a synergistic effect with IAV [11, 22]. Therefore, the results of our screen were consistent with data from other studies, and we expect that our mutant library screen results are still representative of the viral-bacterial interaction. Although fewer genes were identified in this study, we still found the PA gene in our mutant library pool and revealed the importance of metabolome changes after influenza infection in bacterial growth.

Conclusions

Because of the recent increases in antimicrobial-resistant non-pneumococcal conjugate vaccine (non-PCV) *S. pneumoniae* types and new subtypes of influenza that continue to cause global pandemics [46], influenza and *S. pneumoniae* coinfection remains a significant public health concern. Specific bacterial virulence factors that contribute to bacterial-viral interaction may be useful therapeutic targets to control or prevent secondary bacterial pneumonia after influenza infection. The results of the study indicate that bacterial metabolites that cannot be synthesized by the host may serve as potential drug targets to combat secondary bacterial pneumonia induced by serotype 3 pneumococci upon influenza infection.

Abbreviations

CDM: Chemically defined medium; CFU: Colony forming unit; Goi: Genes of interest; IACUC: Institutional Animal Care and Use Committee; IAV: Influenza A virus; LB: Luria broth; MDCK: Madin-Darby Canine Kidney; PA: Putative aminotransferase; PCV13: 13-Valent pneumococcal conjugate vaccine; PFU: Plaque forming unit; THY: Todd–Hewitt broth supplemented with 0.5% yeast extract; UHPLC: Ultra-High-performance liquid chromatography.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12929-021-00756-0.

Additional file 1. Supplemental appendix for Materials & Methods and Results.

Additional file 2. Thirty genes that were identified in the genome-wide screening method in this study, and gene annotation of Taian-S3.

Additional file 3. Metabolome comparison of S3-wild-type strain and S3-ΔPA mutant.

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Authors' contributions

Conceptualization: YYC, TLL, and YCH. Data curation: YYC, CTH, SWL, and YCH. Formal analysis: YYC, CTH, SWL. Funding acquisition: YCH and YJP. Investigation and methodology: YYC, SWL, CTH, YJP, YYH, THL, YCY, and YNG. Supervision and visualization: YYC, CTH, YJP, TLL, and YCH. Writing—original draft preparation: YYC and YCH. Writing—review and editing: YYC, CHT, SWL, YJP, YCH. All authors read and approved the final manuscript.

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Availability of data and materials

The Whole Genome Shotgun project of S. pneumoniae serotype 3 clinical strain, Taian-3, during this study were deposited at DDBJ/ENA/GenBank under the accession JAFLNB000000000. The version described in this paper is version JAFLNB010000000.

Declarations

Ethics approval and consent to participate

The mice were monitored daily for disease manifestation. Mice with severe sickness were euthanized humanely, in accordance with the guidelines provided by the institutional ethical committee and protocols approved by the Animal Care and Use Committee of the Chang Gung Memorial Hospital. All experiments were approved by the local ethical committee for animal research (approval number: 2017-07-2001). All animals were housed in an animal facility at 22 °C, with a relative humidity of 55%, in a 12 h light/12 h dark cycle, with sterile tap water and food available ad libitum.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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