

Single-bacterial cell insights into mechanisms of ceftriaxone resistance in *Neisseria subflava*

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The contribution of airway pathobionts to chronic respiratory disease is increasingly recognized, yet the evolutionary processes that shift commensals to pathogens remain poorly understood. Here we investigate how antibiotic pressure drives adaptation in *Neisseria subflava*, a common airway commensal associated with bronchiectasis. Using serial passage under ceftriaxone exposure, we observe a >300-fold increase in resistance, accompanied by enhanced biofilm formation and genetic reprogramming. Whole-genome sequencing reveals recurrent mutations in the adhesin gene *ataA*, while single-cell transcriptomics identifies six functionally distinct clusters indicating adaptive programs in growth, metal homeostasis, oxidative stress, and cell-wall remodeling. Notably, biofilm integrity is maintained through compensatory upregulation of *comP* and *bamE*, which promotes phagocytic evasion and resistance in experimentally evolved strains and clinical isolates. Iron availability further stabilizes biofilm and modulates antibiotic tolerance, underscoring metal homeostasis as a contributory adaptive axis. Together, these findings reveal a multifaceted strategy by which *N. subflava* exploits antibiotic selection to transition towards pathogenicity. By integrating experimental evolution with single-cell resolution, we establish a framework for understanding the commensal-to-pathobiont transition, with broad implications for the airway microbiome and antimicrobial resistance in chronic respiratory disease.

The dynamic interplay between host and microbiome has reshaped our understanding of airway pathology, highlighting the microbiome as a critical determinant of respiratory health and disease^{1–3}. Advances in high-throughput molecular profiling and computation have enabled holistic analyses of airway microbial communities^{2,4}, uncovering novel mechanisms of pathogenesis that extend beyond canonical pathogens^{5–11}. Within this framework, the concept of pathobionts, microorganisms that are typically commensal but

acquire pathogenic potential under specific conditions, has emerged as a central theme in chronic airway disease^{9,11–14}.

Bronchiectasis exemplifies this complexity. It is a chronic inflammatory disorder of the airways, characterized by recurrent infection, heterogeneous clinical presentation, and notable geographic variation. Large-scale microbiome studies implicate both bacteriome and mycobiome composition, interkingdom interactions, host immune responses, and antimicrobial resistance determinants in its pathophysiology^{15–19}.

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Notable members of the Neisseriaceae, particularly *Neisseria subflava*, have recently been associated with bronchiectasis in Asian populations^{13,20–22}. Long regarded as a commensal, *N. subflava* can disrupt epithelial barriers, impair trans-epithelial electrical resistance, and provoke inflammation, thereby driving airway remodelling and immunopathology distinct from established pathogens such as *Pseudomonas aeruginosa*¹⁸. Related observations in Chronic Obstructive Pulmonary Disease (COPD) further highlighting the complexity of pathobiont biology and the influence of the environment on their pathogenesis²³.

Understanding the commensal-to-pathobiont transition is therefore a critical challenge in microbiology^{10,18}. While *Neisseria meningitidis* illustrates the devastating potential of such shifts in the upper airway, the mechanisms underlying more transitions in organisms such as *N. subflava* remain unresolved^{24–26}. Evolutionary pressures within the host, particularly antibiotic exposure, are hypothesized to accelerate adaptation and virulence in commensals, paradoxically, fostering dysbiosis and resistance while treating acute infections^{10,27,28}. Yet, how airway commensals adapt under antibiotic pressure at the genetic and functional levels remains poorly defined²⁹.

Here, we address this knowledge gap by combining experimental evolution with single-bacterial cell transcriptomics to investigate the adaptive responses of *N. subflava* under ceftriaxone selection. We identify convergent genomic and transcriptional changes involving biofilm maintenance, iron-responsive programs, and cell-wall remodeling that together support increased antibiotic tolerance features consistent with pathogenic potential. By delineating the molecular strategies underlying the commensal-to-pathobiont transition, this work provides new insights into the evolution of the airway microbiome and its implications for chronic respiratory disease.

Results

Experimental evolution of antibiotic resistance and biofilm formation in *Neisseria subflava*

Neisseria subflava, a commensal of the oral cavity and upper airway, has limited documented antibiotic resistance^{30,31}. To establish a baseline antibiotic susceptibility, we performed dose-response assays using seven antibiotic classes on the clinical isolate SG-KL01 (SG001) obtained from a bronchiectasis patient with a high *N. subflava* burden¹⁸ (Supplementary Fig. 1a–k). Due to the absence of established clinical breakpoints and specific PK/PD parameters for *N. subflava*, minimum inhibitory concentration (MIC) values served as the primary reference. Initial profiling showed relatively high MIC values (>0.9 µg/ml) for gentamicin, tetracycline, kanamycin, and ofloxacin, whereas extended-spectrum cephalosporins (ESCs), such as ceftriaxone (Cef) and cefotaxime (CTX), exhibited consistently low MICs, confirming their clinical utility against *Neisseriaceae* infections (Supplementary Table 1).

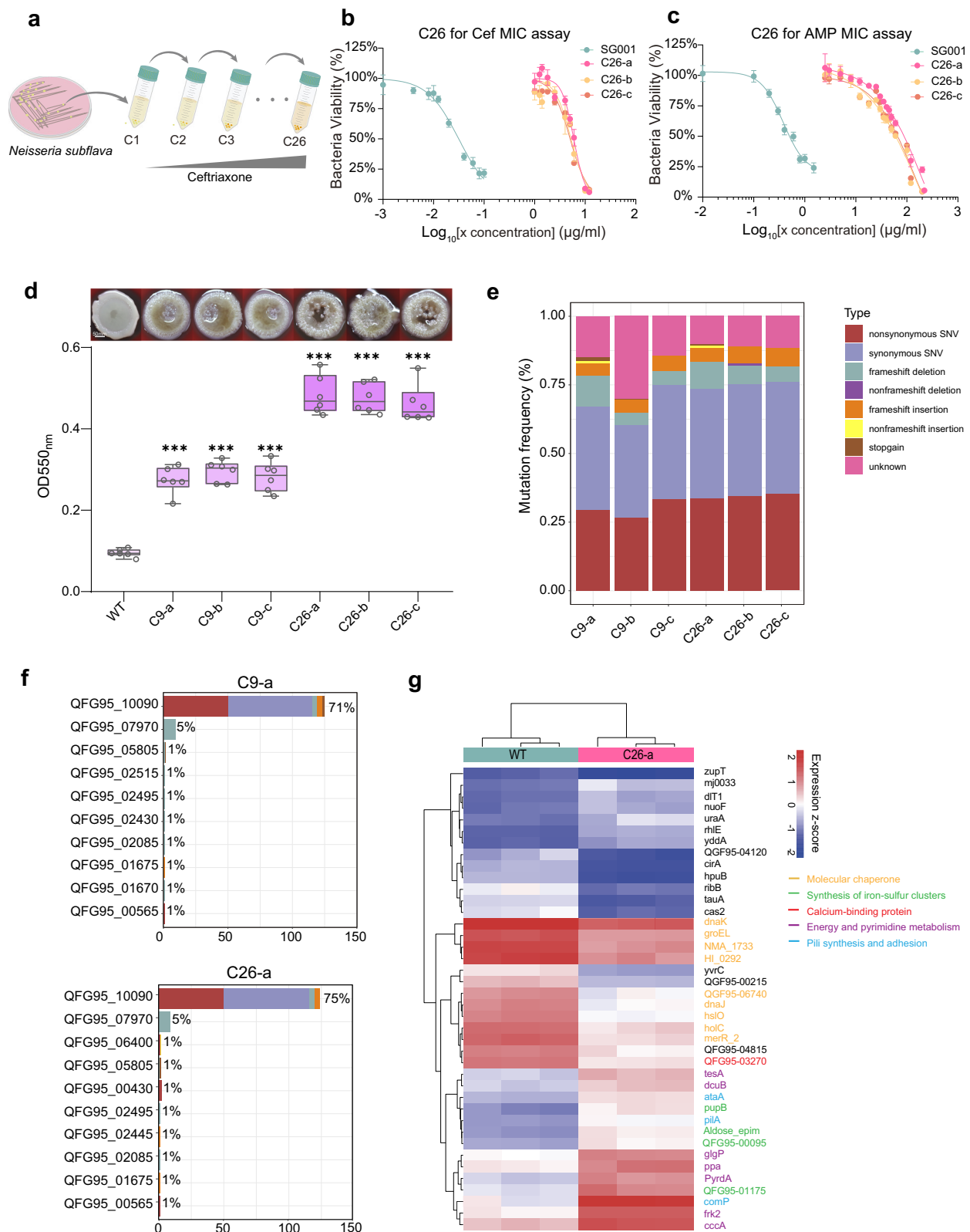
Ceftriaxone is a third-generation cephalosporin widely used in respiratory infections and a key agent in the treatment of *Neisseriaceae*-associated infections^{32,33}. Despite its broad-spectrum activity, serial ceftriaxone exposure of three independent SG001 lineages (Ca, Cb, Cc) over 26 generations (Fig. 1a) resulted in a 300-fold increase in ceftriaxone MICs (>5 µg/ml versus 0.02 µg/ml in the parental strain) (Fig. 1b). These evolved lineages also displayed cross-resistance to other β-lactams (ampicillin and penicillin G) while retaining sensitivity to antibiotics outside this class (Fig. 1c, Supplementary Fig. 2a–h, and Supplementary Table 2), indicating β-lactam-specific resistance development. Concomitantly, all three C26 lineages (C26-a, C26-b, and C26-c) exhibited pronounced colony wrinkling and increased biofilm formation relative to both wild-type and 9th-generation strains (C9-a, C9-b, and C9-c) as confirmed by Congo Red and crystal violet assays (Fig. 1d). Resistance was evident as early as generation 9 (C9-a, C9-b, and C9-c) with MICs of >0.08 µg/ml in these strains (Supplementary Fig. 2i), suggesting that biofilm development and resistance co-evolve under antibiotic resistance pressure^{34–36}.

Systems-level analysis of ceftriaxone-resistant *Neisseria subflava* strains

To investigate the molecular mechanisms underlying resistance, we performed long-read genome sequencing of evolved strains (Fig. 1e). All assemblies were supported by high coverage and were polished with Illumina short reads, ensuring high confidence in mutation calls. We identified predominant mutations in the *ataA* gene (QFG95_10090), accounting for 63–77% of the total mutations (Fig. 1f, Supplementary Fig. 2j, and Supplementary Data 1). The *ataA* gene, involved in bacterial adhesion and biofilm formation³⁷, harbored diverse mutational events, including nonsynonymous and synonymous single-nucleotide variations (SNVs), as well as frame-shift insertions and deletions. Mutations in other genes, including *mltA* (QFG95_07970) and QFG95_05805, indicated parallel genomic adaptations under antibiotic pressure.

To confirm independent evolutionary trajectories, we analyzed a SNP-based dendrogram, which supported related but distinct lineages among the replicates (Supplementary Fig. 3a). Because SNPs, especially synonymous ones, can recur under similar selective pressures, we further profiled structural variants (SVs) in C9 lineages using high-resolution long-read sequencing. Large insertions, deletions, duplications, and complex rearrangements were hierarchically clustered by genomic position and variant types, revealing lineage-specific SV patterns unique to each replicate population (C9-a, C9-b, and C9-c) (Supplementary Data 2, and Supplementary Fig. 3b, c). Such SV patterns are highly unlikely to arise from parallel evolutionary processes, unlike SNPs, thereby supporting independent evolutionary trajectories and excluding the possibility of cross-contamination among replicates^{38–40}. Consequently, the observed convergence in SNP patterns across the lineages thus likely reflected shared selective pressures rather than technical artifacts.

Phenotypically, resistant strains demonstrated enhanced, rather than impaired, biofilm formation, suggesting the presence of compensatory regulatory pathways beyond *ataA* mutations. Bulk transcriptomic profiling supported this, revealing upregulation of genes involved in biofilm development, pilus synthesis (*comP*), iron metabolism (*pubB*), pyrimidine biosynthesis (*pyrDA*), carbohydrate metabolism (*frk2*), and *ataA* itself in the evolved strain compared to wild-type (Fig. 1g, and Supplementary Data 3). Functional gene ontology analyses indicated the coordinated induction of genes involved in cell adhesion and two-component regulatory systems (Supplementary Fig. 2k, Supplementary Data 4), which are essential for biofilm formation and environmental stress responses. Importantly, bulk RNA-seq of the additional evolved lineages (C26-b and C26-c) revealed transcriptional profiles highly consistent with those of C26-a (Supplementary Fig. 3d), reinforcing a common transcriptomic rewiring across independently evolved replicates. Notably, in addition to *comP* and *bamE*, several genes that are directly involved in biofilm matrix construction, such as QFG95_07045 (murein-L,D-transpeptidase), *murD*, *milC*, *murE*, and *pgpA*, were upregulated across resistant lineages. The metal-ion transporter *mntB* (Mn/Fe uptake) was likewise increased, consistent with enhanced metal homeostasis in the resistant populations. Additionally, enrichment analysis further confirmed convergence on biofilm-formation programs in C26-b and C26-c (Supplementary Fig. 3e). Although the composition of upregulated genes differed across lineages, numerous loci involved in biofilm formation, iron metabolism, and cell-envelope biogenesis were recurrently elevated, albeit with lineage-specific magnitudes. Together with our genomic analyses, these results indicate that sustained antibiotic exposure drives complex adaptive responses in *N. subflava*, where genomic mutations and transcriptomic rewiring converge to promote antimicrobial resistance. The convergence of biofilm reinforcement and envelope remodeling highlights the plasticity of this commensal species and its capacity to transition toward antibiotic-resistant pathobiont behavior.



Bacterial heterogeneity drives the adaptive evolution of antibiotic resistance in *Neisseria subflava*

To explore biofilm-related gene expression and functional diversity at single-cell resolution, we performed single-cell RNA sequencing (scRNA-seq) on *N. subflava* cultured under planktonic (shaking) conditions (Fig. 2a). This approach enabled comparison of the transcriptional states of wild-type and ceftriaxone-resistant (C26) strains and

highlighted population heterogeneity during antibiotic adaptation. Recent evidence indicates that biofilm and stress-response genes can be activated under planktonic conditions in response to environmental stress or sub-inhibitory antibiotic concentrations, producing a “primed” transcriptional state that precedes biofilm formation^{41–43}. Consistent with this, our data revealed pronounced activation of gene modules associated with antibiotic resistance, with upregulation of

Fig. 1 | Comprehensive analysis of phenotype variation and gene expression in *Neisseria subflava* under different growth conditions. **a** The diagram illustrates the experimental setup for evolving *Neisseria subflava* (SG001) through 26 serial passages (C1 to C26) under gradually increasing concentrations of ceftriaxone, starting close to the minimal inhibitory concentration (MIC). Each passage represents a two-day cycle, simulating the development of adaptive resistance. **b** MIC assay for ceftriaxone, showing the bacterial viability of resistant strains C26-a, C26-b, and C26-c compared to the control strain SG001 across ceftriaxone concentrations. **c** MIC assay for ampicillin illustrating resistance patterns in the same set of strains at generation 26 (C26). **d** Colony morphology of the indicated strains after 3 days of incubation and growth on Congo Red plates (top); Biofilm formation (bottom) in wild-type (WT), C9, and C26 strains, cultured for 24 h, quantified by optical density at 550 nm (OD₅₅₀) via crystal violet assay. **e** Bar chart showing the distribution of single nucleotide polymorphism (SNP) mutation types across six evolved strains: three from generation 9 (C9-a, C9-b, C9-c) and three from

generation 26 (C26-a, C26-b, C26-c). Mutation types are color-coded by functional category. **f** Mutational landscape in the top 10 genes for the C9-a and C26-a strains, with percentages representing the frequency of each mutation type in key resistance-related genes. **g** RNA sequencing analysis depicted as a heatmap, comparing gene expression between WT and C26-resistant strains. Gene clusters are annotated based on their roles in molecular chaperone functions, energy metabolism, pilus synthesis, adhesion, iron-sulfur cluster synthesis, and calcium-binding activities. In **b**, **c** curves were fitted using a four-parameter logistic regression model (variable slope) in GraphPad Prism. Data represent mean $\pm$ SD from four biologically independent cultures. In **d** data are shown as mean $\pm$ SD ($n = 4$ biological replicates from 3 independent experiments). *** $P < 0.0001$ for all comparisons (one-way ANOVA with Dunnett's multiple comparisons test vs. WT). In the box plots, the centre line indicates the median; the box spans the 25th to 75th percentiles; whiskers extend to the most extreme values within 1.5 $\times$ the interquartile range (IQR).

biofilm-associated genes in the evolved strain, even under planktonic growth conditions.

We successfully captured single-cell transcriptomes from 9661 *N. subflava* cells (3542 wild-type and 6119 C26) with comparable sequencing depths across the two groups. Wild-type bacteria averaged 196 ± 126 reads (SD) per cell and expressed 87 ± 43 genes (SD) per cell, while resistant bacteria had similar average reads per cell (194 ± 113) and slightly more genes per cell (95 ± 43) (Supplementary Fig. 4a).

UMAP visualization resolved six transcriptionally distinct populations with differential representation across conditions (Fig. 2b, c, and Supplementary Data 5). WT bacteria were enriched in clusters 1 (11.5%), 2 (48.1%), and 3 (34.6%), whereas resistant bacteria primarily occupied clusters 1 (34.7%), 4 (34.4%), 5 (16.3%), and 6 (7.9%). Cluster 1, the most abundant shared population, was characterized by outer membrane porin (*porB*) and ribosomal genes (*rplC*, *rpsC*). Clusters 2 and 3, predominantly found in WT bacteria, were marked by genes involved in RNA processing and protein synthesis (*tuf*, *rhlE*), reflecting active growth and metabolic activity. Cluster 3 uniquely expressed *lolA*, adhesins, and chaperones (*dnaK*, *groL*), which play critical roles in outer membrane biogenesis and stress response.

In contrast, resistant-associated clusters exhibited divergent gene expression linked to ceftriaxone resistance. Clusters 4 and 5 shared transcriptional similarities and both upregulated *comP* (pilin formation), *bamE* (outer membrane protein assembly), *bfrA* and *bfrB* (bacterioferritin linked to oxidative stress mitigation)⁴⁴, and *nirK* (a nitrite reductase for detoxification)⁴⁵. Cluster 5 displayed enhanced metabolic capacity (*glyA*, *acnB*, *purl*, *leuS*), suggesting an adaptive rewiring of central metabolism to sustain energy demands under antibiotic stress. Lastly, cluster 6 was characterized by sulfur metabolism genes (*cysP*, *cysI*, *cysJ*, *cysK*), which potentially support alternative redox balance and cell wall modification, enabling evasion of β -lactam targeting.

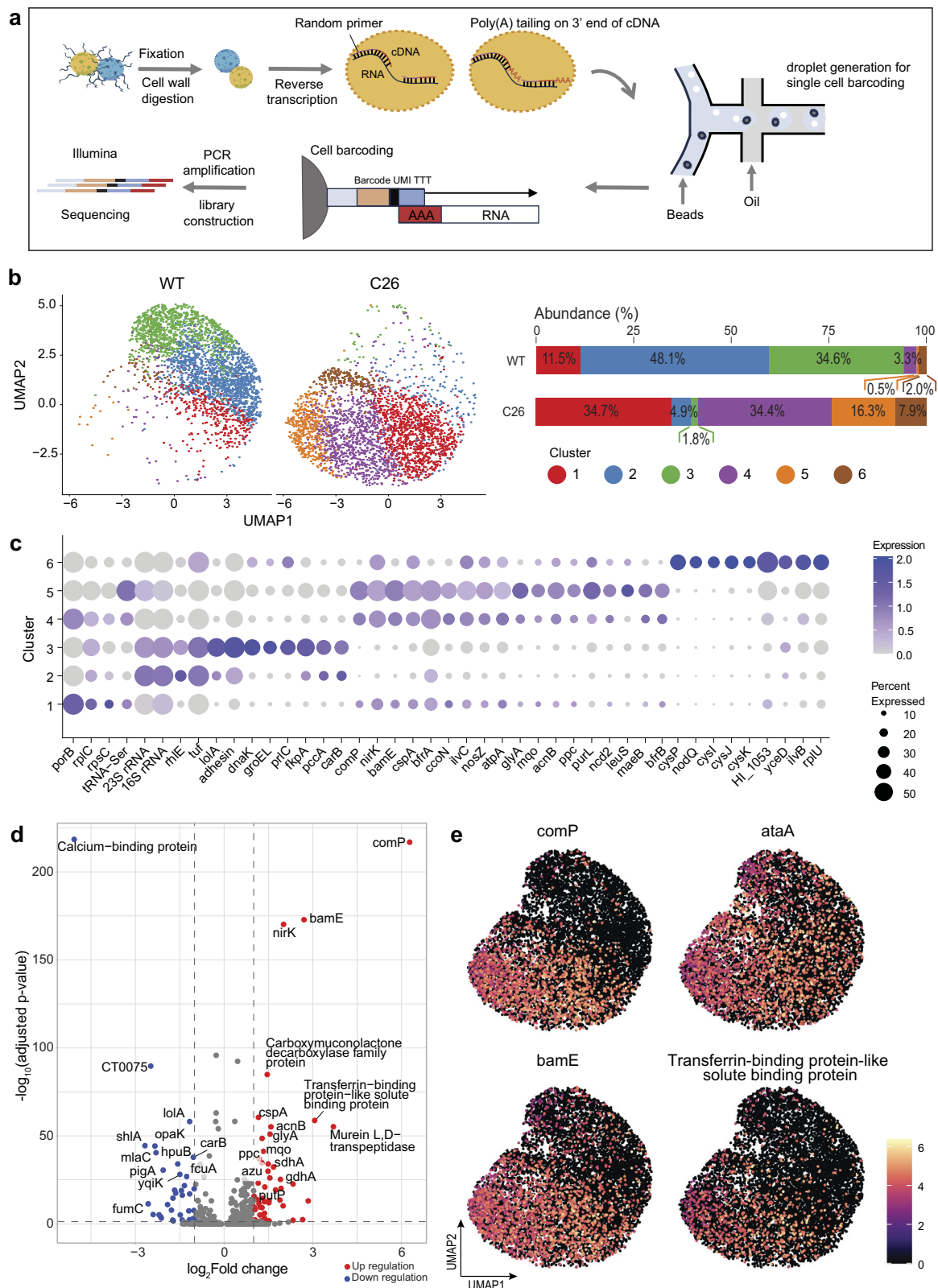
In concordance with the bulk transcriptomic analysis, scRNA-seq profiling of C26-resistant *N. subflava* strains revealed key molecular adaptations that confer ceftriaxone resistance, with marked overexpression of *comP*, *bamE*, *nirK*, *ataA*, as well as carboxymuconolactone decarboxylase family proteins, transferrin-binding proteins and murein-L,D-transpeptidase (Fig. 2d, e, and Supplementary Data 6). Although *ataA* carries a nonsense mutation predicted to produce a truncated, non-functional protein, it nonetheless showed transcriptional upregulation in C26 compared with the WT strain. This suggests a potential compensatory or autoregulatory role in biofilm dysregulation under antibiotic selection. Collectively, these data identify *comP* and *bamE* as key genes enriched in resistant-associated clusters, strongly implicating them in biofilm-associated ceftriaxone adaptation.

Lineage-specific functional adaptations for sustaining resistance
Gene Ontology (GO) enrichment analysis of the cluster-specific marker genes demonstrated that bacterial clusters of the resistant strain

possess enhanced oxidoreductase activity, which may neutralize the oxidative stress induced by ceftriaxone while simultaneously activating stress response regulons that promote survival. Interestingly, the peptidoglycan biosynthesis pathways were enriched in clusters 3 and 5, but exhibited divergent genetic programs, with cluster 5 exhibiting important antibiotic adaptations including metal ion binding and enhanced DNA repair capacity, both relevant to countering ceftriaxone-induced damage to cell wall precursors and DNA (Fig. 3a, and Supplementary Data 7). KEGG analysis indicated heightened macronutrient metabolism in clusters 4 and 5, supporting the energetic demands of efflux activity and cell-wall repair under cephalosporin pressure (Fig. 3b, and Supplementary Data 7). This metabolic rewiring mirrors resistance strategies observed in pathogenic *Neisseria* species, where increased metabolic flux through the TCA cycle and oxidative phosphorylation supports β -lactam resistance⁴⁶. The specialized functions of clusters 4 and 5, particularly their overexpression of denitrification genes (*nirK*, *ncd2*, *norB*), competence and adherence factors (*comP*, *bamE*), SOS and stress response (*sodB*, *cspA*, *rnr*, *recQ*), and metal ion acquisition systems (*bfrA*, *bfrB*, *lutA1*, *pqqL*), collectively reinforce envelope integrity, limit oxidative damage, and maintain metal homeostasis under ceftriaxone exposure (Fig. 3c). By contrast, cluster 6 adopted a metabolically conservative program characterized by sulfate assimilation (*cysP*, *cysI*, *cysJ*, *cysK*), redox activity (*nuoF*, *nuoD*, *nqrB*) and selected efflux transporters (*tet(M)*, *acrB*, *potA*, *tet35*) (Fig. 3b, c). As sulfate assimilation precedes cysteine biosynthesis, this transcriptional profile indicates elevated cysteine production to bolster redox balance and antioxidant defenses during β -lactam-induced envelope stress.

Although *bfrA* and *bfrB* genes primarily function as iron-storage proteins, they may secondarily contribute to enhancing biofilm stability and stress tolerance^{47–49}. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) confirmed increased intracellular Fe levels in resistant versus wild-type bacteria, consistent with elevated expression of iron-related genes, while Mg levels remained unchanged (Supplementary Fig. 4b). We also compared the growth kinetics of wild-type and C26 strains. The C26 strain also showed accelerated growth rates and higher stationary-phase density, consistent with enhanced metabolism and proliferative potential (Supplementary Fig. 4c). Additionally, we established a *bfrA*-gfp genomic reporter system to directly assess iron-dependent gene regulation. Upon iron supplementation (50 μ M FeCl₃), we observed robust induction of GFP fluorescence driven by the *bfrA* promoter, confirming enhanced iron-responsive gene activity in the evolved C26 strain (Supplementary Fig. 4d). These results provide direct support for the adaptive role of metal homeostasis identified by single-cell analyses, reinforcing the crucial role of iron regulation in cephalosporin resistance.

Among these modules, *comP* and *bamE* showed peak expression in the resistance-associated cluster 5, highlighting their central roles within the dominant adaptive lineage and linking biofilm features with



antibiotic tolerance. To assess whether planktonic culture conditions could obscure biofilm-specific expression, RT-qPCR was performed under both planktonic (shaking) and sessile (biofilm-promoting) conditions. The evolved C26 strain maintained similarly elevated expression of both genes under planktonic and sessile conditions, whereas wild-type cells showed no significant difference (Supplementary Fig. 4e). Thus, the resistant lineage maintains a constitutively “primed”

biofilm transcriptome across growth modes. At the population level, *comP* and *bamE* are the principal genetic drivers linking biofilm enhancement to ceftriaxone resistance. *ComP* encodes a DNA uptake minor pilin that mediates natural competence and facilitates incorporation of extracellular DNA (eDNA) into the matrix, a structural component that reinforces *Neisseria* biofilms and stabilizes surface communities under antibiotic stress^{50–52}. *BamE*, a lipoprotein

Fig. 2 Single-cell RNA sequencing analysis of *Neisseria subflava* highlights cellular heterogeneity in response to antibiotic resistance. **a** Schematic of the single-cell RNA sequencing workflow using the M20 Genomics platform for both WT and C26 (antibiotic-resistant) strains, including cell wall digestion, reverse transcription, and droplet-based cDNA synthesis, followed by library construction and sequencing. **b** UMAP visualization (left) of the integrated single-cell transcriptomes from both wild-type (WT) and ceftriaxone-resistant (C26) strains, showing subclusters across the two strains. A stacked bar chart (right) shows the relative abundance of each subcluster. **c** Dotplot illustrating marker gene

expression across distinct clusters in *N. subflava*, integrating WT and C26 strains to highlight cellular heterogeneity linked to antibiotic resistance. **d** Volcano plot showing the top DEGs comparing C26 to WT strains. Differential expression was tested using the two-sided Wilcoxon rank-sum test, with *P* values adjusted for multiple comparisons using the Benjamini-Hochberg method. Volcano plots display $\log_2$ fold changes and $-\log_{10}$ (adjusted *P* values) **e** Subclusters of C26 strains significantly upregulated key antibiotic resistance genes, namely *comP*, *bamE*, *ataA* and transferrin-binding protein-like solute binding proteins.

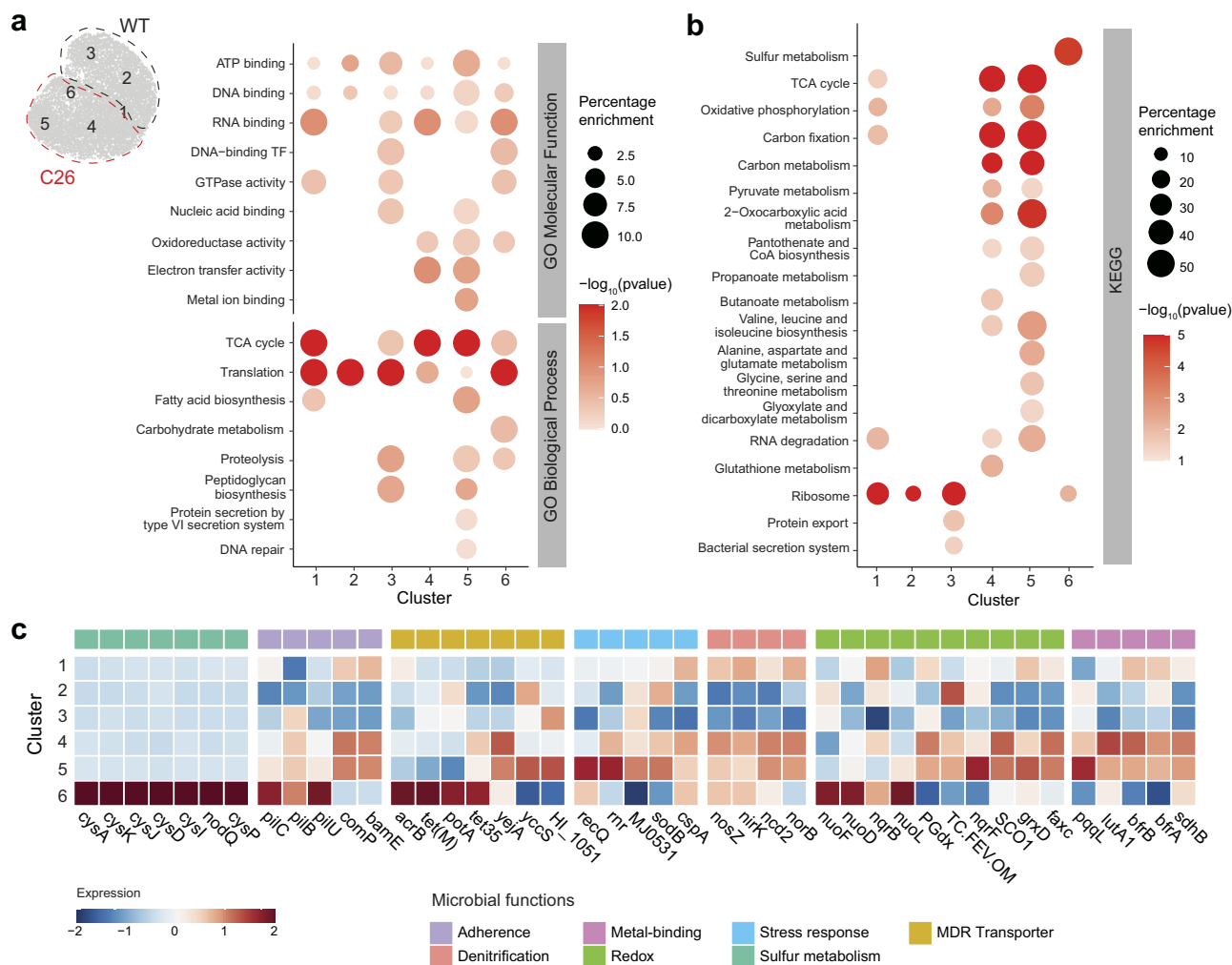


Fig. 3 | Functional enrichment and gene expression profiles across transcriptional subclusters. **a**, **b** GO and KEGG enrichment analyses were performed for each transcriptional subcluster using a two-sided hypergeometric test, with *P* values adjusted for multiple comparisons using the Benjamini-Hochberg correction. Bubble

color represents $-\log_{10}$ (adjusted *P* values), and bubble size indicates the proportion of genes in the gene set (enrichment percentage). **c** Heatmap illustrating differentially expressed genes (DEGs) upregulated in each transcriptional subcluster relative to other subclusters, annotated by functional categories.

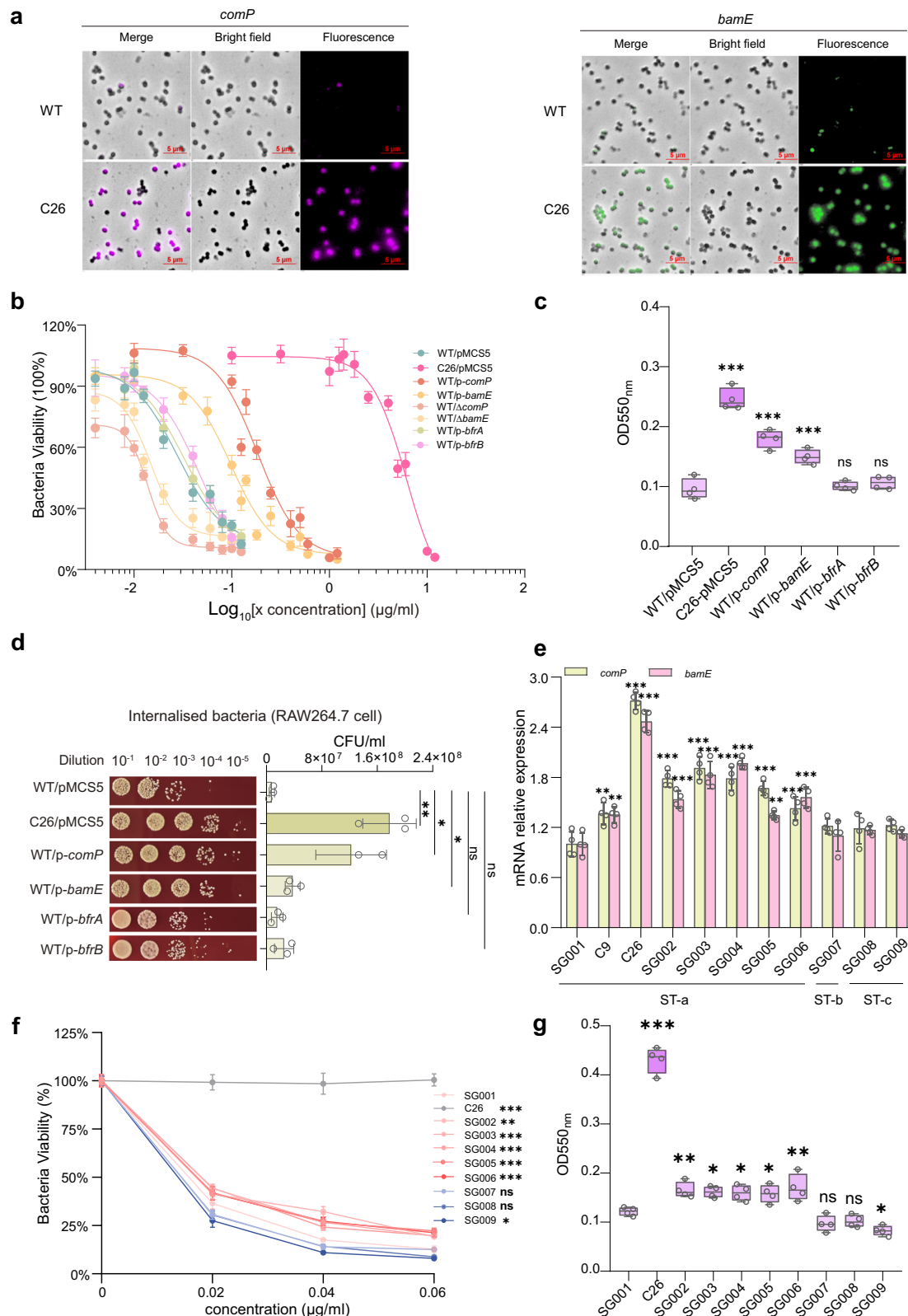
of the β -barrel assembly machinery (BAM), ensures efficient outer-membrane protein assembly, preserving the integrity and architecture of the biofilm surface layer and reducing β -lactam permeability^{53–55}. Together, *comP*-driven eDNA enrichment and *bamE*-mediated envelope stabilization establish a biofilm phenotype that shields cells from cephalosporin penetration and maintains a microenvironment conducive to horizontal gene exchange.

scRNA-seq revealed functional heterogeneity underpinning ceftriaxone resistance in *N. subflava*, which confers a survival advantage by enabling specialized roles, enhancing resilience, and supporting long-term adaptation^{29,56}. This resistance is centred on a biofilm-oriented programme driven by *comP*-mediated extracellular DNA enrichment and *bamE*-dependent outer-membrane

stabilisation. This programme converges with the functional enrichment profiles of clusters 4–6, where stress-response activation, cell-wall remodelling and metal-ion homeostasis form an integrated network that maintains biofilm integrity under sustained β -lactam pressure. These findings support a model in which resistance evolution is intrinsically linked to biofilm optimisation, providing immediate protection from antibiotic challenge while preserving a genetic reservoir for long-term adaptation.

Elevated expression of *comP* and *bamE* drives antibiotic resistance and immune evasion

Building on these observations, we next dissected the functional contributions of *comP* and *bamE* as the principal biofilm-linked drivers,



alongside *bfrA* and *bfrB* as bacterioferritin-associated iron homeostasis modules. To assess gene expression at single-cell resolution, we integrated chromosomally integrated fluorescent reporters (mCherry for *comP* and GFP for *bamE*) in both wild-type and C26 strains. Fluorescence microscopy revealed markedly increased reporter intensity in the evolved C26 strain, with a larger fraction of cells showing high *comP* and *bamE* expression compared with the wild type (Fig. 4a).

MIC assays confirmed that the overexpression of *comP* and *bamE* enhanced ceftriaxone resistance, with MIC values of 0.2 μg/ml and 0.1 μg/ml, respectively, representing a 5–10-fold increase compared to vector controls. Conversely, deletion of *comP* or *bamE* reduced MICs by ~two-fold, confirming their essential roles in resistance (Fig. 4b). Both genes also promoted biofilm maturation. Their overexpression enhanced biofilm mass, whereas deletion markedly impaired biofilm

Fig. 4 | The elevated expression of *comP* and *bamE* drives biofilm formation, enhances antibiotic resistance, and facilitates macrophage evasion in *N. subflava*. **a** Representative images of wild-type and ceftriaxone-resistant (C26) strains with chromosomally integrated *comP*-mCherry and *bamE*-GFP reporters. Images show merged, bright field, and fluorescence channels. Images were acquired using an ECLIPSE Ti2-I fluorescence microscope (Nikon), and processed with NIS-Elements Viewer 5.27. Each image is representative of three biological replicates. **b** MIC assay of ceftriaxone (Cef) in WT/pMCS5, C26/pMCS5, WT overexpressing *comP*, *bamE*, *bfrA*, *bfrB*, and deletion mutants ($\Delta comP$, $\Delta bamE$). Curves were fitted with a four-parameter logistic model. Data represent mean $\pm$ SD from four biologically independent cultures. **c** Biofilm formation (OD550 nm) quantified by crystal violet staining after 24 h of culture in WT/pMCS5, C26/pMCS5, WT strains overexpressing *comP*, *bamE*, *bfrA*, and *bfrB*. **d** CFU were quantified from RAW264.7 macrophage phagocytosis assays. Bacteria were incubated with macrophages for 1 h, followed by lysis, serial dilution, and plating for CFU enumeration. **e** RT-qPCR

analysis showing relative expression levels of *comP* and *bamE* in WT and C26 strains and clinical isolates cultured for 12 h. **f** Bacterial viability of WT (SG001), laboratory-evolved strains (C9, C26), and clinical isolates (SG002-009) under increasing antibiotic concentrations near the MIC for WT (0.02, 0.04, and 0.06 μ g/ml). Bacterial viability was determined by measuring OD600 after 24 h of incubation. **g** Biofilm quantification (OD550 nm) of WT (SG001), experimentally-evolved strains (C9, C26), and clinical isolates (SG002-009) cultured for 24 h by crystal violet assay. All data are shown as mean $\pm$ SD from 3–4 biologically independent replicates. Statistical significance was determined by two-sided unpaired *t*-tests (**d**), one-way ANOVA with Dunnett's test (**c**, **e**, **g**), or two-way ANOVA with Dunnett's test (**f**). In the box plots, the centre line indicates the median; the box spans the 25th to 75th percentiles; whiskers extend to minimum and maximum values within 1.5 $\times$ the interquartile range (IQR) (**c**–**g**). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Exact *P* values for all statistical comparisons in panels c–g are provided in the Source Data file.

formation (Fig. 4c, and Supplementary Fig. 5a). Overexpression of *bfrA* and *bfrB* did not affect ceftriaxone resistance and had a minor impact on biofilm mass, suggesting their limited role. Macrophage phagocytosis assays revealed enhanced resistance to phagocytic clearance in strains overexpressing *comP* and *bamE*, consistent with their roles in biofilm formation and immune evasion (Fig. 4d).

The clinical relevance of these findings was supported by analysis of patient cohorts. In bronchiectasis patients from Shenzhen, China, *N. subflava* was detected in 18.4% of samples (*n* = 38) (Supplementary Fig. 5c), with carriers generally being older and showing lower leukocyte counts (Supplementary Fig. 5d). In an independent cohort, nine clinical isolates were examined by multilocus sequence typing (MLST), which grouped them into three sequence types: ST-a (*n* = 6), ST-b (*n* = 1), and ST-c (*n* = 2). ST-a isolates exhibited significant upregulation of *comP* and *bamE*, which correlated with enhanced biofilm formation and higher ceftriaxone MICs (Fig. 4e, f, and Supplementary Fig. 5b). Whole-genome sequencing further revealed frequent *ataA* mutations across clinical isolates, particularly in ST-a isolates, suggesting that *comP* and *bamE* upregulation may compensate for impaired *ataA* function. This suggests *comP* and *bamE* upregulation potentially compensates for impaired *ataA* function, promoting adaptation under antibiotic pressure. Consistent with this, ST-a isolates formed more robust biofilms than ST-b or ST-c strains, mirroring the phenotypes observed in experimentally evolved resistant lineages (Fig. 4g).

Taken together, our results demonstrate direct involvement of *comP* and *bamE* in ceftriaxone resistance, biofilm formation, and immune evasion in *N. subflava*. By contrast, iron-storage genes *bfrA* and *bfrB* play ancillary roles in this emerging pathobiont.

Iron enhances biofilm formation and antibiotic resistance in ceftriaxone-evolved *N. subflava*

Although the precise roles of *bfrA* and *bfrB* were not resolved functionally, their upregulation in C26 antibiotic-resistant strains suggests that iron homeostasis could modulate adaptation under antibiotic stress. We therefore examined the effect of iron (Fe³⁺) supplementation on bacterial survival and biofilm formation in wild-type (WT) and ceftriaxone-resistant (C26) strains.

In colony-forming unit (CFU) assays, Fe³⁺ supplementation did not alter the viability of wild-type strain at its MIC (0.02 μ g/ml) but markedly improved survival of the C26 strain at its higher MIC (6 μ g/ml) (Fig. 5a). Fe³⁺ supplementation had no impact on WT viability at its MIC (0.02 μ g/ml), indicating iron availability does not influence WT growth at low antibiotic pressure. In contrast, Fe³⁺ markedly enhanced viability of the C26 strain at its higher MIC (6 μ g/ml), significantly reducing ceftriaxone susceptibility. In the absence of iron supplementation, the viability of the C26 strain sharply declined, underscoring the critical role of iron in sustaining resistance under antibiotic exposure (Fig. 5b).

Biofilm formation showed that Fe³⁺ supplementation increased biofilm biomass in both strains under antibiotic-free conditions, with the evolved C26 strain exhibiting greater biofilm formation compared to WT. Under ceftriaxone treatment at MIC levels, biofilm biomass in the WT strain remained unaffected by iron supplementation, whereas the C26 strain showed significantly increased biofilm biomass, indicative of reduced antibiotic susceptibility (Fig. 5c, d).

Further assessment of bacterial survival across a gradient of antibiotic pressures highlighted strain-specific advantages provided by Fe³⁺ supplementation. At ceftriaxone concentrations slightly above WT MIC (0.02–0.04 μ g/ml), WT viability was minimal, irrespective of iron availability. In contrast, Fe³⁺ supplementation substantially enhanced the viability of the C26 strain even at higher ceftriaxone concentrations (6–8 μ g/ml), further confirming iron's role in resistance under significant antibiotic stress (Fig. 5e, f).

These findings highlight iron's dual contribution to biofilm formation and bacterial viability under antibiotic challenge, specifically benefiting the evolved, resistant C26 strain. Although iron broadly supports biofilm development, only the antibiotic-adapted strain effectively utilizes iron-enhanced biofilms to sustain ceftriaxone tolerance. This highlights ceftriaxone resistance as a modifiable trait in antibiotic-adapted *N. subflava*, influenced by iron availability via supplementation or chelation.

Discussion

Our study addresses a critical gap in understanding how antibiotic exposure can drive commensal bacteria towards pathogenicity by employing single-cell RNA sequencing to analyze ceftriaxone-resistant strains of the emerging airway pathobiont *Neisseria subflava*, which have evolved in vitro. We identified a previously uncharacterized resistance mechanism involving functional variation in the *ataA* adhesin gene, linked to complex adaptive traits such as biofilm matrix reinforcement, outer-membrane stabilization, and iron homeostasis reprogramming. These adaptations collectively confer increased resistance to phagocytic clearance and altered biofilm formation, underscoring antibiotic-driven evolutionary pressures that may facilitate commensal-to-pathobiont transitions in vivo.

Resistance mechanisms in *N. subflava* remain poorly defined, and available susceptibility data are limited, highlighting its clinical relevance as an emerging pathobiont in the airway²⁶. Our evolved lineages exhibit a distinct resistance mechanism tied to premature truncation of *ataA*, implicating biofilm-associated pathways in ceftriaxone resistance. This represents a divergence from canonical resistance routes in pathogenic *Neisseria* species^{25,57}, and highlights *ataA* as a potential mediator of both antibiotic tolerance and pathogenic adaptation. Single-cell transcriptomics reveals an upregulation of biofilm-associated genes, including *comP* and *bamE*, in distinct cellular subpopulations, highlighting compensatory and adaptive responses to *ataA* mutational inactivation. While *porB* was

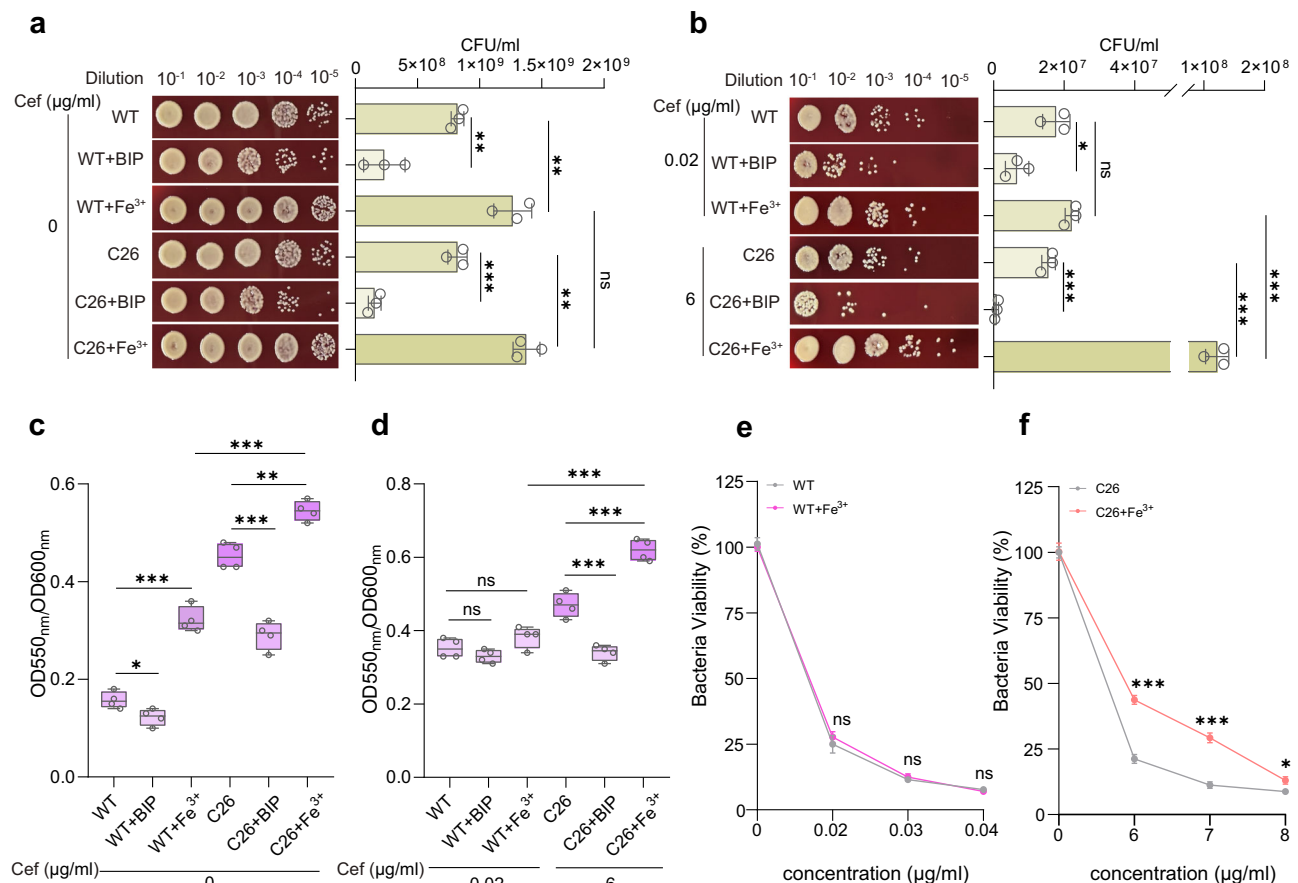


Fig. 5 | Iron-mediated enhancement of biofilm formation in resistant strains reduces susceptibility to ceftriaxone in *Neisseria subflava*. **a** Colony growth of *N. subflava* WT and C26 strains after treatment with iron (Fe^{3+} , 50 μM) or the iron chelator bipyridine (BIP, 50 μM). Viable cells were quantified after 24 h of incubation without ceftriaxone to assess the impact of the treatments, from three independent experiments. **b** Growth comparison of WT and C26 strains under ceftriaxone (Cef) treatment alone (0.02 $\mu\text{g/ml}$ for WT, 6 $\mu\text{g/ml}$ for C26 strains) and in combination with Fe^{3+} (50 μM) or BIP (50 μM). Viable cells were quantified after 24 h incubation to reflect the impact of treatments, based on three independent experiments. **c, d** Quantification of biofilm formation ($\text{OD}_{550\text{ nm}}/\text{OD}_{600\text{ nm}}$) in response to Fe^{3+} (50 μM) and BIP (50 μM) treatments: (c) without and (d) with ceftriaxone exposure (0.02 $\mu\text{g/ml}$ for WT and 6 $\mu\text{g/ml}$ for C26 strains) in strains cultured for 24 h. **e, f** MIC assays of WT and C26 strains under ceftriaxone (Cef) with or without Fe^{3+} (50 μM). Bacterial viability was determined by measuring OD_{600} after 24 h of incubation. In all panels, error bars represent mean $\pm$ SD from 3–4

biologically independent replicates. *P* values were calculated by two-sided unpaired *t*-tests (**a–d**) or two-way ANOVA with Sidak's test (**e, f**). In the box plots, the centre line indicates the median; the box spans the 25th to 75th percentiles; whiskers extend to the most extreme values within 1.5 $\times$ the interquartile range (IQR) (**c, d**). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. In **a**, *P* = 0.0042 (WT + BIP vs WT), 0.0088 (WT + Fe^{3+} vs WT), 0.6101 (C26 + Fe^{3+} vs WT + Fe^{3+}), 0.0002 (C26 + BIP vs C26), 0.0019 (C26 + Fe^{3+} vs C26). Using the same pairwise comparisons as in panel **a**, in **b**, *P* = 0.0197, 0.1495, 0.0008, 0.0002, 0.0007, respectively; in **c**, *P* = 0.0274, < 0.0001, < 0.0001, 0.0002, 0.0015, respectively; and in **d**, *P* = 0.2095, 0.1823, < 0.0001, 0.0006, 0.0005, respectively. In **f**, *P* = 0.9995 (0 $\mu\text{g/ml}$), < 0.0001 (6 $\mu\text{g/ml}$), < 0.0001 (7 $\mu\text{g/ml}$), and 0.0166 (8 $\mu\text{g/ml}$). Using single-bacterial-cell analysis, this study reveals how the airway commensal *Neisseria subflava* acquires ceftriaxone resistance through enhanced biofilm formation and transcriptional heterogeneity, promoting persistence under antibiotic pressure.

overall less differentially expressed than *comP* and *bamE*^{59–65}, changes in porin composition together with efflux could still contribute by limiting outer-membrane permeability and restricting antibiotic entry.

We reveal that *N. subflava* resistance is a multi-layered process involving enhanced metal-binding capacities and bacterial adhesion beyond biofilm formation alone. While bacterioferritins are less characterized in *Neisseria* spp, similar iron-storage mechanisms in *N. gonorrhoeae* are known to influence outer membrane properties, colonization, and pathogenicity⁵⁸. Interventions targeting iron storage have proven effective in sensitizing other pathogens, such as *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*, to antimicrobial therapy, suggesting potential relevance in *N. subflava*-colonized respiratory patients^{44,49,59}.

In addition to iron homeostasis, biofilm formation provides a crucial barrier against antibiotics and host defenses, promoting long-term colonization⁶⁰. scRNA-seq profiling revealed enriched

expression of *comP* and *bamE*^{59–65}, which support competence-linked eDNA accrual and β -barrel assembly, respectively. These two functions are well positioned to stabilize the envelope and strengthen matrix architecture under β -lactam pressure. Notably, these biofilm-associated transcripts were elevated even under planktonic conditions, consistent with a “primed” state observed under environmental stress or sub-inhibitory antibiotics. This accords with prior reports that environmental stress, including sub-inhibitory antibiotic exposure, induces biofilm-like gene expression even in non-sessile states^{41–43}. Even under shaking conditions, transient micro-aggregates and surface-adherent subpopulations can generate local biofilm-like niches; the enrichment of *comP* and *bamE* in resistant clusters is congruent with competence/pilus dynamics and BAM complex assembly that facilitate micro-niche formation. Collectively, these observations are most parsimoniously explained by β -lactam-induced priming of biofilm programs rather than a requirement for pre-formed biofilm architecture.

Subtle transcriptional rewiring within constrained phenotypic landscapes may also underlie the pronounced transcriptional heterogeneity and lineage diversification observed in *Neisseria subflava* populations. Even in homogeneous cultures, subtle spatial microgradients, localized fluctuations in nutrients, oxygen, or antibiotic exposure can establish distinct micro-niches, driving adaptive differentiation. Stochastic gene expression, phase variation, and dynamic regulatory mechanisms (including small RNAs and transcription factors) further amplify functional diversity within clonal populations. While antigenic variation is a well-known driver of phenotypic diversity in pathogenic *Neisseria*⁶⁶, our data suggest that the observed lineage-specific transcriptional programs arise predominantly from adaptive gene regulation rather than large-scale antigenic switching. Such transcriptional heterogeneity and lineage bifurcation constitute flexible strategies for rapid bacterial adaptation to fluctuating environmental pressures.

Notably, key adaptive mutations identified in our experimentally evolved lineages (*ataA*, *compP*, and *bamE*) were also present in clinical isolates from patients with bronchiectasis. Clinical isolates, particularly those of the ST-a lineage, showed enhanced biofilm formation and reduced antibiotic susceptibility. In contrast, mutations in iron metabolism genes (*bfrA*, *bfrB*) appeared more prominent in evolved lineages than in the small set of clinical isolates profiled, pointing to ecological context as a determinant of adaptive routes^{67,68}. This adaptive strategy, akin to that observed in *E. coli*, enhances survival under stress by distributing the adaptive burden across specialized phenotypes^{69–71}. Such heterogeneity contributes to treatment failure and persistent respiratory infections, highlighting the adaptive plasticity and clinical relevance of targeting biofilm and iron-related pathways. These non-canonical resistance mechanisms expand our understanding of the microbial resistome beyond classical pathogens^{19,72}.

Our findings reinforce a dynamic continuum from commensalism to pathogenicity, driven by environmental pressures such as antibiotic exposure and immune compromise. This transition is especially relevant in mucosal niches like the lung and gut, where microbes, including *H. influenzae* and *S. pneumoniae*, may shift toward virulence in immunocompromised hosts^{73,74}. Similarly, in the gastrointestinal tract, *E. coli* strains shift to pathogenic forms (e.g., EHEC), and *Clostridioides difficile* and *Helicobacter pylori* demonstrate pathogenic adaptations under antibiotic stress^{75–77}. Recent studies on *Serratia marcescens* reveal a similar adaptive shift towards persistence through biofilm formation⁷⁸. These findings also hold relevance to our understanding of *N. subflava* pathogenicity in acute infections (e.g., meningitis, pneumonia, endocarditis, bacteremia) and, notably, its emerging association with exacerbations in chronic respiratory disease such as bronchiectasis^{79,80}. Such insights into microbial adaptive strategies underline the need for deeper investigation into resistance mechanisms and the ecological triggers driving pathobiont emergence.

Though insightful, our work is not without limitations. We evolved three replicate lineages (C26 a–c), which is appropriate for exploratory multi-omic comparison but may not capture the full space of trajectories. scRNA-seq was performed on one lineage (C26-a), which limited generalization; bulk RNA-seq partially mitigated this limitation by demonstrating cross-lineage consistency. From a technical perspective, transcriptomic analyses were conducted under planktonic conditions due to the challenges in processing biofilm-derived samples for single-cell RNA sequencing. While we validated biofilm-associated gene expression under static conditions, the planktonic transcriptome may not fully represent mature biofilms. Functional annotations of single-cell clusters are interpretive, as direct cluster-resolved measurements remain technically challenging in bacteria. The absence of unique surface markers, multi-gene regulatory complexity, and low RNA recovery from mature biofilms further constrain functional validation at the single-cell level. In addition, the use of stable fluorescent

proteins (mCherry/GFP) may lead to signal accumulation influenced by growth dynamics, limiting their precision in quantifying expression heterogeneity. Single-cell functional enrichment relied solely on KEGG/GO and, given the organism's novelty and the large fraction of unannotated genes, should be interpreted cautiously. Finally, in terms of clinical relevance, while key mutations (e.g., in *ataA*) and transcriptional shifts mirrored those in clinical isolates, some laboratory-evolved adaptations (e.g., iron metabolism changes) may be context-dependent and less prevalent in natural populations. Expanded sampling of clinical and environmental strains will clarify the ecological relevance of these mechanisms.

In conclusion, ceftriaxone resistance in *N. subflava* emerges from complex, multi-layered adaptive mechanisms that integrate biofilm reinforcement, envelope stabilization, and metal-responsive programs with genetic variation in *ataA* and compensatory upregulation of *compP/bamE*. These insights argue against single-target strategies and instead support multi-pronged interventions that combine β -lactam therapy with approaches that disrupt biofilms, perturb envelope assembly, and modulate iron availability. This foundational framework motivates *in vivo* studies to define when and how these programs operate within the airway microbiome and how they can be leveraged therapeutically.

Methods

All experiments were performed using standard microbiological techniques unless otherwise specified. A complete list of bacterial strains, plasmids, and reagents used in this study is provided in Supplementary Table 3.

Bacterial culture media

Neisseria subflava was initially recovered from glycerol stocks stored at -80°C and cultured on Columbia blood agar plates (Columbia agar supplemented with 5% defibrinated sheep blood) at 37°C in a 5% CO_2 incubator for 14 h. Subsequently, a single colony was transferred to Brain Heart Infusion (BHI) liquid medium (Cat#024053, HKM, China) supplemented with 5% fetal bovine serum (FBS) for culture. Bacteria were incubated at 37°C in a 5% CO_2 incubator for an additional 12–16 h to ensure optimal growth.

MIC determination (Minimum Inhibitory Concentration)

The overnight bacterial cultures were re-inoculated at a 1:1000 dilution into fresh medium and allowed to grow to the mid-log phase in a shaking incubator (cat#MQT-60R, Minquan, China) at 37°C , 220 rpm. The bacterial solution was added to a transparent 96-well plate at a final concentration of OD_{600} 0.01 per well, and a gradient dilution of antibiotic concentration using BHI, with four parallels for each concentration. After culturing the bacteria in various concentrations of antibiotics for 24 h, the absorbance at 600 nm was measured to determine the antibacterial effect. Fitting curves by comparing different concentrations of antibiotics and absorbance to obtain IC₉₀ (Inhibitory Concentration 90). Use GraphPad Prism 8 (GraphPad Software) to calculate S-type dose response (slope variation) nonlinear fitting.

Biofilm formation assay

Overnight cultures were diluted to OD_{600} of 0.01 and the 96-well plates were cultured at 37°C for 24 h, then cell plates were washed three times with PBS to remove unbound bacteria. Then biofilm was evaluated by 0.05% crystal violet (CV: 150 μl ; Sangon Biotech, China) staining for 30 minutes⁸¹. Gently rinsed unbound dyes with sterile dd H_2O and blown dried at room temperature. After that, 200 μl of 30% glacial acetic acid was added to each well. Relative biofilm formation was determined by measuring the absorbance at 550 nm with Tecan Infinity 200 PRO microplate reader. Biofilm inhibition experiments were conducted in three independent trials, with four replicates per condition.

Congo red plate assay

Columbia agar was autoclaved, cooled to 50–55 °C, mixed with 5% sterile defibrinated sheep blood, supplemented with 10 µg/mL Coomassie brilliant blue G-250 and 20 µg/mL Congo red (CR), and poured into plates to solidify. Fresh overnight bacterial culture (5 µl per spot, 5 replicates) was spotted onto the plates, which were incubated at room temperature in the dark for 72 h, and then colony staining characteristics were observed and recorded.

Growth curve assay

To assess growth kinetics of wild-type and evolved isolates, overnight cultures were initially adjusted to an OD₆₀₀ 1.0 in BHI broth. The standardized suspensions were diluted into fresh BHI medium supplemented with 5% FBS to a starting OD₆₀₀ of approximately 0.02. Aliquots of 200 µl were transferred to flat-bottom 96-well microtiter plates (Corning), with at least three technical replicates per strain. Plates were incubated at 37 °C in TECAN Spark microplate reader, which recorded OD₆₀₀ every 30 minutes for 48 h with continuous double orbital shaking between reads. Blank wells containing medium only were included for background correction. Growth curves were plotted and analyzed using GraphPad Prism 8. Parameters including lag phase duration, exponential growth rate, and maximal OD₆₀₀ were extracted to compare relative growth fitness between strains.

Preprocessing for single-cell sequencing

Wild-type and antibiotic-resistant strains were cultured at 37 °C with shaking at 220 rpm. Cultures were grown for ~6 h until the optical density at 600 nm (OD₆₀₀) reached 1.0. To minimize technical artifacts and preserve in vivo transcriptomic states, all subsequent steps were performed on ice (4 °C), and all buffers were pre-chilled. The time from harvest to fixation was strictly limited to under 10 minutes for all samples. Wild-type and C26 strains were processed in parallel under identical conditions to ensure comparability. 1 ml of each bacterial culture was collected and centrifuged at 6010 × g for 5 minutes. The supernatant was discarded, and the pellet was washed with 1 ml of phosphate-buffered saline (PBS). The washed bacterial pellet was resuspended in 1 ml of ice-cold 4% paraformaldehyde (PFA) in PBS. The suspension was placed in a rotary mixer at 4 °C and rotated for 9 h to cross-link intracellular RNA, DNA, and proteins. After fixation, the samples were centrifuged at 10,000 × g for 10 minutes at 4 °C to remove the PFA. The pellet was washed twice with 1 ml of PBS-T (PBS containing 0.1% Tween-20 and 1 µl RNase inhibitor). The bacterial pellet was then resuspended in 200 µl of PBS-T, with an additional 5 µl of RNase inhibitor added. The tubes were sealed and stored at 4 °C until further use. Prior to single-cell sequencing, quality control of the samples was performed to ensure a cell count of ≥ 10 million, minimal debris, no visible clumping, and an aggregation rate of ≤ 20%.

Single-cell RNA sequencing

Single-cell encapsulation, droplet generation, and nucleic acid capture were conducted using the VITAcrizur Single-Cell Partitioning System (M20 Genomics), following the VITApilote High-Throughput Single-Bacterial Transcriptome Kit protocol (Cat#R20114124, M20 Genomics). Fixed bacterial cells underwent permeabilization and cell wall digestion, followed by reverse transcription with random primers to produce full-length cDNA. After adaptor ligation, cell barcodes and unique molecular identifiers (UMIs) on microbeads were combined with adaptor-ligated cDNA and encapsulated in droplets. Droplet encapsulation and extension generated labeled cDNA, which was then PCR-amplified. The final library, incorporating P5 and P7 adaptors, was sequenced on an Illumina NovaSeq 6000 platform.

Single-cell data preprocessing

Single-cell RNA sequencing data were processed using VITaseer V1.2 software (M20 Genomics), designed specifically for

transcriptomic data generated by the VITA platform. Briefly, raw sequencing reads were trimmed using Cutadapt (v3.7). For each Read1, unique molecular identifiers (UMIs) and cell-specific barcodes were extracted, with acceptable barcodes defined as those with a Hamming distance ≤ 2 nt and a unique match. Read2 was aligned to the reference genome of the species of interest using STAR (v2.7.10a) under default parameters, retaining only uniquely mapped sequences for downstream analysis. Subsequent data analysis was performed using Seurat (v4.3.0.1) under default parameters unless specified otherwise. Cells expressing fewer than 10 genes were excluded, and genes present in fewer than one cell were filtered out. Data were log-normalized using NormalizeData function, followed by FindVariableFeatures function to identify highly variable features for dimensional reduction. The data were scaled with ScaleData function. Dimension reduction was performed by principal component analysis (PCA) to reduce dimensions to 8. Uniform Manifold Approximation and Projection (UMAP) was constructed using these principal components. Clustering was performed using the Louvain algorithm (FindClusters) at a resolution of 0.5, and differential gene expression was assessed with a log-fold change threshold of 0.25 and adjusted *p* value of < 0.05 (FindMarkers function).

GO and KEGG enrichment analysis of DEGs

Based on the hypergeometric tests, GO and KEGG enrichment analysis of DEGs was performed to identify the significantly enriched terms using clusterProfiler package (v4.16.0) in R (v4.5.1).

Genomic DNA extraction and genome sequencing

Neisseria subflava SG-KL01 (SG001) were grown to 10⁹ CFU/ml, and total DNA was extracted from each 2 ml bacterial suspension using the TIANamp Bacterial DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Qualified genomic DNA was fragmented with G-tubes (Covaris, Woburn, MA, USA), followed by end-repair using the NEBNext FFPE DNA Repair Kit (M6630, NEB, USA) and NEBNext End Repair/dA-Tailing Module reagents (E7546, NEB, USA). The fragmented DNA was subsequently ligated to adapters to construct sequencing libraries using NEBNext Quick Ligation Module reagents (E6056, NEB, USA). After purification with the AMPure XP system (Beckman Coulter, Brea, CA, USA), library sequencing was performed on the Oxford Nanopore PromethION long-read sequencing platform (Oxford Nanopore Technologies, Oxford, UK) following standard protocols. For Illumina sequencing, qualified genomic DNA was first randomly sonicated, then subjected to end-repair, A-tailing, and adapter ligation using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's protocol. DNA fragments of 300–400 bp (in length) were enriched by PCR. Finally, PCR products were purified using the AMPure XP system (Beckman Coulter, Brea, CA, USA). Library size distribution was analyzed with the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA), and quantification was performed by real-time PCR. Genome sequencing was conducted on the Illumina NovaSeq 6000 platform using paired-end technology (PE150).

Genome assembly and quality control

Raw ONT (.fast5) signals were base-called with Oxford Nanopore Guppy v6.4.6 (HAC), followed by quality control to remove reads with Q < 7 and trim adapter-containing reads, and the filtered data were used for assembly and alignment. Raw Illumina reads were processed with FASTP v0.20.0 to (i) remove adapters (trimming adapters and downstream sequence; reads < 50 nt after trimming were discarded), (ii) remove poly-A reads, (iii) remove reads with N > 10%, and (iv) remove low-quality reads where Q ≤ 20 bases constituted ≥ 50% of the read; the resulting clean reads were aligned to the assembly with BWA v0.7.17-r1188 and used for iterative polishing with Pilon v1.23 to obtain the final genome sequence. Per-base coverage was obtained with

samtools depth (v1.16.1; ONT) and bedtools genomecov (v2.30.0; Illumina), and coverage breadth at thresholds ($\geq 1\times - \geq 200\times$) was derived from these per-base depths (Supplementary Data 8). Inter-assembly variant calling was performed by whole-genome alignment with MUMmer v3.1 and regional structural variation interpreted with SyRI v1.4; SNPs/indels were derived from MUMmer/nucmer alignments (parameters: --maxmatch -c 100 -b 200 -l 50), and SVs were detected with SyRI (parameters: --unic 100 -k).

Bulk RNA-seq library preparation, sequencing and analysis

For RNA extraction, overnight cultures of SG001 and ceftriaxone-evolved lineages were diluted to an optical density (OD) at 600 nm of 0.01 and grown at 37 °C with shaking at 220 rpm for 12 h. Total RNA was extracted from whole cells with EASYspin Plus Bacterial RNA Rapid Extraction Kit (cat#RN43, Aidlab, China), according to the manufacturer's protocol. RNA purity and quantity were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). After rRNA depletion, RNA was fragmented in fragmentation buffer and subjected to first- and second-strand cDNA synthesis, followed by end repair, A-tailing and adaptor ligation (with USER treatment where applicable). Libraries were PCR-amplified, purified with AMPure XP beads, and size-selected to 370–420 bp prior to final QC. Qualified libraries were sequenced on an Illumina NovaSeq 6000 to generate 150-bp paired-end (PE150) reads. Adapters and low-quality bases were trimmed with cutadapt; reads < 50 nt or with > 10% N were discarded, and reads mapping to rRNA were removed to obtain clean reads. Per-sample metrics (Raw reads, Adapter trimmed, rRNA removed, Clean rate, Q20/Q30, GC%) were summarised. Clean reads were aligned to the *Neisseria subflava* reference genome using STAR (default parameters), retaining uniquely mapped reads. Gene-level raw counts were obtained with featureCounts; TPM values were additionally computed for display/visualisation only and were not used for statistical testing. Differential expression was performed on raw counts using edgeR (negative-binomial modelling with multiple-testing correction); unless otherwise specified, genes with $p < 0.05$ and $|\log_2\text{fold change}| \geq 1$ were considered differentially expressed, and FDR values were reported. For visualisation, the TPM matrix of DEGs was transformed as $\log_2(\text{TPM} + 1)$, row-wise Z-scored, and subjected to hierarchical clustering of genes and samples to generate heatmaps.

Bacterial strains and genomic sequencing

The clinical isolate *Neisseria subflava* strains (SG001–009) were isolated from patients with bronchiectasis or other chronic lung diseases in the Singapore-Kuala Lumpur region (SG-KL, Malaysia)¹⁸. Genomic DNA was extracted from overnight cultures using the TIANamp Bacteria DNA Kit (cat#GDP302-02, Tiangen, China) according to the manufacturer's protocol. High-throughput sequencing was conducted on the Illumina NovaSeq 6000 platform, generating paired-end reads with a read length of 150 bp. Raw sequencing reads were assessed for quality using FastQC (version 0.11.9) and subsequently trimmed using Trimmomatic (version 0.39) to remove low-quality bases and adapter sequences. De novo genome assembly was performed using SPAdes (v3.15.4) under default settings, and genome annotations were generated using Prokka (v1.14.6).

ICP-MS analysis of intracellular metal content

Bacterial cultures of wild-type and evolved (C26) *Neisseria subflava* strains were grown to mid-log phase in BHI broth at 37 °C with shaking. 1×10^9 colony-forming units (CFU) were harvested by centrifugation at $5000 \times g$ for 10 min at 4 °C. Washed pellets were resuspended in 200 μl of ultrapure water and subjected to acid digestion by adding 500 μl of 65% trace-metal-grade nitric acid (HNO_3) in metal-free Teflon tubes. Digestion was carried out at 95 °C for 1 h in a closed heating

block or using a microwave digestion system where available, following validated protocols for biological samples. After cooling to room temperature, the digests were diluted to 5 ml with ultrapure water and filtered through a 0.22 μm PTFE filter to remove particulates. Metal ion concentrations (Fe, Mg) were quantified by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7900) under standard operating conditions with helium collision mode. Calibration was performed using certified multi-element standards. Three biological replicates were analyzed for each strain.

RAW264.7 cell phagocytosis resistance assay

RAW264.7 murine macrophage cell line was maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified incubator with 5% CO_2 . Cells were seeded at a density of 5×10^5 cells per well in 12-well plates and incubated overnight to allow adhesion. *Neisseria subflava* strains were grown overnight in BHI medium at 37 °C with shaking. Cultures were diluted to an OD600 of 0.6 in fresh BHI medium and grown to mid-log phase. Bacterial cells were washed twice with PBS, resuspended in antibiotic-free DMEM, and added to the RAW264.7 cells at a multiplicity of infection (MOI) of 100:1. Plates were centrifuged at $300 \times g$ for 5 minutes to synchronize infection and then incubated for 1 h at 37 °C with 5% CO_2 . Following infection, cells were treated with 100 $\mu\text{g}/\text{mL}$ gentamicin for 30 min at 37 °C to eliminate extracellular bacteria after PBS washing, then washed again and lysed with 0.1% Triton X-100 in PBS. The lysates were serially diluted. Dilutions were plated on BHI agar and incubated at 37 °C for 24 h to determine colony-forming units (CFU). CFU counts were normalized to the initial RAW264.7 cell density, and results were expressed as mean $\pm$ SD from three independent experiments.

Sequence typing (ST) analysis

Multilocus sequence typing (MLST) was conducted using the MLST scheme for *Neisseria* species. Seven housekeeping genes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm*) were amplified and aligned against the PubMLST database. The resulting ST profiles were clustered using goeBURST to determine phylogenetic relationships. SG001–006 were assigned to the ST-a group, SG007 was classified as the ST-b group, and SG008 and SG009 were grouped into the ST-c group.

RT-qPCR

Total RNA was extracted using EASYspin Plus Bacterial RNA Rapid Extraction Kit, (cat#RN43, Aidlab, China). RNA concentrations were measured using NanoDrop 2000c (Thermo Fisher, USA) and normalized among the experimental sample set. cDNA was reverse transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Bio, Japan) according to the manufacturer's instructions. The SYBR Green Pro Taq HS qPCR Premix (Cat#AG11701, AG, China) and reactions were run on ABI Step One Plus Real-Time PCR equipment in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The expression levels of target genes were normalized to *gyrB*, and relative expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Three candidate genes (*compP*, *bamE*, *bfrA*, and *bfrB*) were randomly selected from the transcriptome data to verify the accuracy of the transcriptome data; the primers are listed in Supplementary Table 4.

Gene knockout in *Neisseria subflava*

Approximately 700 bp sequences flanking the upstream and downstream regions of the target gene were PCR-amplified from *N. subflava* genomic DNA, using primers listed in Supplementary Table 7. The gentamicin resistance cassette, containing its own promoter and terminator, was PCR-amplified from a plasmid template. These three fragments (upstream arm, gentamicin cassette, downstream arm) were fused into a single linear knockout cassette by overlap extension PCR and verified by agarose gel electrophoresis. For transformation, *N.*

subflava was streaked on blood agar plates and incubated overnight at 37 °C. Fresh colonies were scraped and resuspended in ice-cold 0.3 M sucrose, washed three times, and finally resuspended in 0.3 M sucrose to adjust the suspension to an OD₆₀₀ of ~1.5. Fifty microliters of electrocompetent cells were mixed with ~1 µg of purified knockout cassette and transferred into a pre-chilled 0.2 cm electroporation cuvette. Electroporation was performed using a Bio-Rad Gene Pulser at 2.5 kV, and cells were immediately recovered in 1 ml of pre-warmed BHI broth with 15% FBS at 37 °C with gentle shaking (110 rpm) for 2–6 h. Transformants were selected on blood agar supplemented with 15 µg/ml gentamicin and incubated at 37 °C for 36–48 h. Gentamicin-resistant colonies were screened by PCR using primers flanking the homologous arms, and correct gene replacement was further confirmed by Sanger sequencing. Colonies were screened by colony PCR with primers outside the homologous regions, and positive mutants were confirmed by Sanger sequencing.

Fluorescent tagging via homologous recombination

For in situ fluorescent tagging (*comP*::mCherry, *bamE*::gfp, and *bfrA*::gfp), the double-crossover homologous recombination strategy described above was employed, with the fluorescent marker gene (mCherry or gfp) inserted in-frame at the target locus along with a gentamicin resistance cassette for selection. Fusion constructs consisted of relatively long homologous arms flanking the target site, along with external primers to enhance transformation efficiency. Electroporation, recovery, and selection were performed as described for gene knockout mutants. Correctly tagged clones were verified by fluorescence microscopy, PCR, and Sanger sequencing. Strains were cultured overnight on blood agar, then inoculated into BHI broth and grown at 37 °C with shaking (220 rpm) to mid-log phase (OD₆₀₀ ≈ 0.5–0.8). Bacterial cells were harvested by centrifugation, washed once with PBS, and resuspended in PBS. A 5 µl aliquot was spotted onto a glass slide for microscopy. In bacterial live-cell localization experiments, the ECLIPSE Ti2-I microscope was used to capture both mCherry and GFP channel fluorescence, as well as bright-field images of bacteria. NIS-Elements Viewer 5.27 was employed for subsequent image processing. For each condition, three independent fields of view were analyzed. Primers for fluorescent reporter fusions (*comP*::mCherry, *bamE*::gfp, *bfrA*::gfp) are provided in Supplementary Table 6.

Fluorescence imaging of *bfrA*::gfp

To assess iron-dependent expression of the *bfrA*::gfp reporter, single colonies were inoculated from blood agar into BHI broth and cultured at 37 °C with shaking (220 rpm) to mid-log phase (OD₆₀₀ ≈ 0.5–0.8). Cultures were divided and supplemented with FeCl₃ (0 or 50 µM final concentration), followed by continued incubation for 6 h. Bacterial cells were harvested by centrifugation, washed with PBS, and resuspended in PBS. A 5 µl aliquot was placed on a glass slide for microscopy. The ECLIPSE Ti2-I microscope was used to capture fluorescent images of bacteria, and NIS-Elements Viewer 5.27 was employed for subsequent image processing. Three independent fields of view were analyzed per group.

Cloning procedures and overexpression of genes

DNA cloning and plasmid construction were performed using standard molecular biology techniques. PCR primers were designed to incorporate BamHI and HindIII restriction sites at their 5' ends, enabling the subsequent digestion and ligation of the target gene into the pMCSS vector, which harbors a tetracycline resistance gene. Seamless cloning was performed according to the manufacturer's guidelines. The recombinant plasmid was introduced into *Escherichia coli* DH5α and *Neisseria subflava* via electroporation. Positive clones were selected on 5% sheep blood agar plates supplemented with 15 µg/ml tetracycline (TC), and all plasmids were verified by Sanger sequencing. *Neisseria subflava* strains harboring the recombinant plasmid were cultured in

medium containing 15 µg/ml TC until reaching logarithmic growth, at which point gene expression was induced with 1 mM IPTG. The over-expressed gene is in Supplementary Table 5.

Bacterial isolation from bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage fluid (BALF) samples were collected from 38 patients with bronchiectasis undergoing fiberoptic bronchoscopy at Shenzhen Third People's Hospital, based on clinical indications. During the procedure, sterile 0.9% saline was instilled into the right middle lobe or lingular segment of the bronchus and recovered using low-pressure suction (≤100 mmHg). Samples were immediately placed on ice to preserve microbiome composition, then centrifuged at 3000 × *g*, 4 °C for 10 min, and the pellet was resuspended in sterile PBS. To maximize bacterial recovery, aliquots were plated onto chocolate agar and sheep blood agar (37 °C, 5% CO₂, 48 h) for common respiratory pathogens, MacConkey agar (37 °C, aerobic incubation, 48 h) for Gram-negative bacteria, and CDC anaerobic agar (37 °C, anaerobic conditions, 5–7 days) for anaerobic species. After single-colony purification, individual bacterial isolates were selected for species identification using 16S rRNA sequencing and confirmed by Sanger sequencing.

Statistical analyses

All data analysis was expressed as the mean ± SD. Each experiment was repeated a minimum of three times, with statistical analysis based on biological replicates. Detailed information regarding replicates and statistical methods for each experiment can be found in the figure legends and the Source data file. Differences were considered significant when the *P* value was <0.05. GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA) was used to analyze the experimental data.

Ethics

The study involving human clinical samples was approved by the Institutional Review Board of Shenzhen Third People's Hospital (Ethics approval number: 2024-153). Informed consent was obtained from all participants involved in the study.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

These datasets are publicly accessible and provide detailed information to support data validation, reproducibility, and further analysis by the research community. The transcriptomic and single-cell RNA sequencing datasets generated in this study have been deposited in the NCBI Sequence Read Archive (SRA). The single-cell RNA-seq and bulk RNA-seq datasets for wild-type and C26-a strains are available under BioProject accession [PRJNA1209901](#), and the whole-genome sequencing data are under [PRJNA1208745](#). To support lineage-level validation, additional bulk RNA-seq datasets for independently evolved C26-b and C26-c strains have been deposited under BioProject accession [PRJNA1292455](#). All data are publicly available as of the date of publication. Source data are provided with this paper.

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Competing interests

S.H.C. is on advisory boards for CSL Behring, Pneumagen, Sanofi and Boehringer Ingelheim, serves on Data and Safety Monitoring Boards for Inovio Pharmaceuticals and Imam Abdulrahman Bin Faisal University, and has received personal fees from Chesi Farmaceutici and Astra-Zeneca, all outside of the submitted work. All other authors declare no competing interests.

Additional information

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