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An integrated respiratory microbial gene catalogue to better understand the microbial aetiology of Mycoplasma pneumoniae pneumonia

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Abstract:	Background The imbalanced respiratory microbiota observed in pneumonia causes high morbidity and mortality in childhood. Respiratory metagenomic analysis demands a comprehensive microbial gene catalogue, which will significantly advance our understanding of host–microorganism interactions. Results We collected 334 respiratory microbial samples from 171 healthy children and 76 children with pneumonia. The respiratory microbial gene catalogue we established comprised 2.25 million non-redundant microbial genes, covering 90.52% of prevalent genes. The major oropharyngeal microbial species found in healthy children were Prevotella and Streptococcus. In children with Mycoplasma pneumoniae pneumonia (MPP), oropharyngeal microbial diversity and associated gene numbers decreased compared to those of healthy children. The concurrence network of oropharyngeal microorganisms in patients predominantly featured Staphylococcus spp. and M. pneumoniae. Functional orthologues, which are associated with the metabolism of various lipids, membrane transport and signal transduction, accumulated in the oropharyngeal microbiome of children with pneumonia. Several antibiotic resistance genes and virulence factor genes were identified in the genomes of M. pneumoniae and 13 other microorganisms reconstructed via metagenomic data. Although the common macrolide/beta-lactam resistance genes were not identified in the assembled M. pneumoniae genome, a single nucleotide polymorphism (A2063G) related to macrolide resistance was identified in a 23S rRNA gene. Conclusions The results of this study will facilitate exploration of unknown microbial components and host–microorganism interactions in studies of the respiratory microbiome. It will also yield further insights into the microbial aetiology of MPP.
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An integrated respiratory microbial gene catalogue to better understand the microbial aetiology of Mycoplasma pneumoniae pneumonia

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Abstract

Background: The imbalanced respiratory microbiota observed in pneumonia causes high morbidity and mortality in childhood. Respiratory metagenomic analysis demands a comprehensive microbial gene catalogue, which will significantly advance our understanding of host–microorganism interactions. **Results:** We collected 334 respiratory microbial samples from 171 healthy children and 76 children with pneumonia. The respiratory microbial gene catalogue we established comprised 2.25 million non-redundant microbial genes, covering 90.52% of prevalent genes. The

major oropharyngeal microbial species found in healthy children were *Prevotella* and *Streptococcus*. In children with *Mycoplasma pneumoniae* pneumonia (MPP), oropharyngeal microbial diversity and associated gene numbers decreased compared to those of healthy children. The concurrence network of oropharyngeal microorganisms in patients predominantly featured *Staphylococcus spp.* and *M. pneumoniae*. Functional orthologues, which are associated with the metabolism of various lipids, membrane transport and signal transduction, accumulated in the oropharyngeal microbiome of children with pneumonia. Several antibiotic resistance genes and virulence factor genes were identified in the genomes of *M. pneumoniae* and 13 other microorganisms reconstructed via metagenomic data. Although the common macrolide/beta-lactam resistance genes were not identified in the assembled *M. pneumoniae* genome, a single nucleotide polymorphism (A2063G) related to macrolide resistance was identified in a 23S rRNA gene. **Conclusions:** The results of this study will facilitate exploration of unknown microbial components and host–microorganism interactions in studies of the respiratory microbiome. It will also yield further insights into the microbial aetiology of MPP.

Keywords: Pneumonia; *Mycoplasma pneumoniae*; Oropharynx; Microbiome; Respiratory microbial gene catalogue

Background

Several microorganisms have been identified as indispensible for the respiratory microbiota (RM) [1–5]. In cases of pneumonia, an imbalance in the levels of the microorganisms within the RM [6, 7] is a leading cause of high morbidity and mortality [8, 9] worldwide, especially in children under 5 years old [10, 11]. In recent years, the number of cases of *Mycoplasma pneumoniae* pneumonia (MPP) in Chinese children has been increasing [12] and the microbial aetiology of this disease remains poorly understood.

Previous studies by our group have revealed altered RM in children with MPP [13, 14]. However, current RM research has mainly focused on analysis of 16S rRNA [6, 7, 15, 16], which only provides cues about known bacterial components at the genus level. Emerging 16S rRNA analysis has revealed an imbalanced microbial structure in the respiratory tracts of children with pneumonia [7, 17, 18]; however,

changes in microbial functions and the species-level microbial components of the RM of patients with MPP remain unexplored. In addition, current multi-omics studies are limited to explorations of known bacterial genomes in the RM [15]. Nevertheless, the RM includes many unknown microbial species [1–3, 5, 6] that require further exploration.

A comprehensive catalogue of reference genes is crucial for in-depth functional metagenomic analysis such as species/gene profiling, discovery of microbial biomarkers and functional annotation. Given that the RM varies with the environment [19], age [1, 2, 4] and disease [6, 7, 15, 16], we took samples from the nasopharynx (NP), oropharynx (OP) and lungs of 76 children with pneumonia and OP samples from 171 healthy children in China. These samples were used to establish an integrated respiratory microbial gene catalogue (RMGC), with which to study the imbalanced RM in Chinese children with MPP. Using this catalogue, we assessed the microbial components and functions in the OP microbiota of healthy children and children with MPP, as well as the characteristics of recovered microbial genomes.

Data Description

Between 3 July 2016 and 27 August 2016, 247 patients were recruited from the hospitalization zone in the Department of Respiratory Diseases of Shenzhen Children's Hospital, China. Inclusion criteria were characteristic chest radiographic abnormalities consistent with pneumonia, exclusion of asthma and clearance of respiratory infections or exposure to antibiotics within one month of sampling (Table 1). NP (25-800-A-50, Puritan, Guilford, ME, USA) and OP (155C, COPAN, Murrieta, CA, USA) swabs were collected from 76 hospitalized patients within 24 hours of hospitalization and before the administration of antibiotics. Broncho-alveolar lavage fluid (BALF) was collected 2–15 days after hospitalization (Supplementary Table 1).

Healthy children were recruited during physical examinations at Shenzhen Children's Hospital, China, between July and August of 2016. OP swabs were collected from 171 healthy children meeting the following inclusion criteria: no diagnosis of asthma or family history of allergy; no history of pneumonia; a lack of wheezing, fever, cough or other respiratory/allergic symptoms at sampling 1 month prior to the study and 1 week after sampling; no exposure to antibiotics 1 month prior to sampling.

All samples were collected by an experienced clinician. Samples were stored at −80°C within 20 minutes of collection and DNA was extracted within 10 days of sampling. A TGuide S32 Magnetic Swab Genomic DNA Kit (DP603-T2, TIANGEN Biotech (Beijing) Co., Ltd., Beijing, China) was utilized to extract the DNA. Metagenomic sequencing was performed using the Illumina Hi-Seq platform (San Diego, USA) according to the manufacturer's instructions. Unused swabs and DNA extraction kits from the same batch served as negative controls to assess DNA contamination.

Sample information and data output

Two hundred forty-seven children aged <13 years were enrolled in this study (Table 1 and Supplementary Table 1). After removing host contamination and low-quality data, metagenomic sequencing produced 4,765,288,986 reads, with an average of 14,267,332 reads per sample. The DNA concentration of unused sampling swabs and DNA extraction kits was less than 0.01 ng/μl, whereas the DNA concentration was greater than 80 ng/μl in sampling swabs and BALF. Furthermore, the DNA

amplification results of extracted bacterial DNA were less than 0.01 nmol/l for the enveloped sampling or extraction materials (Supplementary Figure 1)

Construction of the RMGC

By applying metagenomics sequencing data from 247 children and three respiratory-related microbial genome/gene resources (Figure 1), we constructed a comprehensive RMGC with 2,245,343 non-redundant ORF. Data are freely accessible on our website [20]. The total length of ORFs in the RMGC was 1.71 Gbp and the average length was 760 nt (range: 102–32,241 nt). We selected 241 samples with ≥650 Mb data to examine the coverage of the microbial genes in the RMGC. In accordance with the rarefaction curve, 90.52% of prevalent microbial genes were captured in the RMGC (Figure 2a and 2b).

Taxonomic assessment and functional annotation of the RMGC

Based on taxonomic profiling, 1,281,673 genes (57.08% of RMGC) were assigned to phyla and 1,143,382 genes (50.92% of RMGC) were assigned to genera, representing

56.58% and 51.75% of the sequencing reads, respectively. A total of 617,968 genes (25.92% of RMGC) were annotated to known bacterial species, representing 33.49% of the sequencing reads. The RMGC was dominated by the phyla Firmicutes, Bacteroides, Proteobacteria, Actinobacteria and Fusobacteria, while the most prevalent microbial genera were *Staphylococcus*, *Streptococcus*, *Haemophilus*, *Corynebacterium*, *Dolosigranulum*, *Prevotella*, *Blautia*, *Rothia*, *Porphyromonas*, *Lactobacillus*, *Veillonella*, *Fusobacterium* and *Leptotrichia*. Unknown microbial species accounted for 9.62–55.50% of the RMGC. Detailed taxonomic information of the RMGC is deposited on our website [20].

Metagenomic analysis revealed a genus-level microbial structure resembling the results of the 16S rRNA analysis (Supplementary Figure 2). Notably, a greater proportion of microbial genera remained unclassified in the metagenomic analysis than in the 16S rRNA analysis; this might be attributed to the wide detection by metagenomics sequencing and limited reference microbial genomes.

By aligning the RMGC to the KEGG database, 6,408 KEGG orthologous (KO) groups were identified, including 853,446 genes representing 37.85% of the total

sequencing data. As more samples were included, known microbial functions (annotated by KEGG) saturated quickly to 6,346 groups (Figure 2c). Combining novel gene families, the rarefaction curve plateaued when 12,924 groups were detected (Figure 2c). Upon alignment to the eggNOG database, 53.95% of genes in the RMGC were assigned to known functional categories.

Core microbial species in the OP microbiota of healthy children

We acquired 67 core species across five dominant phyla: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria and Fusobacteria (Figure 3). *Prevotella melaninogenica* (4.38 ± 2.91%, mean ± sd), *Prevotella sp.* (3.06 ± 1.92%), *P. histicola* (3.23 ± 3.58%), *P. pallens* (2.31 ± 1.88%) and *Veillonella atypical* (1.60 ± 1.44%) were the top five microbial species. In addition, *Streptococcus pseudopneumoniae* (1.26 ± 0.96%), *Haemophilus influenzae* (0.60 ± 0.68%), *S. pneumoniae* (0.60 ± 0.50%), *H. parainfluenzae* (0.42 ± 0.49%) and *Staphylococcus aureus* (0.27 ± 1.52%), which were generally defined as opportunistic pathogens, were also prevalent in the OP microbiota of healthy children (Figure 3).

Microbial structure and functions of the OP microbiome of children with MPP differed from those of healthy children

Based on permutational analysis of variance (PERMANOVA), onset of pneumonia is the most significant factor (adjusted *P*-value <0.001) explaining the variations observed in OP microbial samples. This is followed by feeding pattern (adjusted *P*-value = 0.037) and age (adjusted *P*-value = 0.048). Compared with healthy children, children with MPP exhibited significantly decreased microbial gene numbers and alpha diversity of the OP microbiota (Figure 4a and 4b). Thirty co-abundance gene groups (CAGs) accumulated significantly in the OP microbiota of children with MPP, comprising six unknown and 24 known microbial species. These species were primary respiratory pathogens such as *M. pneumoniae*, *Staphylococcus epidermidis* and *S. aureus* (Figure 5a). Ninety-five CAGs were enriched in the OP microbiota of healthy children, including prevalent colonizers such as *Prevotella* species (Figure 5a). The microbial co-occurrence networks of children with MPP were simpler than those of healthy children; negative correlations were only identified between

193 healthy-enriched and MPP-enriched CAGs (Figure 5a). For example,
194 healthy-enriched *Prevotella spp.* negatively correlated with MPP-enriched *S.*
195 *epidermidis* ($r < -0.60$, adjusted *P*-value ≤ 0.05, Figure 5a).

196 By comparing functional KEGG annotations (Supplementary Table 2), we
197 assessed the functional alterations of the OP microbiota in children with MPP.
198 Microbial functions relateing to lipid metabolism, membrane transport and signal
199 transduction were slightly enriched in children with MPP (Figure 5b). In contrast, the
200 OP microbiota of healthy children was significantly enriched in orthologues involved
201 in glycan biosynthesis and metabolism, biosynthesis of secondary metabolites, and
202 cell growth and death (Figure 5b and Supplementary Table 2). Host
203 homeostatis-associated functions, such as the immune system, digestive system,
204 circulatory system and environmental adaptation, were also significantly abundant in
205 the OP microbiota of healthy children (Figure 5b and Supplementary Table 2).

206

207 **Characterization of the *M. pneumoniae* genome and 13 other reconstructed**
208 **microbial genomes**

We re-assembled 14 qualified microbial CAGs (Supplementary Table 3), representing the *M. pneumoniae* genome (0.80 Mbp) and the genomes of 13 other microorganisms (average genome size: 2.30 Mbp). The *M. pneumoniae* genome accumulated significantly in the OP microbiota of MPP patients and had high similarity with the reference genome (97.79% genome coverage; Supplementary Table 3). The *M. pneumoniae* genome included four antibiotic resistance genes (ARGs) against common antibiotics including peptide, rifamycin and fluoroquinolone (Figure 6, Supplementary Table 4). In eight patients who had been given experimental macrolides or beta-lactams (such as azithromycin, erythromycin or sulbactam), a SNP mutation, A2063G, which is related to macrolide resistance, was identified in the 23S rRNA (Supplementary Table 1). In addition, 136 virulence factor genes (VFGs) were found along its reassembled genome sequence (Supplementary Table 5), as well as redundant *M. pneumoniae* VFGs enriched in the secretion of adhesin P1, cytadherence protein and community-acquired respiratory distress syndrome (CARDS) toxin (Figure 6 and Supplementary Table 5).

Of the 13 other microbial genomes included in our study, five could be annotated

as specific species, one was annotated at the genus level (*Ralstonia*), and the remaining seven were novel microbial genomes (average genome size 1.74 Mbp) (Supplementary Table 3). For the five annotated microbial species, *S. aureus* and *S. epidermidis* increased significantly in MPP patients, while the other three *Prevotella spp.* mainly accumulated in healthy children (Figure 7, Supplementary Table 3). The largest reassembled *Ralstonia* genome (5.89 Mbp) carried numerous ARGs, including 13 beta-lactam ARGs, 21 tetracycline ARGs, and 11 macrolide ARGs. *P. histicola*, *P. shahii* and CAG00068 all had one copy each of a macrolide resistance gene and a beta-lactam antibiotic resistance gene. These genomes also harboured abundant VFG resources, ranging from 105 to 808 copies of relative genes. Correlation analysis revealed no significant correlation between these 14 reassembled microbial genomes and five clinical indices (Supplementary Table 6).

Discussion

Morbidity and mortality related to MPP is increasing in Chinese children. The development of RM studies has improved our understanding of the microbial

241 aetiology of MPP by revealing infection-associated RM imbalances [13, 14]. However,
242 microbial functions and host–microbiota interactions in the RM of patients with MPP
243 remain to be explored, particularly for novel microbial species.

244 In recent years, several reference gut microbial catalogues have been constructed
245 to promote understanding of host–microorganism interactions. Qin et al. built a global
246 view of the human gut microbiome (GM) and revealed a comprehensive functional
247 potential of the prevalent gut microbial genes [21]. Li et al. upgraded the gut gene
248 catalogue in 2014 [22], enabling studies to associate microbial genes with human
249 health conditions. These frameworks have allowed researchers to deepen their
250 understanding of the correlations between GM and various diseases, such as
251 gastrointestinal and cardiovascular diseases [23, 24].

252 Like the reference gene catalogues that have been developed for the GM, our
253 RMGC will further understanding of microbial aetiology in respiratory diseases. The
254 development of a well-established RMGC is crucial for the functional metagenomics
255 analysis needed to improve our knowledge of host–microbiota interactions in MPP.
256 By aligning metagenomics data directly with the RMGC we have established,

257 researchers will be able to profile all microbial species, as well as explore microbial
258 functions in both known and unknown microbial species. The fact that microbial
259 assignment using the RMGC proved similar to that by 16S rRNA analysis also
260 suggests that the RMGC may be promising for taxonomic studies using our
261 constructed gene sets. Our data on the core microbial species of the OP microbiota in
262 healthy children will provide a reference for exploring microbial and host–
263 microorganism interactions in RM studies [25]. More generally, the RMGC provides
264 a comprehensive respiratory-associated microbial profile to further microbiota
265 analysis at the species level; functional profiling will facilitate further, more in-depth
266 multi-omics analyses [26, 27], such as associations between proteins products and
267 metabolites with known and novel microbial genomes. This capability would help to
268 clarify interactions between the host and alterations to the RM during the progression
269 of disease in MPP.

270 The OP microbiota of children with MPP has a simpler structure than that of
271 healthy children. Previous studies have revealed that the bacteria-like *M. pneumoniae*
272 is able to deplete levels of other bacteria through direct competition, and activates

bacterial clearance factors in host responses [28, 29]. This leads to decreased numbers colonising *Prevotella spp.*[30] and reduced proliferation of pathogens such as *S. aureus* and *S. epidermidis*. MPP patient-enriched genes have functions involved in membrane transport and the metabolism of various nutrients, which may partly explain the reduced tight junction proteins and increased respiratory mucosa permeability after infection [31]. Several studies have also identified increased glucose concentration in airway surface liquids [32–34] and associated pathogen proliferation [35]. This also corroborate the existence of enriched nutrient uptake pathways in the OP microbiota of children with MPP. Although the mechanism of *M. pneumoniae* clearance in the respiratory system remains unclear, these findings would provide new insights into host–microbiota interactions in MPP infection.

As well as a variety of well-known microorganisms, respiratory tracts also harbour numerous undiscovered microbial species [36]. Moreover, recent reports have demonstrated that a single bacterial genome can be recovered via reference gene sets and metagenomics data [37, 38]. It is difficult to culture *M. pneumoniae,* thus it is rarely done in clinical diagnosis, and this limits our understanding of antibiotic

289 resistance and virulence [39] in this species. Reconstruction of the *M. pneumoniae*
290 genome using the RMGC and metagenomics data has indicated the existence of
291 various ARGs related to RNA transcription [40], DNA replication [41] and protein
292 synthesis [42]. According to clinical practice guidelines [43–45], most children with
293 MPP are treated with azithromycin, erythromycin or sulbactam; none of these were
294 associated with ARGs identified in the *M. pneumoniae* genome. Increasingly, reports
295 demonstrate that specific dominant bacteria are associated with severe acute
296 respiratory infections (ARIs) [6, 46, 47], but there have been no meaningful
297 correlations identified between disease severity and *M. pneumonia* – or indeed
298 between the other species of reassembled bacteria found in the OP microbiota of
299 children with MPP. In our previous studies, we confirmed the succession of *M.*
300 *pneumoniae* infection in NP to OP and lung, and determined an association between
301 *M. pneumoniae* load in the lung microbiota with disease severity [14].

302 Although no macrolide/beta-lactam resistance genes were discovered in the *M.*
303 *pneumoniae* genome, one SNP mutation (23S RNA, 2063A–>G) associated with
304 macrolide resistance was identified in children with MPP. Meanwhile, in

patient-enriched microbial genomes such as *Ralstonia*, plenty of ARGs related to macrolide, beta-lactam and tetracycline resistance were found. Given rigorous antibiotic selective pressure and complex microbial interactions, environmentally redundant genetic components can be rapidly transferred into the pathogen genome by horizontal gene transfer [48, 49], causing the emergence of several diseases, such as happened in the European enterohemorrhagic *Escherichia coli* breakout [50] and the emergence of scarlet fever caused by *Streptococcus pyogenes* in Hong Kong [51]. Considering these findings, it should be recognised that current regimes for the treatment of *M. pneumoniae* hold the potential to trigger emerging drug-resistant strains, whether in *M. pneumoniae* or other novel microbial species. Indeed, macrolide resistance has already been reported in *M. pneumoniae*-PCR-positive children [52–54].

In OP microbiota samples from healthy children, several bacterial genomes were recovered, including the key player *Prevotella spp.* [55, 56], and several other novel microorganisms, such as *Vampirovibrio*, which might function as pathogen competitors [57]. Microbial genomes recovered from respiratory tracts hold the

potential to improve our understanding of the microbial aetiology in MPP pneumonia.

There are several limitations of this study. Given that there are currently no effective medicines for MPP, the patients in our study received empirical treatments that might have slightly shifted their airway ecology [58]. Despite promising applications for the RMGC, unclassified CAGs and novel gene families in the RMGC must be annotated and further explored. The copy numbers of several genes require further assessment because of the potential for inaccuracies caused by the low respiratory bacterial biomass, NGS sequencing and assembly methods. In this study, the respiratory microbial samples were obtained from Chinese children; therefore, like the continual updates made to the GM reference genes, metagenomics data from a more diverse sample will be incorporated into the RMGC in the future to more broadly characterise microbial components and functions [22, 59, 60]. This will incrementally improve studies of the imbalanced RM in patients with respiratory diseases. Alterations in the OP microbiota of Chinese patients with MPP will also provide extensive insights into the microbial aetiology of acute respiratory infection.

Potential implications

The respiratory microbial gene catalogue established here will help to deepen respiratory micro-ecology research, and has the potential to elucidate respiratory microbial communities at the microbial species level. In addition, by aligning metagenomics data with the reference catalogue, our work will facilitate assembly of the genomes of novel microbial genera or species, allow exploration of microbial functions and their associated microbial components, and allow the construction of whole microbial networks within the respiratory microbial community. Established reference gene sets can be employed to deepen multi-omics analysis, which will further our understanding of host–microorganism interactions in acute respiratory infection. One example of how these gene sets might be used is in the comparison of the OP microbiota of healthy and diseased children.

Methods

Clinical detection of infectious pathogens

Broncho-alveolar lavage fluid was used to identify common microorganisms of the microbiota. Culturing was conducted to detect *Streptococcus pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Staphylococcus haemolyticus*. The D3 Ultra DFA Respiratory Virus Screening and ID Kit (Diagnostic

Hybrids, Inc., Athens, OH, USA) was used to detect common viruses, including adenovirus (AdV), respiratory syncytial virus (RSV), influenza virus and parainfluenza virus. Cytomegalovirus (CMV) and Epstein–Barr virus (EBV) were detected using the Diagnostic Kit for Quantification of Human CMV DNA and EBV Polymerase Chain Reaction (PCR) Fluorescence Quantitative Diagnostic Kit, respectively (DaAnGene, Guangzhou, China). *M. pneumoniae* and *Chlamydia pneumonia* were diagnosed using a diagnostic kit for *M. pneumoniae* DNA (PCR Fluorescence Probing; DaAnGene) and Anti *C. pneumoniae* ELISA (IgM) (EUROIMMUN AG, Lübeck, Germany), respectively.

Construction and annotation of the RMGC

Sequencing data were filtered using a previously reported method [61]. Samples with ≥650 Mbp data (Figure 1, Supplementary Figure 3) were selected for genome assembly using SOAPdenovo [62] (SOAPdenovo, RRID:SCR_010752, v2.07, –F –K 39 –M 3 –d 1). For samples with <650 Mbp data, data from the same respiratory site were mixed and assembled (Figure 1). Assembled contigs with ≥500 bps were

372 selected for gene prediction with MetaGeneMark [63] (v3.26, default parameters). We
373 applied Glimmer3.02 [64] (Glimmer, RRID:SCR_011931, default parameters) to
374 predict genes from the 1,384 respiratory bacterial genomes in the Integrated Microbial
375 Genomes and Microbiomes (IMG) database (2016-12-21 [65]). Gene sequences were
376 also retrieved from the genomes of 73 respiratory bacteria in the Pathosystems
377 Resource Integration Center (PATRIC) database (2017-3-25 [66]) and 450,204 open
378 reading frames (ORFs) of respiratory bacteria in Human Microbiome Project (HMP)
379 (2016-10-20 [67]). Genes with a length ≥100 bp and without Ns (unidentified
380 nucleotides) were selected to construct non-redundant gene sets using CD-HIT [68]
381 (CD-HIT, RRID:SCR_007105, v4.66, -c 0.95 –aS 0.9). Genes with ≥2 mapped reads
382 were retained in the established RMGC.
383 Taxonomic annotation of genes was conducted as follows:
384 i) Bacterial and viral genome sequences were retrieved from IMG
385 (2016-12-21), PATRIC (2017-03-25) and National Center for
386 Biotechnology Information (NCBI) NT database (2016-08-09 [69]);. The
387 genome sequence with the longest N50 was selected as the representative

genome for each bacterial species. Non-redundant viral genomes were produced by CD-HIT (v4.66, -aS 0.95 -aL 0.9 -M 0). Gene sets in the RMGC were aligned to 6,869 representative bacterial genomes and 18,916 non-redundant viral DNA genomes using BLASTN (BLASTN, RRID:SCR_001598, v2.5.0, default parameters except –e 0.01)

ii) The top 10% highest-scoring alignments of each gene were retained, with ≥65% identity and ≥80% gene length coverage

iii) Assignment of each gene was determined based on ≥50% consensus above the similarity threshold for a specific rank: ≥65% for phylum, ≥85% for genus and ≥95% for species.

The functional annotation of each gene was determined by searching protein sequences in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (v78.1) database and eggNOG (version 4.0) with BLASTP (BLASTP, RRID:SCR_001010, v2.5.0, default parameters, except -e 1e-5). The best-hit alignment (identity ≥30% and coverage ≥70%) was selected as the functional annotation for the gene. Genes without annotations in KEGG were identified as novel gene families by the Markov Cluster

Algorithm (MCL) [70] (inflation factor=1.1, bit-score cut-off=60).

Comparing taxonomic assessments of 16S rRNA gene analysis and metagenomic analysis

Seventy-two OP microbial samples with ≥650 Mb metagenomic sequencing data were aligned to establish the RMGC to determine taxonomic assignments. The same samples were also sequenced via the V3–V4 region of the 16S rRNA gene [13]. Microbial compositions obtained by these two methods were compared to assess the accuracy of taxonomic assignments via metagenomic analysis.

Rarefaction analysis

We downsized the number of mapped reads to 3 million per sample to eliminate variable influence caused by the quantity of sequencing data. Estimation of total gene richness was done by randomly sampling five individuals 1,000 times using gene counting and Chao2 richness estimator [71].

To produce rarefaction curves of KEGG orthologous groups (KOs) and novel

gene families, saturation was evaluated by randomly sampling five individuals 1,000 times. Relative rarefaction curves were visualized using R software (v3.3.2).

Calculation of gene relative abundance in RMGC

Filtered reads of metagenomics data from each sample were aligned to the RMGC using BWA (BWA, RRID:SCR_010910, v0.7.13, default parameters, except for the mem and identity ≥95%). Alignments meeting the following two criteria were accepted: i) paired-end reads mapped onto the same gene with the correct insert size; and ii) one end of a paired-reads was mapped onto the end of a gene, while the other was located outside of the gene.

If the number of genes in a given sample was n, the relative abundance was calculated using the following steps:

1. The copy number of the gene *i* ($c(i)$) was calculated as:

$$c(i) = \frac{t(i)}{l(i)}$$

where $t(i)$ is the total number of mapped reads of gene *i* in a given sample, and $l(i)$ is the length of the gene *i*.

2. The relative abundance of gene *i (Ab_g(i))* was defined as:

$$Ab\_g(i) = \frac{c(i)}{\sum_{1}^{n} c(i)}$$

3. If m genes can be assigned to the phylogenetic assignment s, the abundance of this phylogenetic assignment *(Ab_p(s))* was calculated using the following equation:

$$Ab\_p(s) = \sum_{1}^{m} Ab\_g(j)$$

Phylogenetic and functional profile of the OP microbiome

All filtered reads of the OP microbiota were aligned to the RMGC using BWA with same parameters as described above. The relative abundance of each phylogenetic assignment was calculated as shown above, while the abundance of KOs in the functional profiling table was determined as described in a previous report [59].

Identification of core OP microbial species in healthy children

Microbial species were selected as 'core' species if they existed in more than 50% of healthy children and had more than 1% relative abundance in one OP microbial sample. Distributions of core microbial species in the OP of healthy children were

described using ggplot2 in R.

Comparison of the OP microbiota between healthy children and children with MPP

Thirty-three healthy children were chosen, with a similar age distribution to that of the 34 children with MPP (data size ≥650 Mbp). Genes in the OP microbiota of selected microbial samples were clustered into co-abundance gene groups (CAGs) via Capony-based algorithms [72] (default parameters). Selected CAGs containing more than 700 genes were regarded as being derived from the same bacterial genome and were selected to construct a correlation network using Spearman's rank coefficient (≤–0.6 or ≥0.6). The co-occurrence network was visualized using Cytoscape (v3.4.0) [73]. If ≥50% of the included genes had consensus phylogenetic annotations, the corresponding CAG was assigned to a related microbial taxonomic assignment.

The relative abundance of each CAG in our microbial samples was calculated as previously reported [60]. Intergroup comparisons between CAGs and KEGG functions were performed using the two-tailed Wilcoxon rank-sum test and corrected

via the Benjamini-Hochberg method (adjusted *P*-value ≤0.05). Confounding factors including pneumonia, sex, age, delivery mode and feed pattern were also assessed using permutational analysis of variance (PERMANOVA) by vegan package (v2.3-4) in R.

Single microbial genome assembly from OP metagenomic data

OP metagenomic data were aligned to the filtered CAGs (those containing ≥700 genes) by BWA (v0.7.13, identity ≥95%). The mapped reads of each CAG were extracted for microbial genome assembly with Velvet [74] (Velvet, RRID:SCR_010755, kmer: from 45 to 75, cov_cutoff: auto, exp_cov: auto). The assembled sequences with the longest contig N50 were selected as representative draft genomes. Assembly quality was assessed using the following six criteria [24]: (i) 90% of the genome assembly included in contigs >500 bp; (ii) 90% of the assembled bases at >5× read coverage; (iii) contig N50 >5 kb; (iv) scaffold N50 >20 kb; (v) average contig length >5 kb; and (vi) >90% of core genes present in the assembly. Fourteen draft microbial genomes passed five or six of these criteria (Supplementary Table 3). The assembly quality

estimation standard published by the Genomic Standards Consortium (GSC) was then applied (Supplementary Table 3) [75]. The microbial species designation of 14 assembled genome sequences followed these standards: 1) concordance with taxonomical assignment of CAGs [72]; 2) aligned to the genome sequences published by IMG, NCBI and PATRIC via BLASTN (v2.5.0, default parameters except –e 0.01), with ≥95% nucleotide identity and ≥95% genome coverage; and 3) assigned by CheckM (CheckM, RRID:SCR_016646, v1.0.12, default parameters) from the Genome Taxonomy database [76]. Furthermore, gene prediction was executed with Glimmer3.02 (Glimmer, RRID:SCR_011931), while related annotations of antibiotic resistance and virulence genes were acquired through CARD [77] and VFDB [78]. The SNP mutation associated with macrolide resistance of *M. pneumoniae* was identified by mapping sequencing reads against 23S rRNA genes [79] using BWA.

Correlations between reassembled microbial genomes and disease severity in MPP patients

The correlation between reconstructed microbial genomes and hospitalization

duration and fever peak was assessed using R software, a well as serum CRP, PCT and eosinophil levels at 24 hours after hospitalization. The distributions of relative abundance of 14 reassembled genomes in children ith MPP and healthy children were showed via scatter plot.

Availability of supporting data and materials

The BioProject ID for this study is PRJNA413615. The sequencing data supporting the results of this article are available in the GenBank repository under accession number SRP119571. The gene profiles are freely accessible on RMGC database [20]. All supporting data and materials are available in the *GigaScience* GigaDB database [80].

Declarations

List of abbreviations

AdV, Adenovirus; ARI, Acute respiratory infection; BALF, Broncho-alveolar lavage fluid; CAG, Co-abundance gene group; CMV, Cytomegalovirus; EBV, Epstein–Barr

virus; GM, Gut microbiome; KEGG: Kyoto Encyclopedia of Genes and Genomes; KO, KEGG orthologous group; MCL, Markov cluster algorithm; NP, Nasopharynx; OP, Oropharynx; ORF, Open reading frame; PCA, Principal component analysis; PCR, Polymerase chain reaction; PERMANOVA, Permutational multivariate analysis of variance analysis; PP, Paediatric pneumonia; RM, Respiratory microbiome; RMGC, Respiratory microbial gene catalogue; RSV, Respiratory syncytial virus.

Ethics approval

Ethical approval for this study was obtained from the Ethical Committee of Shenzhen Children's Hospital (Shenzhen, Guangdong Province, China) under registration number 2016013. All experiments were performed under the relevant guidelines and regulations. Guardians of all children included in this study provided their informed consent to participate.

Consent for publication

All authors have given their consent to publish the manuscript.

Competing interests

The authors declare no that they have no competing interests.

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Author contributions

Y.Z., Y.Y. and K.Z. managed the project. Z.L., G.X. and Y.B. collected samples and information. W.W. and Q.Y. prepared the DNA extraction. D.L., Q.Z., X.F. and Z.Y. performed the bioinformatics analysis. C.Q., Y.L. and Y.L. optimized the graphs. X.X. and M.L. optimized the data curation. S.L. and Y.Y. guided data interpretation. X.F.

developed the website. H.W. and W.D. mined the data and wrote the paper. K.S. and K.Y. polished the article. All authors read and approved the final version of the manuscript.

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804 **Tables**

805 **Table 1.** Sample information

Characteristics	Pneumonia patients (n=76)	Healthy children (n=171)
Gender		
Female	24	87
Male	52	84
Age (years)	2.9 (0.2–12.7)	4.3 (0.1–8.9)
Sampling site		
OP	75	171
NP	42	-
Lung	46	-
Delivery mode		
Vaginally born	46	102
Caesarean section	30	69
Feeding pattern		
Breast	48	84
Breast + milk	12	66
Milk	16	21
Family history of allergy	1	-
History of pneumonia	14	-
Asthma	-	-
Clinical symptoms		
Lung consolidation, atelectasis, infiltration	76	NA
Fever	44	-
Cough	72	-
Wheezing	20	-
Hospitalization time (days)	9 (2–37)	-
CRP (<0.499 mg/l)	22	NA

PCT (<0.5 ng/ml)	73	NA
Eosinophils (0.5–5%)	44	NA

-, no detection result; NA, not available; CRP, C-response protein; PCT, procalcitonin

Figure Legends

Figure 1. **Construction of the human Respiratory Microbial Gene Catalogue**.

Genome assembly was performed for each sample with ≥650 Mbp of data. For samples with <650 Mbp of data, data from the same respiratory site (NP, OP or lung) were mixed and assembled. Gene predictions were conducted for all assembled contigs with ≥500 bp and respiratory bacterial genomes in the Integrated Microbial Genomes and Microbiomes (IMG) database. Genes with ≥100 bp were retained. Respiratory gene sets from the Human Microbiome Project (HMP) and Pathosystems Resource Integration Center (PATRIC) were combined to construct the non-redundant Respiratory Microbial Gene Catalogue (RMGC) containing 2,245,343 genes.

Figure 2. Rarefaction curves for genes and KEGG orthologous groups/gene families.

a) Rarefaction curve for gene count.

b) Rarefaction curve for Chao2. The Respiratory Microbial Gene Catalogue (RMGC) captured 90.52% of the prevalent genes.

c) Rarefaction curve for KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologous groups/gene families. Known functions saturate quickly to 6,346 groups. After including novel gene families, the rarefaction curve plateaus when 12,924 groups are detected. Boxes represent the interquartile ranges (IQRs) between the first and third quartiles and the line inside the box represents the median value. Whiskers represent the lowest or highest values within values 1.5 times the IQR from the first or third quartiles. Circles represent data points located outside of the whiskers.

Figure 3. Core microbial species in healthy OP microbiota.

Upper bar plot represents the prevalence of each core species; lower box plot shows the relative abundance of each core species. Different colours refer to different phyla.

Figure 4. Differentiation of oropharyngeal microbial samples between healthy

838 **children and children with *Mycoplasma pneumoniae* pneumonia.**

839 **a)** Gene counts in the oropharyngeal (OP) microbiotas of healthy children and

840 children with *Mycoplasma pneumoniae* pneumonia (MPP).

841 **b)** Alpha diversity of the OP microbiota in healthy children and children with MPP.

842 Boxes represent the interquartile ranges (IQRs) between the first and third quartiles

843 and the line inside the box represents the median. Whiskers represent the lowest or

844 highest values within values 1.5 times the IQR from the first or third quartiles. Points

845 represent data located outside of the whiskers. *** $P \leq 0.001$.

846

847 **Figure 5. Phylogenetic and functional alterations in children with pneumonia.**

848 **a)** Size of the circle represents the average relative abundance of co-abundance gene

849 groups (CAGs) in healthy children or children with *Mycoplasma pneumoniae*

850 pneumonia (MPP). A line between two circles indicates a Spearman's rank correlation

851 coefficient ≥0.6 and an adjusted *P*-value ≤0.05. The phylum and genus corresponding

852 to each CAG are indicated by the information listed on the left.

853 **b)** X-axis represents level 2 functional categories in the Kyoto Encyclopedia of Genes

and Genomes (KEGG); colours of the characters represent level 1 functional categories (listed on the right) Y-axis shows the relative abundance of level 2 functional categories. Adjusted P-values at *, ≤0.05; **, ≤0.01; and ***, ≤0.001, respectively.

Figure 6. Virulence factor genes and antibiotic resistance genes on the *Mycoplasma pneumoniae* genome.

Tracks from outside to inside represent antibiotic resistance genes (ARGs), genes on the plus strand, genes on the negative strand and GC skew, respectively. Virulence factor genes (VFGs) painted with different colours refer to the different types of VFGs.

Figure 7. Comparison of relative abundance of 14 re-assembled genomes between healthy children and children with *Mycoplasma pneumoniae* pneumonia.

Blue circles and red triangles represent the microbial relative abundance of healthy children and children with *Mycoplasma pneumoniae* pneumonia (MPP)**.** Solid dot and

paired whiskers represent the mean and SD of each microbial relative abundance. P-values at *, ≤0.05; **, ≤0.01; and ***, ≤0.001, respectively. NS, not significant.

Figure 1

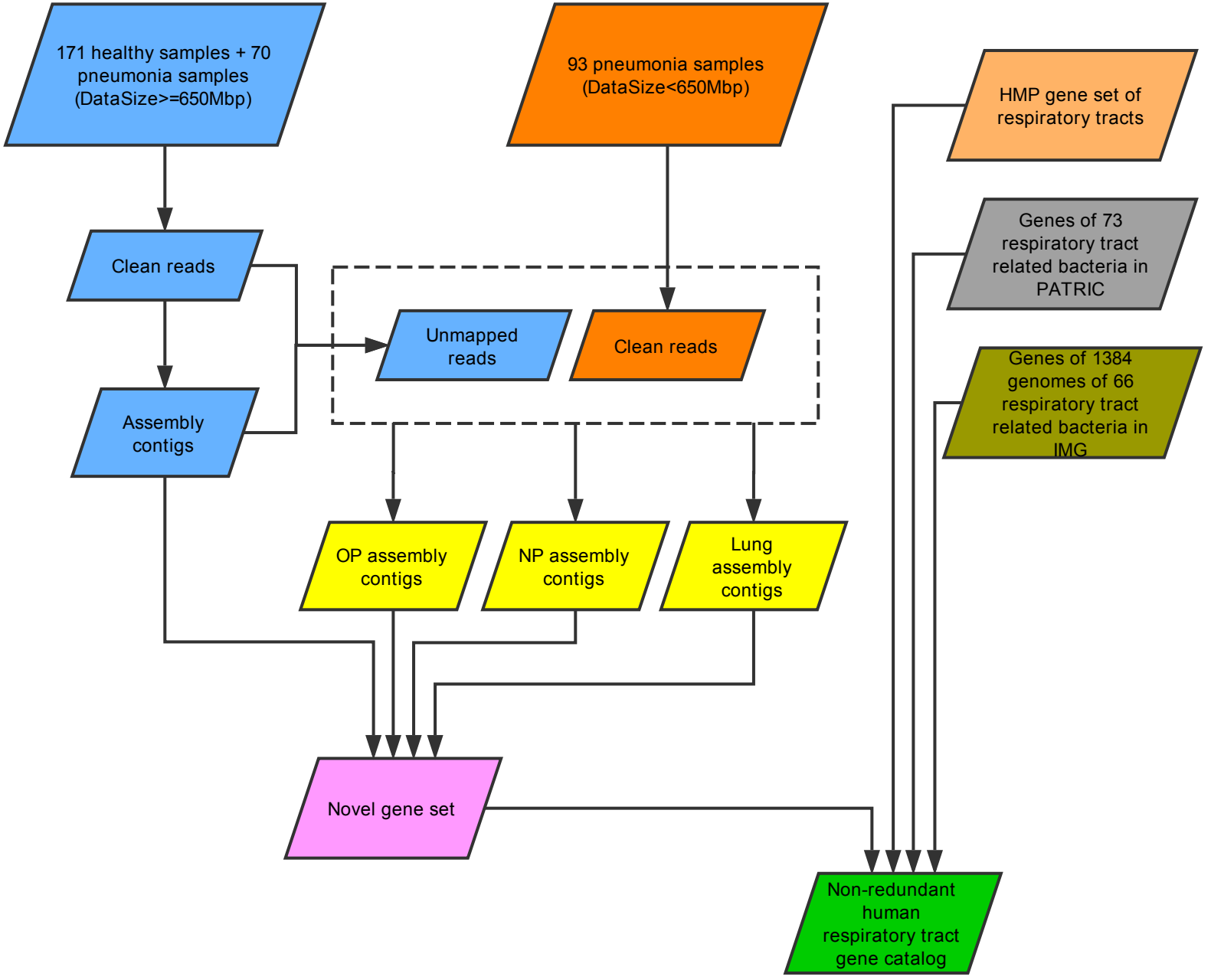

Figure 2

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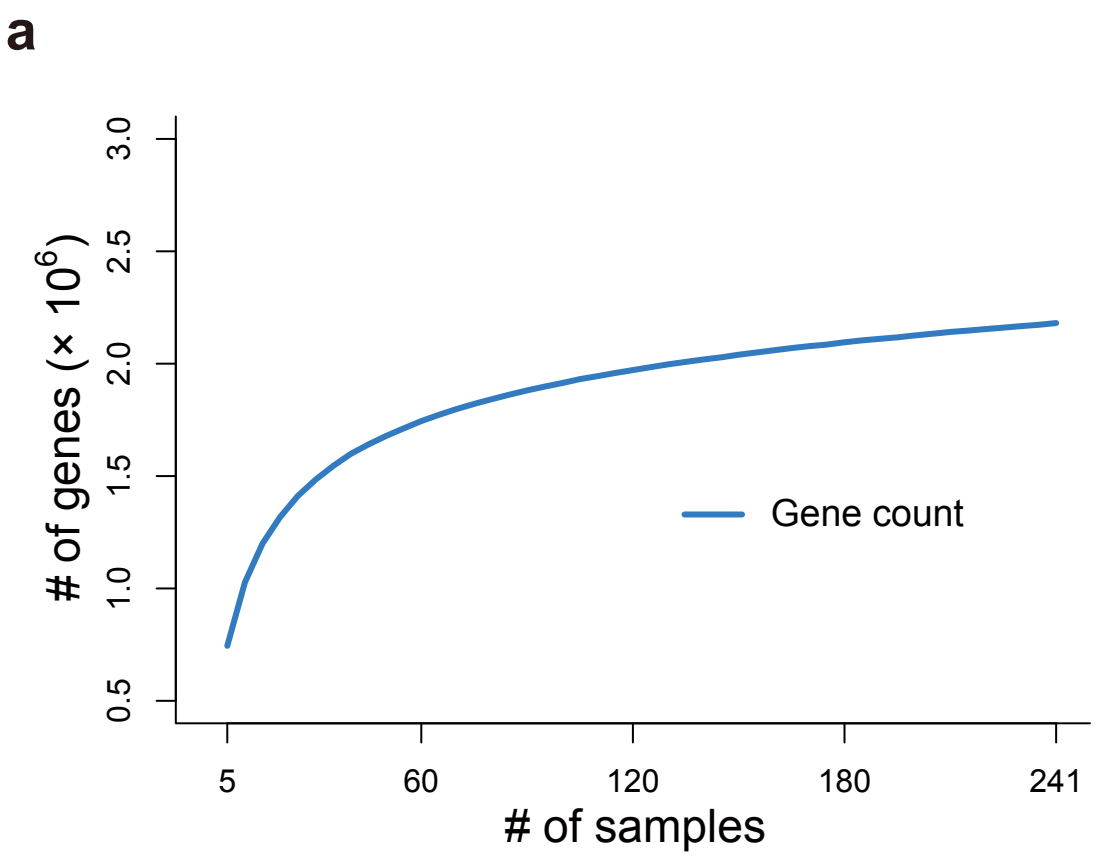
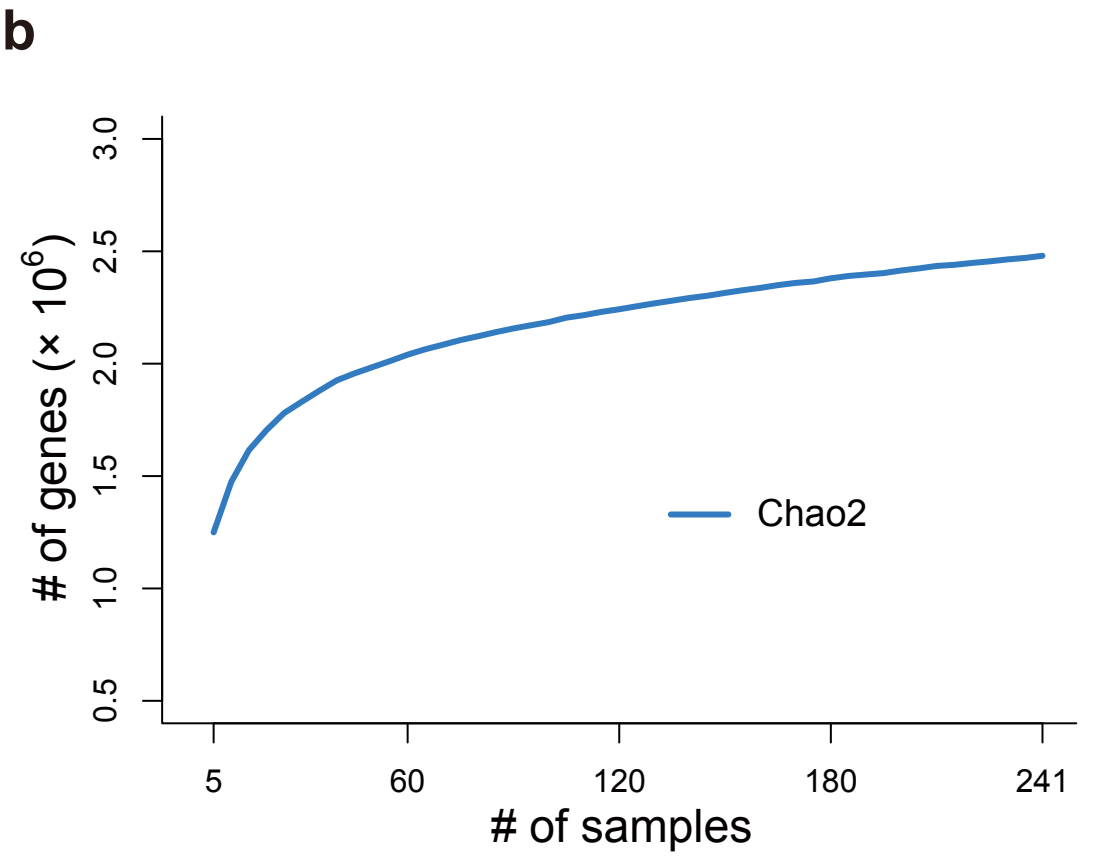




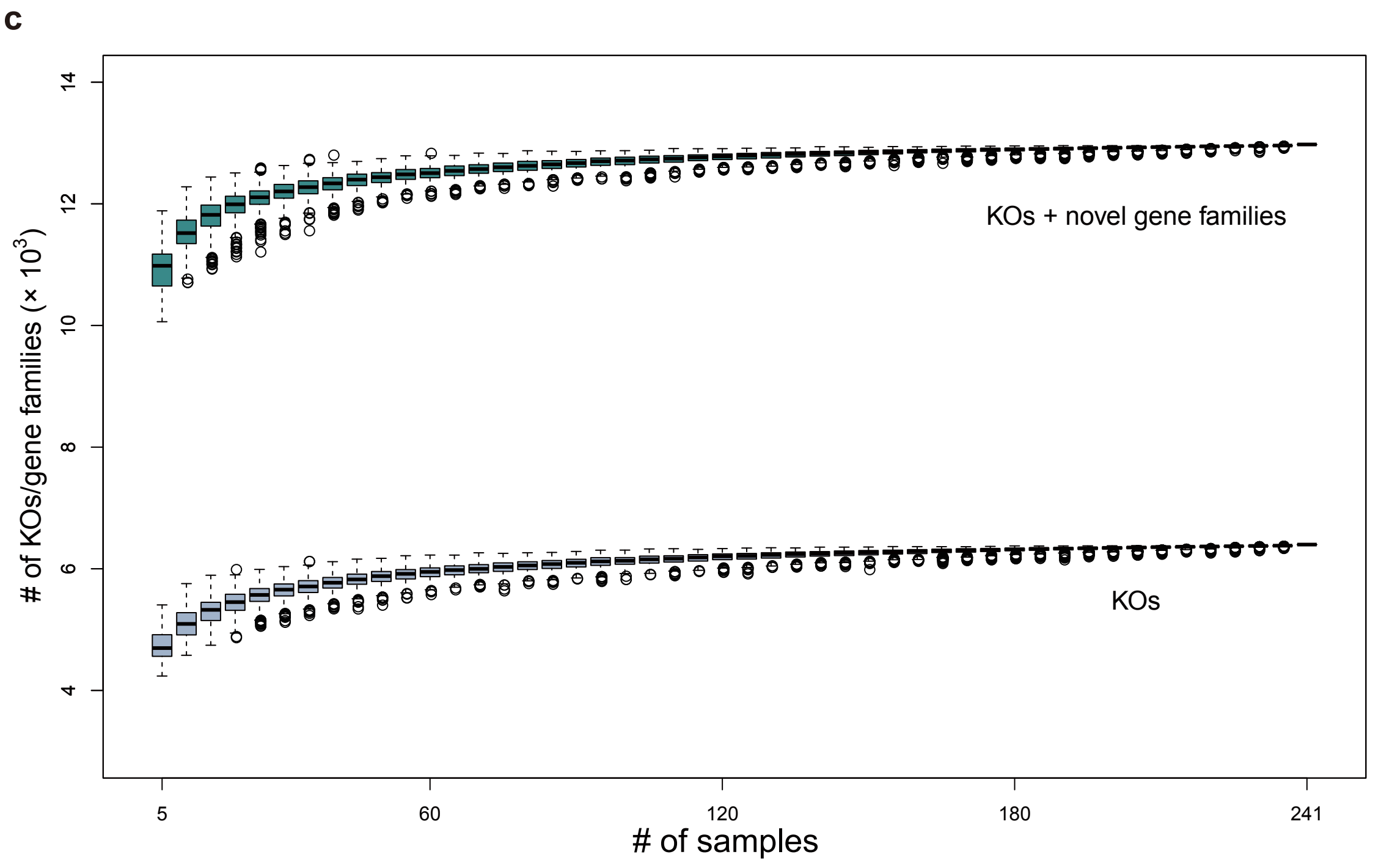
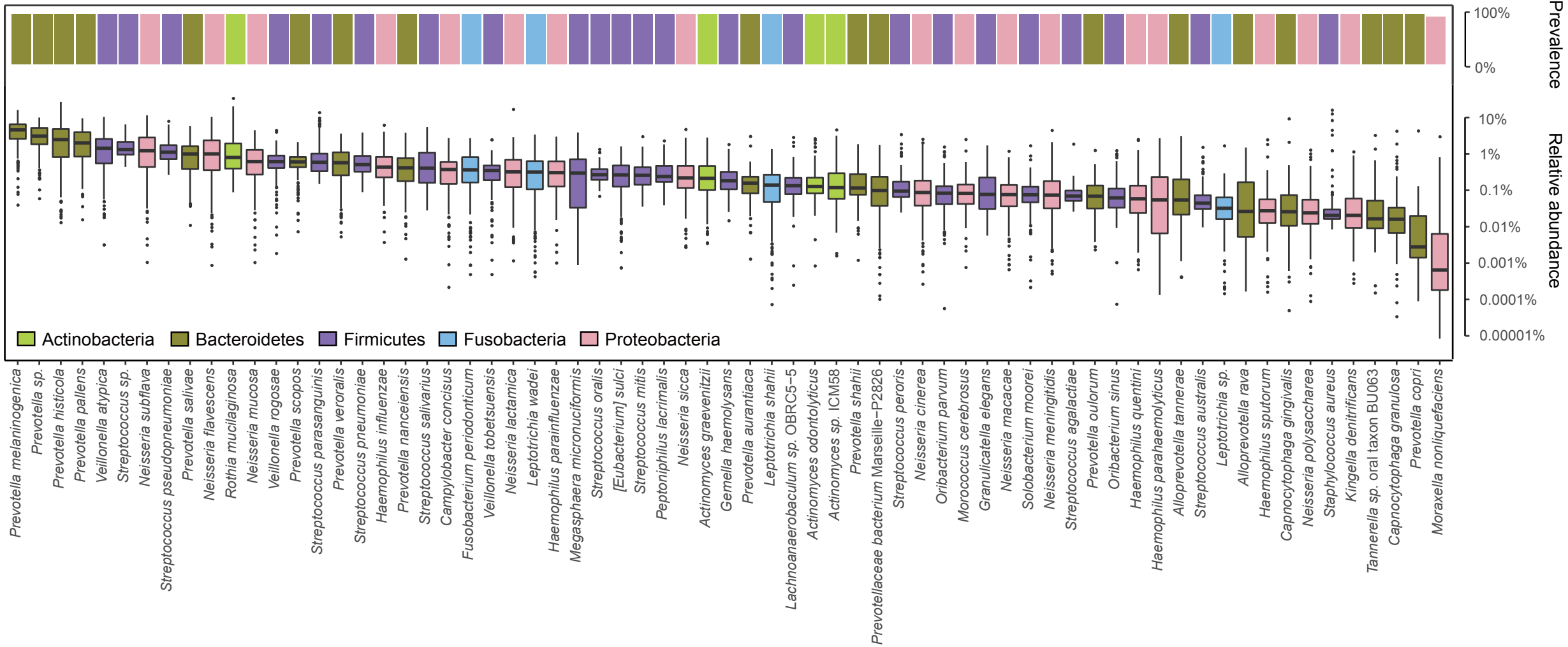


Figure 3

Core microbial species of the healthy children's oropharynx



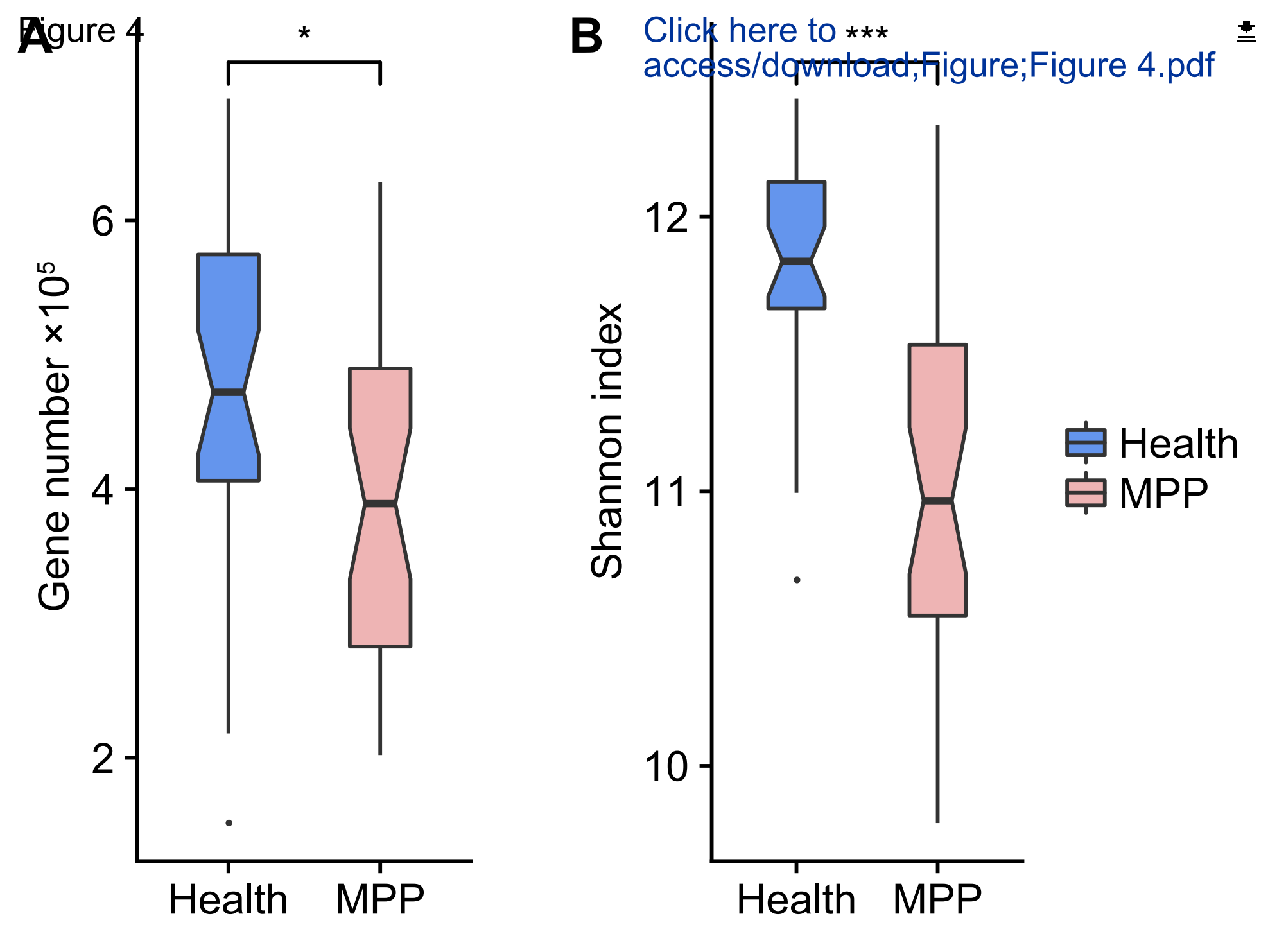


Figure 5

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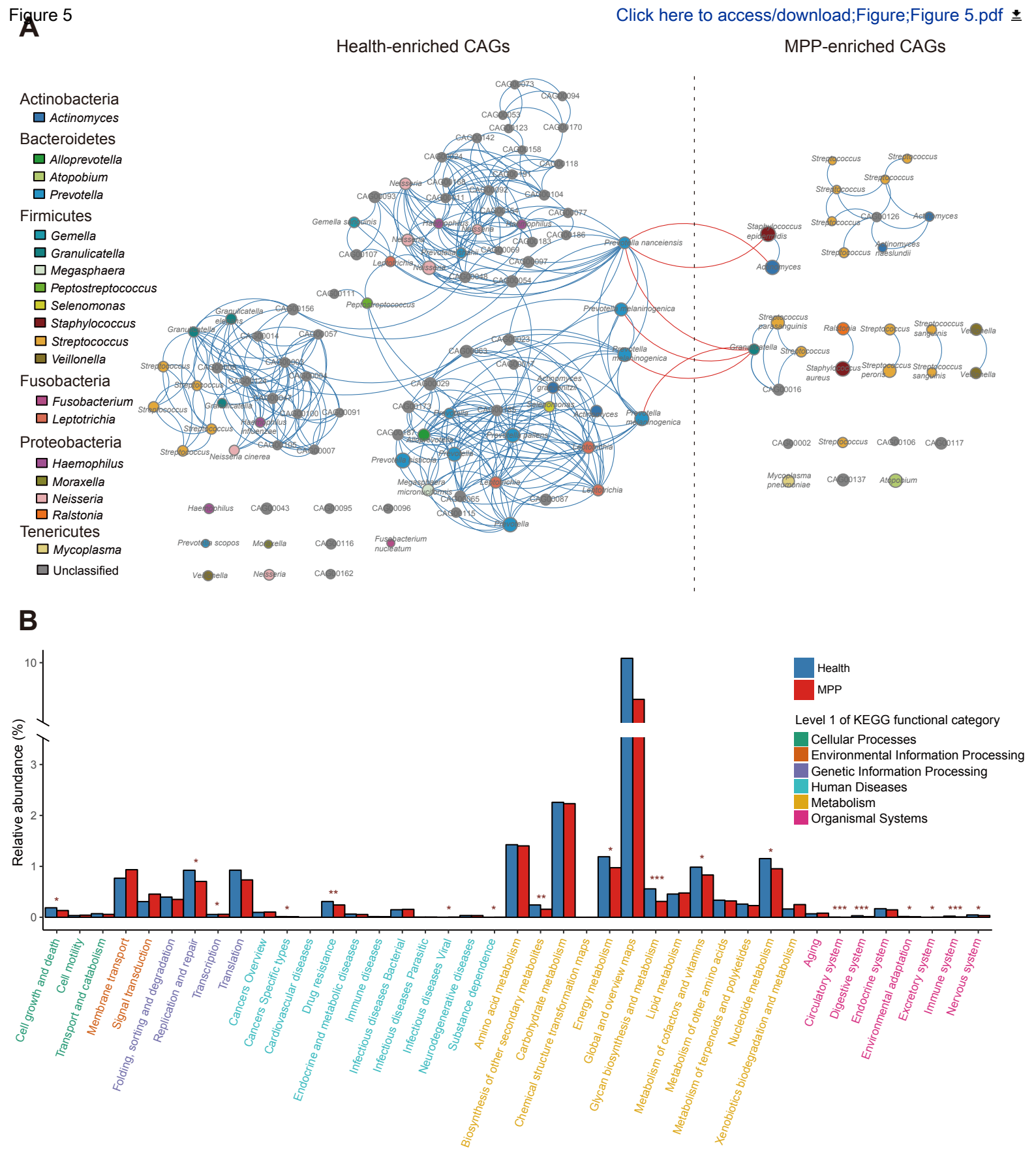

Figure 6

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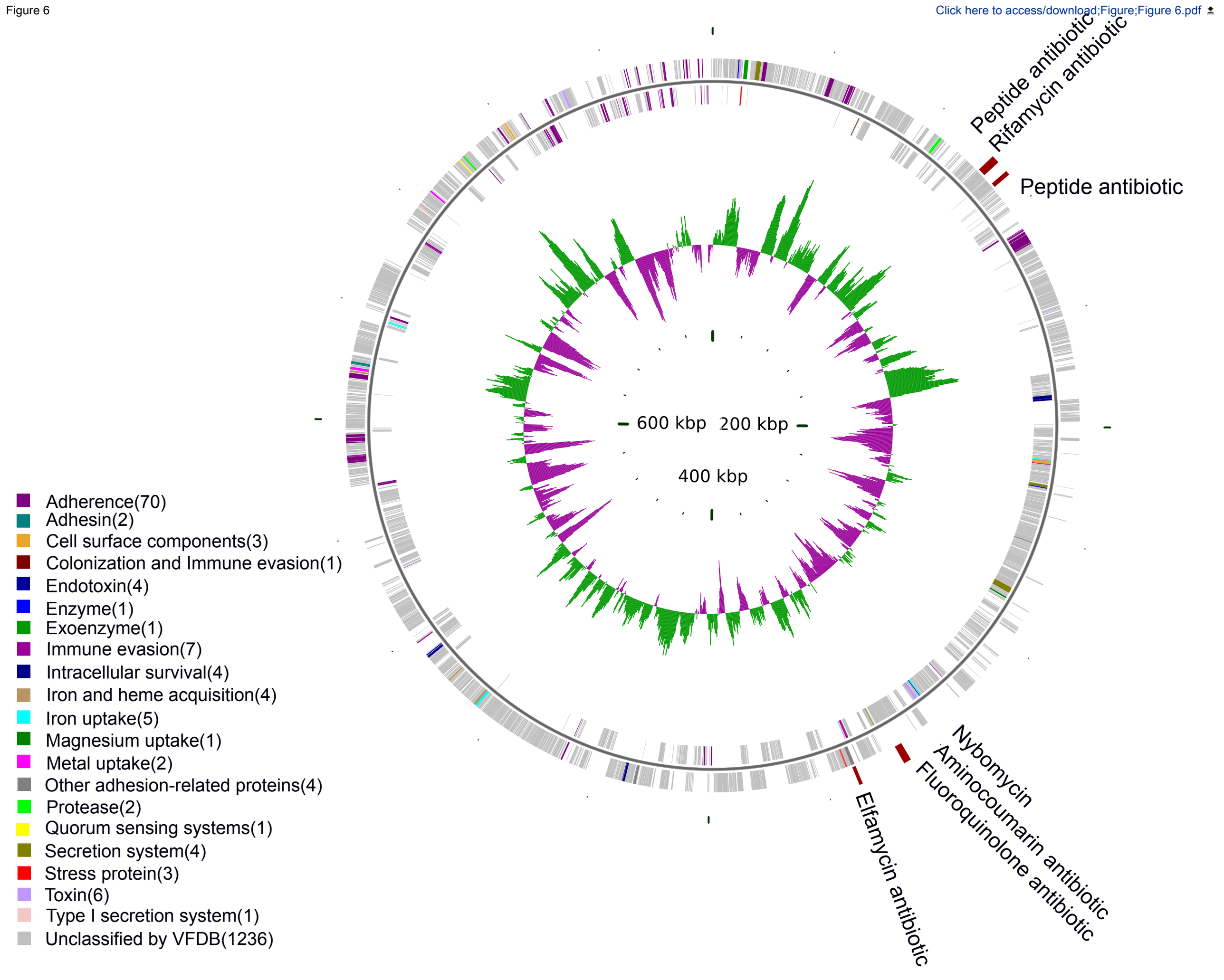

Figure 7

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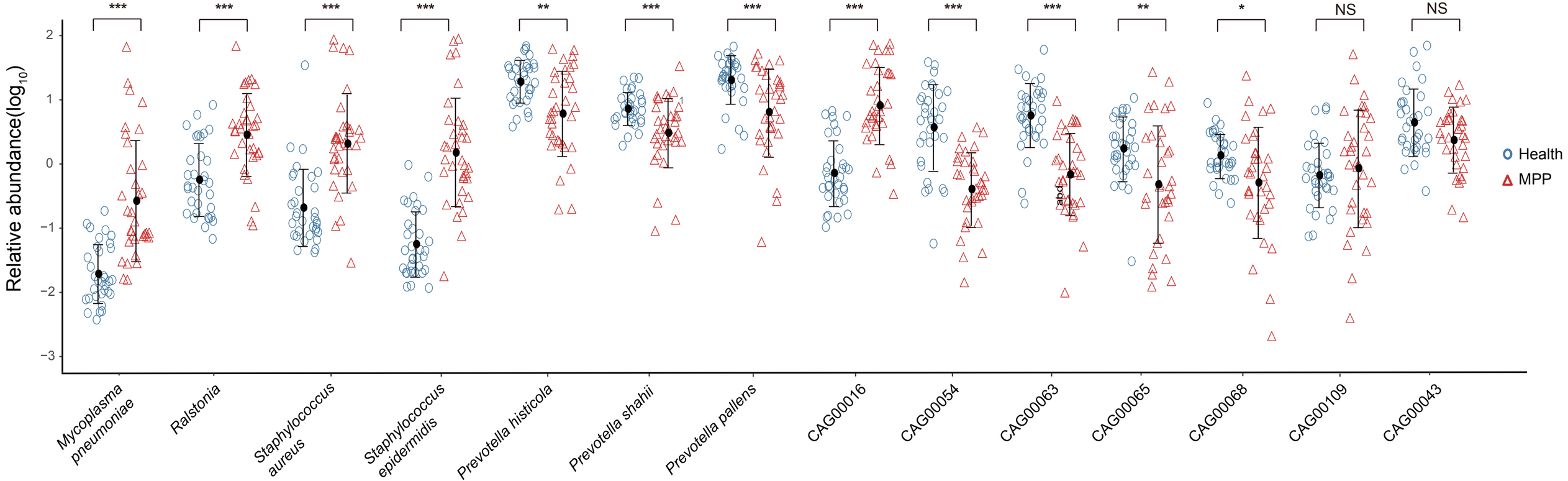

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

Supplementary Table 1

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