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Comparative genomics of *Rothia* species reveals diversity in novel biosynthetic gene clusters and ecological adaptation to different eukaryotic hosts and host niches

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Abstract

Rothia species are understudied members of the phylum Actinobacteria and prevalent colonizers of the human and animal upper respiratory tract and oral cavity. The oral cavity, including the palatine tonsils, is colonized by a complex microbial community, which compete for resources, actively suppress competitors and influence host physiology. We analysed genomic data from 43 new porcine *Rothia* isolates, together with 112 publicly available draft genome sequences of *Rothia* isolates from humans, animals and the environment. In all *Rothia* genomes, we identified biosynthetic gene clusters predicted to produce antibiotic non-ribosomal peptides, iron scavenging siderophores and other secondary metabolites that modulate microbe–microbe and potentially microbe–host interactions. *In vitro* overlay inhibition assays corroborated the hypothesis that specific strains produce natural antibiotics. *Rothia* genomes encode a large number of carbohydrate-active enzymes (CAZy), with varying CAZy activities among the species found in different hosts, host niches and environments. These findings reveal competition mechanisms and metabolic specializations linked to ecological adaptation of *Rothia* species in different hosts.

DATA SUMMARY

All datasets generated for this study are included in the manuscript and/or in the Supplementary Files. The whole-genome sequencing data is available at the European Nucleotide Archive (ENA) under BioProject ID PRJEB49523.

INTRODUCTION

The oropharyngeal cavity including the palatine tonsils is colonized by a complex microbial community influencing pig health and physiology [1]. *Rothia* bacteria (phylum Actinobacteria) are prevalent members of the upper respiratory tract microbiota of humans [2], pigs [1], rats [3] and birds [4] and early colonizers of the oropharyngeal cavity including the tonsils, tongue and teeth of healthy humans and piglets [5–7]. It is therefore possible that specific *Rothia* species contribute to the establishment of the microbial communities colonizing healthy animals in early life. Although several different *Rothia* species have been described, there is little knowledge about genomics in relation to species physiology and their ecological niches.

Rothia bacteria are all Gram-positive rods and to date 14 species have been identified (https://www.bacterio.net/genus/rothia): *Rothia aeria* [8], *Rothia aerolata* [9], *Rothia amarae* [10], *Rothia arfidiae* [11], *Rothia dentocariosa [12], Rothia endophytica* [13], *Rothia halotolerans* [14], *Rothia koreensis* [14], *Rothia kristinae* [14], *Rothia marina* [15], *Rothia mucilaginosa* [16], *Rothia nasimurium* [16], *Rothia nasisuis* [17] and *Rothia terrae* [18]. Furthermore, unclassified and uncultured *Rothia* genomes have been reconstructed from metagenomic data [19]. Members of the *Rothia* genus, have been isolated from different ecosystems, including environmental samples such as air and water, and host-associated niches including the skin, gut and oral cavity.

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Abbreviations: ARGs, antimicrobial resistance genes; BGC, biosynthetic gene cluster; CAZy, carbohydrate-active enzymes; NRPs, non-ribosomal peptides; NRPS, non-ribosomal peptide synthetase; PKs, polyketides; RiPPs, ribosomally synthesized and post-translationally modified peptides.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. One supplementary figure and five supplementary table are available with the online version of this article.

Impact Statement

Rothia species are abundant members of the upper respiratory tract microbiota in humans and animals but are relatively understudied. Like other notable members of the Actinobacteria phylum, *Rothia* encode novel bioactive compounds in their biosynthetic gene clusters (BGCs) and are an untapped potential source of new antimicrobials. Additionally, our work advances knowledge about the genetics and physiology of *Rothia* species revealing correlations between BGCs, carbohydrate metabolic activities and the colonization of specific ecological niches.

Microbiota association studies have shown that the genus *Rothia* mainly correlates with healthy individuals [19–21] whereas specific strains from the species *R. aeria*, *R. dentocariosa* and *R. mucilaginosa* are associated with caries and dental pits, fissures and plaque [22, 23].

Recently, we showed that *Rothia nasisuis* colonizing the palatine tonsil epithelium of piglets produces the antimicrobial, antiviral and antiparasitic ionophore valinomycin *in vivo*, via a large multimodule non-ribosomal peptide synthetase (NRPS) enzyme complex encoded by genes tandemly arranged in a biosynthetic gene cluster (BGC) [24]. Biosynthetic gene clusters (BGCs) produce diverse metabolites including natural antimicrobial products that fall into three major metabolite groups: ribosomally synthesized and post-translationally modified peptides (RiPPs), non-ribosomal peptides (NRPs) and polyketides (PKs) [25]. RiPPs form a large heterogeneous group of peptides that are usually classified into post-translationally modified peptides and unmodified peptides [26]. NRPs are relatively small peptides (2–30 amino acids) with hallmarks of nonproteinogenic amino acids and extensive 'post-translational modifications'. Substrates and intermediates are covalently bound during the assembly pathway and the order of the catalytic domains in the NRPS often parallels the order of their biosynthetic pathway [27, 28]. Finally, PKs are categorized into different classes based on their biochemical mechanisms and enzyme architecture [29]. The presence of specific BGCs in *Rothia* species can be used as markers to group and distinguish between species, and BGC annotations help to predict ecological traits.

Carbohydrate-active enzymes (CAZy) are widely present in all organisms and play important roles in biological processes and ecological adaptations. CAZy are classified into several families and their annotation can be used to predict the ability of organisms to assemble and break down complex carbohydrates [30], important ecological traits for niche exploitation and colonization of novel environments. Bacteria colonizing different environments, display a range of CAZy metabolic capacities linked to the diversity, structure and composition of carbohydrates available in the habitat [31]. *Rothia* species in the oral microbiota have been reported as nitrate-reducers [32, 33]. The conversion of nitrate to nitrite and nitric oxide can promote oral health by reducing the acidification of the saliva and inhibiting species contributing to periodontal disease through the antimicrobial activities of nitric oxide. Furthermore, nitrite is swallowed and taken up into the blood circulation where it is converted into nitric oxide, a signalling molecule, which is reported to improve cardiovascular and metabolic health [32, 34].

Here, we report on genomic analyses of 43 novel *Rothia* strains that we isolated and cultured from the tonsillar microbiota of piglets between 1 and 3 weeks of age. To place the genomic and phylogenetic analysis of these novel porcine strains in a broader ecological context, we analysed these *Rothia* strains together with publicly available draft genome sequences from human, animal and environmental *Rothia* strains. We inferred phylogenetic trees based on genomic data that we annotated with origin (niche) and presence/absence of genes of interest including biosynthetic gene clusters, carbohydrate-active enzymes, and antibiotic resistance and nitrate–nitrite metabolism genes, in order to discover ecological adaptations of *Rothia* species to different mammalian hosts (pigs and human) and other environments.

METHODS

Samples

We sampled the palatine tonsil of six random piglets at two high-health status farms in Catalunya, Spain, 1 week before weaning (timepoint −1) and 3 weeks after weaning (timepoint +3). Tonsil biofilms were collected using Puritan HydraFlock Swabs and stored at −80 °C in transport medium [buffered peptone +15% (v/v) glycerol].

Rothia spp isolation, identification and culture conditions

Dilutions of the tonsil samples between 10^{-1} and 10^{-3} were plated onto Sheep Blood Agar [35] (Becton Dickinson, Heidelberg, Germany) and Brain Heart Infusion (BHI) Agar [36] (Becton Dickinson) media. Single isolates were selected based on the morphology (size, shape and colour) of the colonies and transferred to BHI liquid medium (Fig. S1, available in the online version of this article), incubated at 37 °C in the presence of 5% CO_2. Isolates were purified using Gram staining and the quadrant streak plate method [37] and then stored at −80 °C into cryotubes containing BHI and 15% (v/v) glycerol.

Table 1. Indicator bacteria and culture conditions

Indicator micro-organism	Source/strain	Culture medium/temp (°C)
Streptococcus suis	P1/7	THB/37 °C
Streptococcus suis	S10	THB/37 °C
Streptococcus suis	J28	THB/37 °C
Staphylococcus aureus	ATCC 6538P	BHI/37 °C
Escherichia coli	L 4242	LB/37 °C
Streptococcus porci	DSM 23759	THY/37 °C
Streptococcus parasuis	DSM 29126	THY/37 °C
Streptococcus porcinus	DSM 20725	THY/37 °C

DSM, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures; ATCC, American Type Culture Collection; L prefix are from the NAICONS pathogens library, Italy. The strains of *S. suis* strain P1/7 [36], *S. suis* strain S10 [37] are zoonotic pathogen and the *S. suis* strain J28 [38] is less pathogenic, an unencapsulated mutant. *Escherichia coli* L4242, a ΔtolC mutant derivative from MG1061 [39], THB, Todd Hewitt broth (Becton Dickinson); BHI, Brain Heart Infusion (Becton Dickinson); LB, Luria–Bertani (Becton Dickinson); THY; THB enriched with 0.2% (w/w) yeast extract.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for screening and identification of pure colonies. The samples were spotted in the MALDI-TOF MS target plate and covered with 1 µl of matrix solution (saturated α-cyano 4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) [38]. The samples were analysed using a MICROFLEX spectrometer (Bruker Daltonics) according to the manufacturer's recommendations. For each spectrum, 90–100 peaks were compared with reference databases at the Department of Medical Microbiology, University Medical Centre Groningen, the Netherlands. An isolate was considered correctly identified at the species level when at least one MALDI-TOF MS spectrum score was ≥1.9 and at the genus level with a score ≥1.7 [38].

Bacterial indicator strains and culture conditions

The following culture media and bacteria were used in overlay inhibition assays (Table 1) to identify *Rothia* isolates with antagonistic activity.

Overlay inhibition assays

Rothia isolates were cultured in BHI broth medium and incubated overnight at 37 °C. Using a 96-well replicator (Boekel Scientific), overnight cultures of *Rothia* were spotted onto BHI agar media in Petri dishes (Fig. S1, available in the online version of this article) and grown for 18 h. Bacterial colonies were inactivated by exposure to UV light for 20 min and overlayed with 20 ml soft agar (0.75% w/v agar) containing approximately 1×10^5 c.f.u. ml^{-1} of the indicator bacteria (Table 2). Antimicrobial activity was determined by the presence of visible zones of growth inhibition ('halos') around the *Rothia* colonies after overnight incubation [39].

Genomic DNA extraction and sequencing

Rothia spp. strains were grown overnight in BHI broth at 37 °C. The next day, cells were pelleted by centrifugation, and genomic DNA was extracted using the PowerSoil Genomic Purification Kit (Qiagen) according to the manufacturer's recommended protocol for Gram-positive bacteria. Recovery of high molecular weight DNA was assessed on a 0.8% agarose gel (Sigma-Aldrich) in 1× TAE buffer [Tris-HCl 40 mM, 20 mM acetic acid and 1 mM EDTA (pH 8)], stained with 25 µg ml^{-1} of SYBR Safe and quantified using the Qubit dsDNA Broad-Range (BR) assay and Invitrogen's Qubit Fluorometer.

Genome sequencing, assembly, annotation and phylogenetic analysis

We obtained genome sequences from 43 tonsil isolates of *Rothia* from five piglets, randomly selected from different litters on two farms with a high-health status. Genome sequencing was performed on an Illumina HiSeq 2000 platform (Illumina) at MicrobesNG, Birmingham, UK. However, due to lower sequencing depth and shorter read lengths, a few genome sequence assemblies had a high number of contigs (Fig. S3, available in the online version of this article). Reads were trimmed using Trimmomatic 0.30 software with a sliding window set at Q15 [40]. Genome assembly was performed using SKESA 2.4.0 [41]. In total, 112 genome sequences of publicly available *Rothia* species were downloaded from NCBI's Reference Sequence database (Table. S1, available in the online version of this article) (download 27 April 2021). All genomes were annotated using Prokka 1.14.6 [42]. The 'core' genes were determined based on 90% protein similarity using Roary 3.13.0 [43] (Table S2, available in the

Table 2. *In vitro* inhibitory activity of the 43 *Rothia* sp. obtained from porcine palatine tonsil

		Indicator bacteria								
ID	**Species**	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptocuccus parasuis DSM 29126*	*Sthaphylococcus aureus ATCC 6538P*	*Escherichia coli L4242*	**BGCs from WGS (AntiSMASH 6.0)**
159RC1	*Rothia aerolata*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
56QC2O2	*Rothia endophytica*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing/RiPPs /terpene
63RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing/
67.3RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/NRPS
68RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/NRPS
110RC1	*Rothia nasimurium*	+/-	–	–	+/-	–	–	–	–	NRPS
136RC1	*Rothia nasimurium*	–	–	–	+/-	–	–	–	–	NRPS
207RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	NRPS
8QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
15QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
22QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
28QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
38QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone /RRE-containing
48QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
54QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing /RiPP-like
64QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing /NRPS
2QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
3QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/NRPS
4QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing /RiPP-like
6QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing /RiPP-like
7QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
19QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
34QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone /NRPS
36QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing/RiPP-like
37QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing/RiPP-like
46QC2O2	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing/RiPP-like
63QC2CO	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
65RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/NRPS
65.2RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/NRPS

Continued

Table 2. Continued

		Indicator bacteria								
66RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	NRPS
67RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/NRPS
123RC1	*Rothia nasimurium*	+/-	–	–	+/-	–	–	–	–	NRPS
124RC1	*Rothia nasimurium*	+/-	–	–	+/-	–	–	–	–	NRPS
213RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	Betalactone/RRE-containing
206RC1	*Rothia nasimurium*	–	–	–	–	–	–	–	–	NRPS
15QC4O2	*Rothia nasimurium*	–	–	–	–	–	–	–	+	RRE-containing
18QC4O2	*Rothia nasimurium*	–	–	–	–	–	–	–	+	RiPP-like/RRE-containing
31RC1	*Rothia nasisuis*	+	+/-	+	+	+	+	–	–	Betalactone/RRE-containing/NRPS (valinomicin)
69RC1	*Rothia nasisuis*	–	–	+	+	+	+	–	–	Betalactone/RRE-containing/NRPS (valinomicin)
141RC1	*Rothia nasisuis*	+	+/-	+	+	+	+	–	–	Betalactone/RRE-containing/NRPS (valinomicin)
152RC1	*Rothia nasisuis*	+	+/-	+	+	+	+	–	–	Betalactone/RRE-containing/NRPS (valinomicin)
196RC1	*Rothia nasisuis*	+	+/-	+	+	+	+	–	–	Betalactone/RRE-containing/NRPS (valinomicin)
107RC1	*Rothia nasisuis*	+/-	–	–	–	–	–	–	–	Betalactone/RRE-containing/NRPS (valinomicin)

Inhibition zones were scored after overnight incubation in at least two assays. (–) absence of inhibitory activity; (+/–) inhibition zone between 3 and 6 mm diameter (weak); (+) inhibition zone ≥7 mm diameter [14]. NRPS: non-ribosomal peptide synthetase; RRE-containing: recognition element containing NRPs and/or PKs clusters; RiPPs-like: category of ribosomally synthesized and post-translationally modified peptides.

online version of this article). A maximum-likelihood tree was inferred using IQ-TREE 2.1.4 on default parameters from the core gene alignment obtained from Roary 3.13.0. Tree visualization was done in R 4.0.5 using ggtree 2.4.2 [44].

Carbohydrate-active enzyme analysis

Carbohydrate-active enzymes (CAZy) present in the genomes were determined using dbCAN2 2.0.11 using default parameters [45]. CAZy were annotated as auxiliary activities (AA), carbohydrate-binding modules (CBM), carbohydrate esterase (CE), glycoside hydrolases (GH), polysaccharide lyases (PL) or glycosyltransferases (GT). To limit false positives, a minimum of two hits was considered reliable. Hierarchical clustering based on Bray–Curtis dissimilarity was used to cluster the genomes. Five clusters were determined to be the optimal number based on gap statistics. Principal component analysis (PCA) was used to visualize the distribution of the CAZy across the collection genomes in our dataset.

Antimicrobial resistance genes (ARGs) and nitrate–nitrite metabolism genes

Antimicrobial resistance genes (ARGs) were identified using Abricate 1.0.1 (https://github.com/tseemann/abricate) with the ResFinder database [36] at 80% DNA identity and coverage cut-off for both. The sequences taxonomically related to nitrate/nitrite metabolism in *Rothia* [32] were compared by similarity using the programme BLASTX [46] against all the genomes used in this study to identify the potential hits with filtering of e-value $<10^{-5}$ and as threshold for sequence identity and coverage ≥50%.

Mining of antimicrobial peptide encoding genes

For identification and annotation of biosynthetic gene clusters (BGCs), draft genomes of all *Rothia* strains were analysed using antiSMASH 6.0.1 using default detection criteria for the analysis [47]. The BGCs from the strains were sorted into groups based on their predicted activity. In addition, the Biosynthetic Genes Similarity Clustering and Prospecting Engine (BiG-SCAPE) software was used to define a distance metric between gene clusters using a combination of three indices: Jaccard Index of domain types, Domain Sequence Similarity, and the Adjacency Index [48]. Networks were visualized using Cytoscape 3.8.2 [49]. Clinker 0.0.21 was used for the alignment and comparison of BGCs [50].

RESULTS

Rothia spp. isolation and identification corroborated and complemented previous *Rothia* distribution reports

To obtain upper respiratory isolates of the *Rothia* genus from pigs we cultured tonsil swabs from five piglets on sheep blood agar plates and purified over 500 isolates by repeated steaking of single colonies on fresh plates. Ninety-three isolates were identified as *Rothia* spp. by MALDI-TOF MS analysis (data not shown). Forty-six *Rothia* isolates were then selected for genome sequencing based on differences in colony morphology, growth characteristics and inhibitory activity against different target bacteria in the same niche. The assembled genomes ranged between 2.31–2.79 Mb in size, with a GC-content between 56.7–60.3% (Table S3, available in the online version of this article). We identified 43 *Rothia* isolates to the species level using full-length sequences of 16S small subunit ribosomal RNA (rRNA); *R. nasimurium* (*n*=36), *R. nasisuis* (*n*=5), *R. aerolata* (*n*=1) and *R. endophytica* (*n*=1).

Genomic analyses and screening the genomic dataset to discover candidate genes involved in colonization and adaptation of the different species in the environment

To identify accessory genes involved in antibiotic resistance, secondary metabolite biosynthesis, and carbohydrate utilization genes, which might play crucial roles in niche adaptation, competition, and persistence, we analysed the genomes of our 43 isolates and 112 publicly available draft genomes of *Rothia* species and strains (as of April 2021). In total, 155 *Rothia* genomes were included along with the metadata, sample origin and disease-associated or commensal species. (Fig. 1). The relatedness of the 155 genomes was investigated using core-genome phylogenies and mined for the presence of (i) BCGs, (ii) antimicrobial resistance genes, and (iii) carbohydrate-active enzymes (CAZy) encoded in the genome.

We constructed phylogenetic trees based on 90% core protein similarities (encoded by 28 core genes that were shared by the *Rothia* strains (Table S2, available in the online version of this article) and observed three major clades that overall corresponded to their origin; human, environmental/food and, porcine/other animals (sponge, *Mus musculus*, duck and marmot). We annotated the phylogeny with the presence/absence of different classes of BGCs and antibiotic resistance genes (Fig. 2).

Genome mining for antibiotic resistance, nitrate/nitrite reduction genes and BGCs

On average *Rothia* genomes contain between two to eight antibiotic resistance genes (ARGs) including variants of the gene 'families' *aac, aad, ant, aph, cmx, dfr, erm, sul* and *tet*. ARGs were identified mainly in *Rothia* isolates belonging to the species *R. nasimurium* and *R. mucilaginosa* sampled from pigs and humans (Fig. 2). Most of the *Rothia* genomes encode genes involving nitrate/nitrite metabolism as previously reported [51]. The predicted gene functions and number of genes associated with nitrate/nitrite reduction vary across *Rothia* species and their ecological niche (Table S5, available in the online version of this article).

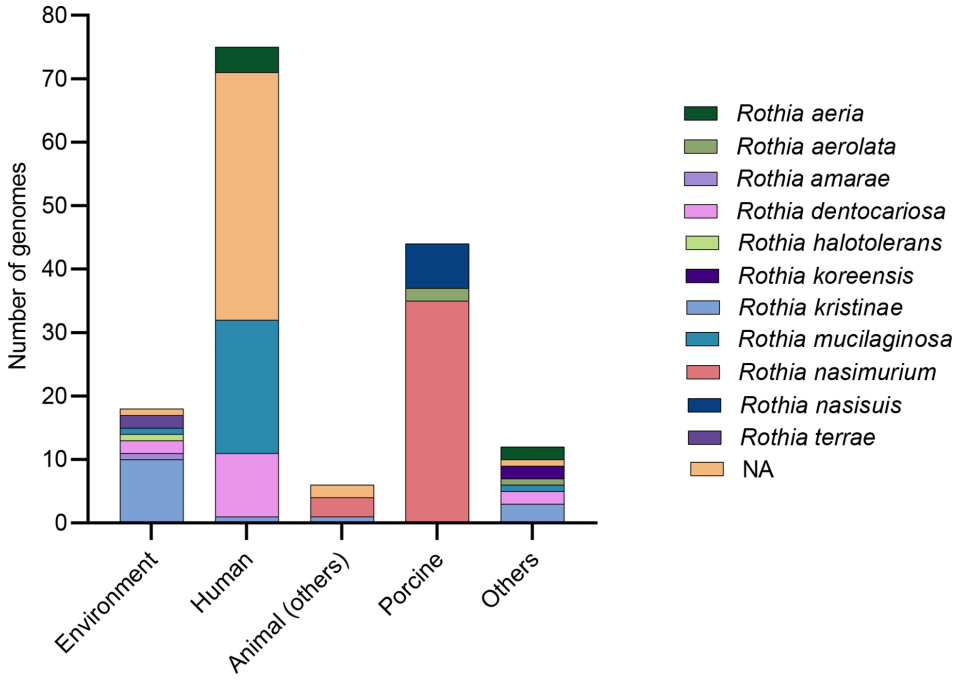


Fig. 1. *Rothia* genomes including the species *R. aerolata, R. aeria, R. amarae, R. kristinae, R. dentocariosa, R. halotolerans, R. mucilaginosa, R. terrae, R. nasimurium, R. nasisuis, R. koreensis* uncultured*Rothia* sp identified in metagenomic data from mainly human clinical samples in the NCBI Reference Sequence Database. The bar chart shows the number of genomes analysed for each *Rothia* species per sampling site (i.e. origin of isolates); *'others'* correspond to isolates from food and unspecified samples. *Animal* (others) corresponds to diverse animal groups (sponge, *Mus musculus*, duck and marmot) but not pigs. NA no assigned species (*Rothia sp*).

Human-associated *Rothia* species carry the largest number of genes involved in nitrate and nitrite reduction metabolism, followed by animal-associated *Rothia* species.

Overall, 386 BGCs were predicted in the 155 genomes used in this study. In 80% of the *Rothia* genomes, at least one BGC was annotated as non-ribosomal peptide synthetase (NRPS) or hybrid non-ribosomal peptide-polyketide (NRP/PK) synthase, which together account for 146 of all the predicted clusters (Fig. 2). Other common BGC types were gene clusters annotated as producing betalactone; multidomain polyketide synthases (PKSs), and ribosomally synthesized and post-translationally modified peptides (RiPPs) with a broad range of biological functions including antimicrobial acticvity (Table S3, available in the online version of this article).

In the 43 porcine Rothia isolates, antiSMASH mainly predicted the presence of NRPs, RiPPs and NRPs (Table 2). NRPS gene cluster (family FN2043), found only in *R. nasisuis* is predicted to produce the natural antibiotic valinomycin (Fig. 3c). Genomes of *R. nasimurium,* possess NRPs clusters (family FN0062); members of this family are predicted to produce secondary metabolites (Fig. 3a). These 18 BGCs had low similarity showing potential novelty based on the BigScape analyses.

Overlay inhibition assays

Inhibition assays were performed using a panel of eight indicator bacteria with all the *Rothia* strains from this study (n=43) (Table 2). The indicator bacteria included strains of three zoonotic pathogen *S. suis*, which colonizes the oropharyngeal cavity and tonsil epithelium of pigs, as well as related streptococci *S. porci*, *S. porcinus*, *S. parasuis, S. aureus* and *E. coli* as a representative Gram-negative bacterium (Table 1). Fourteen *Rothia* strains were able to inhibit the growth of at least one species of Gram-positive bacteria tested (Table 2). All *R. nasisuis* and six strains of *R. nasimurium* showed growth inhibitory activity against streptococci but had no activity against *S. aureus* and two strains of *R. nasimurium* had low inhibitory activity against *E. coli*.

Carbohydrate active enZymes (CAZy)

We discovered more than 5040 diverse carbohydrate active enZyme (CAZy) genes, which were classified in different families and subfamilies (Table. S4, available in the online version of this article), suggesting there may be a link between CAZy functionality ('toolbox') and the ability to colonize and persist in novel niches under varying environmental conditions.

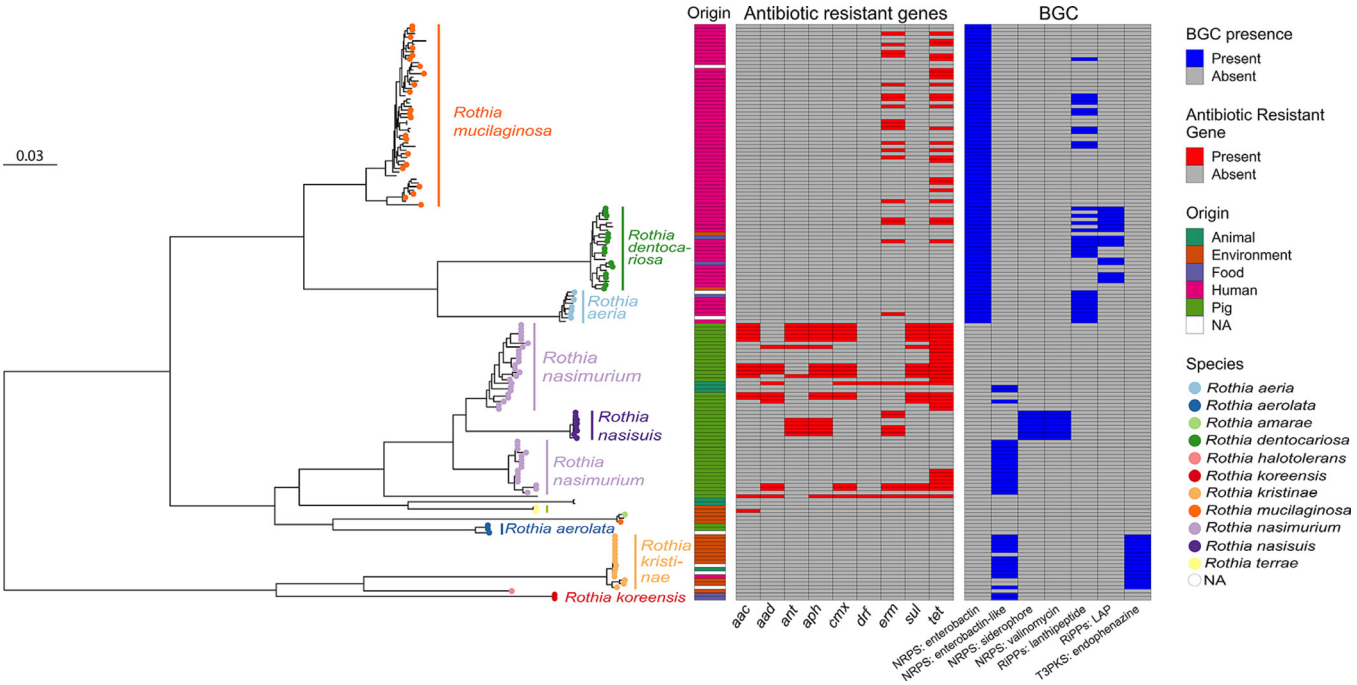


Fig. 2. Overview of the biosynthetic gene clusters (BGCs) and antimicrobial resistance genes (ARG) in the species of *Rothia* analysed in this study from different sampling sites. ARG represents multiple variants of the gene 'families'. The compilation follows: the sampling-sites are represented by the colours shown in the inner colourful column. The rooted tree was constructed based on the core genes (*n*=28), the species are named and highlighted with the same colour. The antibiotic resistance genes are displayed as presence (red) and absence (grey), which could lead to the selection of potential producers of bioactive compounds. The outermost columns display the presence/absence of biosynthetic gene clusters; distinct clusters were annotated according to antiSMASH in grey (absence) and dark blue (presence). The scale bar indicates nucleotide substitution per site.

Using the gene annotations of *Rothia* species, our analyses revealed that *Rothia* species contain five major families of genes predicted to participate in carbohydrate metabolism and energy conversion systems, including genes predicted to encode glycosyl hydrolases (GHs), glycosyl transferases (GT), carbohydrate-binding modules (CBM), carbohydrate esterases (CE), auxiliary activities [36], and other carbohydrate metabolic pathway components (Fig. 4).

Overall, 41 CAZy families were annotated among the *Rothia* genomes (21 GHs, 14 GTs, 2 CBMs, 3 CE and one AA) at different abundances per species and niche (Fig. 4). The GHs had highest gene sequence diversity and abundance followed by the GTs. Rothia species have large numbers of GTs representing >65% of the total CAZy enzymes toolbox. GT families are highly diverse in the CAZy database but in the *Rothia* genomes just two families, GT2 and GT4, represent a large portion of the total number of GT genes (*n*=3372) identified all strains and niches. The first horizontal blocks of the heatmap (Fig. 4) shows clustering between the families CBM5-GT87, which contains enzymes prevalent in several species of *Rothia* in all niches, suggesting the presence of essential enzymes responsible for carbohydrate metabolism. The CAZy families GH33, GH36, GT5, GT35, CBM48 +GH13-11 and CBM48 +GH13-9 (CAZy Group 1) were mostly discovered in the animal-associated species *R. nasimurium* and *R. nasisuis*. The families GT25 and CBM50 +GH23 were more often identified in human-associated *Rothia* species; these enzymes clustered in CAZy groups 2 and 3. Specifically, the family GT25 was mainly discovered in the genomes of *R. dentocariosa* and the family complex CBM50 +GH23 was present in 85% of the *Rothia* species isolated from human microbiota. On the other hand, CAZy enzymes GH65, CBM50 and GT20 were only discovered in *Rothia* species sampled from the environment (Fig. 5).

To investigate CAZy distribution across the 155 *Rothia* genomes representing 12 species originating from different niches, a principal component analysis (PCA) was conducted (Fig. 6). The first two principal components explained 66.7% (50.3 and 16.4% for PC1 and PC2, respectively) of the total variance.

The CAZy-based PCA plot clustered species largely by taxonomy; isolates and genomes from the species (Fig. 6a). *R. mucilaginosa* and *Rothia* sp. (unclassified *Rothia*'s) mainly clustered in the upper right area. Most isolates and genomes of the species *R. kristinae, R. halotolerans* and *R. koreensis* clustered in the lower-left area. Most isolates and genomes of the species *R. aeria* and *R. aerolata* clustered in the middle. Finally, most isolates and genomes of the species *R. nasimurium* and *R. nasisuis* clustered in the upper left area of the plot. Those *Rothia* species clustering together in the PCA also clustered closely in the phylogenetic analysis or colonized very similar niches (Fig. 6b).

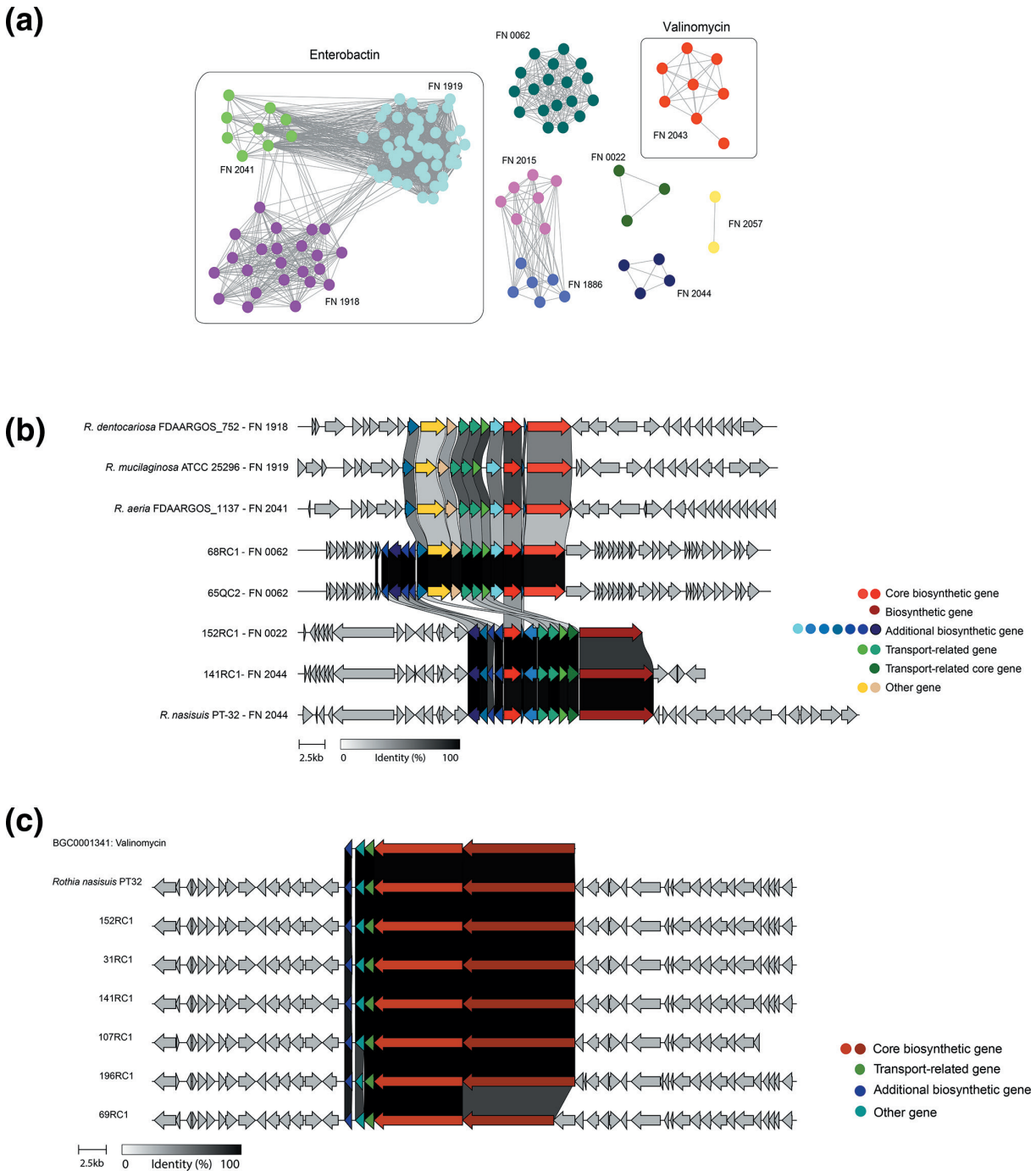


Fig. 3. Overview of NRPs-type BGCs from *Rothia* species. (a) Similarity network of known and putative BGCs annotated as NRPs based on antiSMASH results. The small groups of NPRS families (FN) with unknown clusters containing fewer common genes are drawn outside the boxes: FN2015, FN0022, FN2044 and FN1086. Highlighted groups include widely distributed clusters in many different species. The network containing red nodes represents the valinomycin (FN2043) cluster that was present in all porcine *R. nasisuis* isolates. The network containing light blue, green and purple nodes represent BGCs corresponding to Enterobactin cluster families FN1918, FN1919, and FN2041 that were present in the species of *R. mucilaginosa*, *R dentocariosa* and *R. aeria* that were mainly sampled from the upper respiratory tract of humans. The network containing dark green nodes represents an NRP-like cluster annotated as family FN0062 that was prevalent in *R. nasimurium;* this cluster contained conserved genes from the Enterobactin cluster. The families represent the group of BGCs that encode biosynthesis of highly similar or identical metabolites classified in arbitrary numbers based on the outcome of the analyses. (b) Overview of representative species that contain conserved Enterobactin BGCs, or NRP-like cluster annotated as family FN0062 (*R. nasimurium*) that shares genes with Enterobactin BGC as well as the families FN0022 and FN2044. (c) Overview of the conserved valinomycin cluster (BIG-SCAPE family FN2043) in the species *R. nasisuis*. BIGSCAPE output is visualized as a network using Cytoscape and the cluster organization visualized using Clinker (https://github.com/gamcil/clinker).

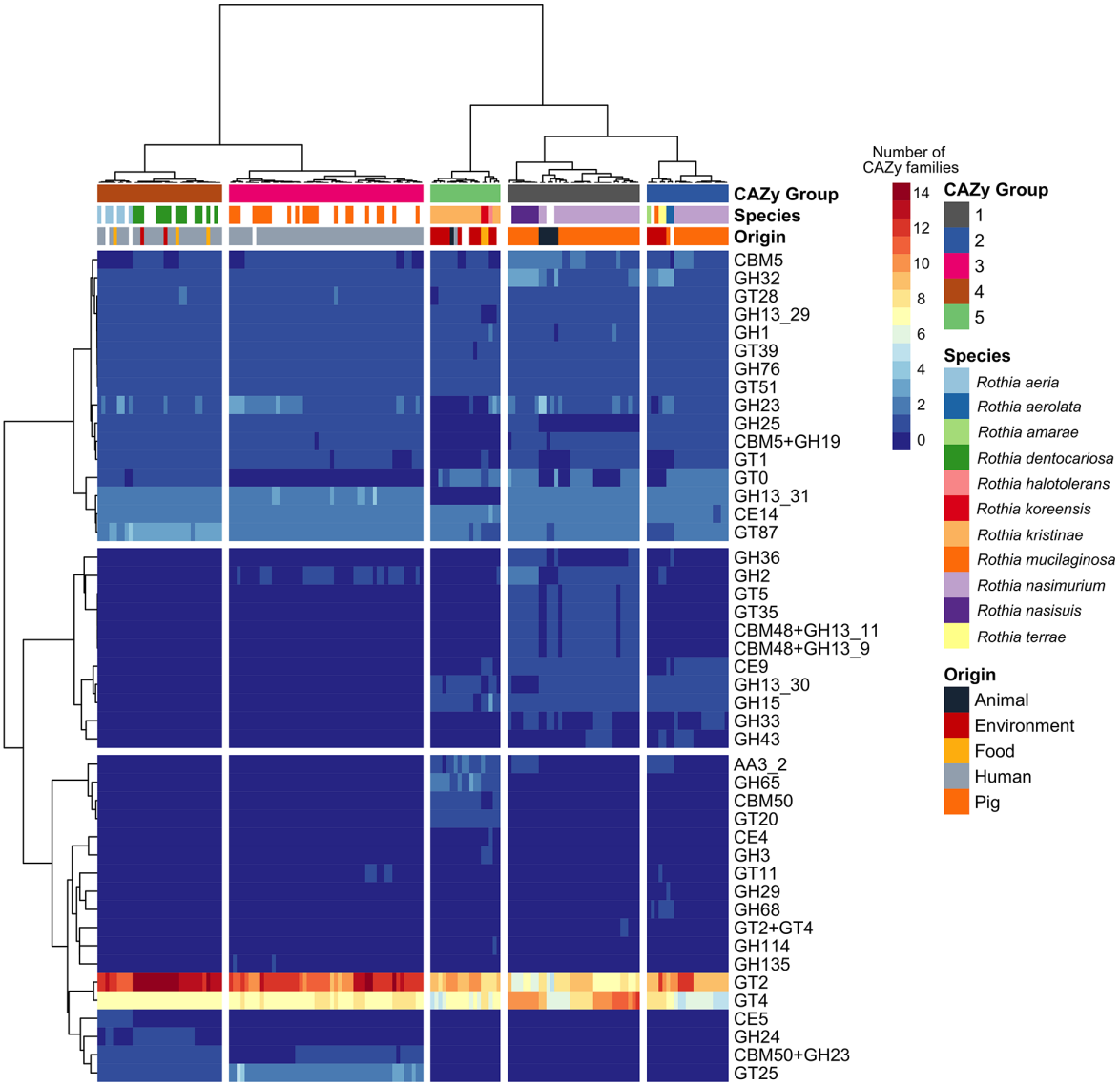
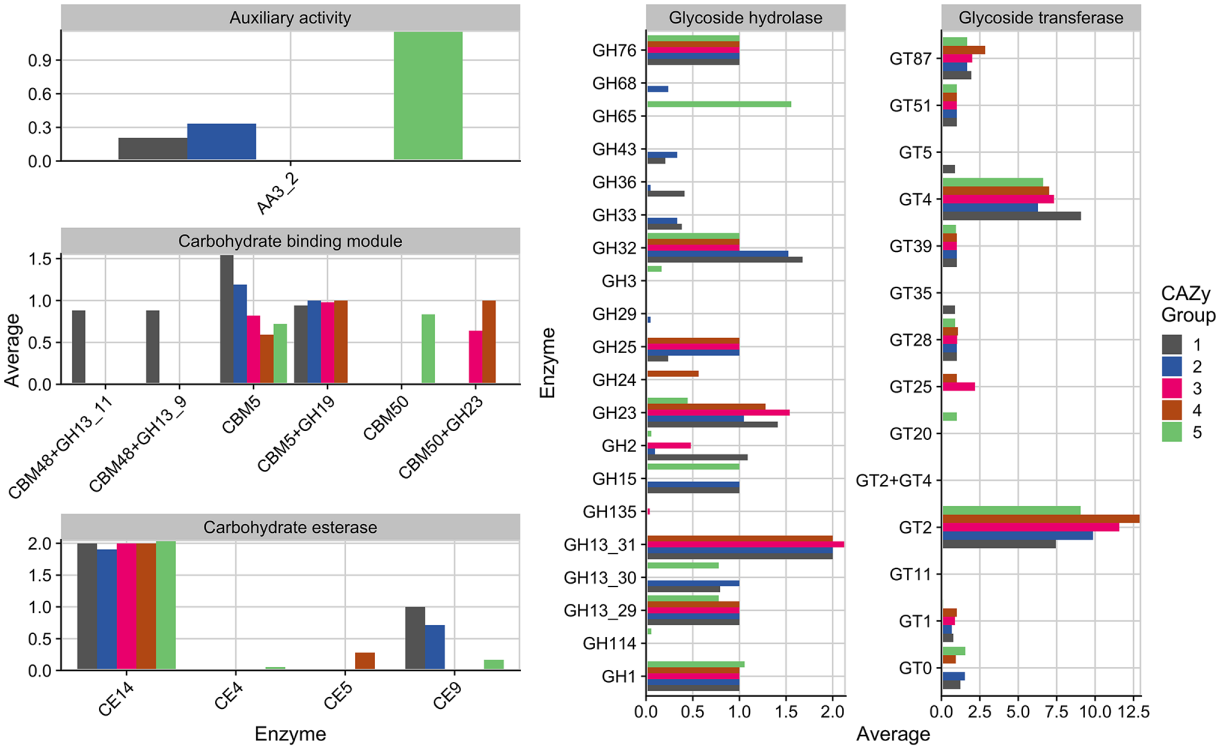


Fig. 4. Heatmap displaying presence/absence of CAZy families across *Rothia* genomes. The heatmap includes data from 41 CAZy families (rows); the sampling site and distribution of CAZy families across *Rothia* species are displayed in bars above the heatmap. 'CAZy group' (columns) represents the enzymes present in a specific group of species clustered according to our analysis. All displayed CAZy gene data and annotations were retrieved from the CAZy enzyme database (accessed October 2021). Glycosyl hydrolase (GH), glycosyl transferases (GT), carbohydrate-binding modules (CBM), carbohydrate esterases (CE).

DISCUSSION

Recently, *Rothia* was listed as no. 17 in the top 20 most abundant and prevalent bacterial genera discovered in the human oral cavity [2]. Our recent work on porcine tonsil microbiota revealed *Rothia* as one of the most prevalent genera with at least 0.98% mean abundance across individuals; the genus was detected in more than 98% of the piglets from a data set with over 160 samples, across 11 farms sampled in Europe (unpublished).

The habitat specificity of *Rothia* species suggest adaptation to certain oral niches such as the teeth surface and the oral mucosa [2]. Here we characterized the genomes of several species of the poorly characterized genus *Rothia* to gain a better understanding of the interactions and colonization in the upper respiratory tract and the potential ecological role of specialized metabolites and CAZy enzymes. Genome mining of the sequences from the databases of over a hundred *Rothia* genomes and the recent sequenced porcine isolates identified several BGCs with potential to produce natural antimicrobial compounds. The metabolites predicted to be produced by these gene clusters include compounds that function in competitive interactions between microbes such as antibiotics and siderophores, but also compounds that may function in the host, including immunosuppressants and anticancer



CAZy group	Species	Sampling site
1	*R. nasisuis*, *R. nasimurium* and NA	Porcine and other animals
2	*R. nasimurium*, *R. aerolata*, *R. amarae*, *R. terrae*, *R. mucilaginosa* and NA	Environment, porcine and NA
3	*R. mucilaginosa* and NA	Human and NA
4	*R. aeria*, *R. dentocariosa* and NA	Human, Environment, food and NA
5	*R. kristinae*, *R. koreensis* and *R. halotolerans*	Environment, other animals, human, food and NA

Fig. 5. Overview of CAZy enzyme families prevalent in the *Rothia* genus. Based on the distribution of species and sampling origins, CAZy enzymes were clustered in five groups displayed in the first column of the table displayed at the bottom. Glycosyl hydrolases (GHs), glycosyl transferases (GT), carbohydrate-binding modules (CBM), carbohydrate esterases (CE). The bottom table is the overall of the 'CAZy group' that represents the enzymes present in a specific group of species clustered according to our analysis (Fig. 4).

drugs. Potentially, the NRPS, PKS and hybrid NRPS-PKS gene clusters could produce groups of bioactive compounds that form a source of antimicrobial products with broad applications [52]. NRPs, PKs as well as other compounds produced by BGCs are released into the surrounding environment upon their biosynthesis. The synthesis is regulated by proteins that are activated by physical and environmental factors, including carbon and nitrogen source availability [52]. Once released, the molecules produced by NRPSs and PKSs or their hybrid gene clusters play roles in niche colonization, including optimization of bacterial proliferation, *quorum sensing* activity, and providing higher substrate affinity or faster growth rates [53].

Valinomycin, identified only in *Rothia nasisuis* species, is an NRP ionophore, forming ion channels in membranes that allow free movement K^+ ions thereby, altering membrane potential that may lead to disruption of the normal K^+ ion membrane gradient [24, 54]. All isolates of *R. nasisuis* containing the valinomycin-producing NRPs showed inhibitory activity *in vitro* against Gram-positive target bacteria including different species of *Streptococcus* sp. and closely related *Rothia* species as previous reported [55] (Table 2). The presence of the valinomycin clusters in *R. nasisuis* likely provide this species with a competitive advantage to prevail in the porcine tonsil microbiota and influence the composition of the microbiota in the first weeks of piglet life [7, 54]. We identified 18 novel NRPS gene clusters in the genomes of *R. nasimurium* species using BigScape. These clustered as family FN0062 and contain genes also present in the FN1919, FN1918 and FN2041 families that have been predicted to produce enterobactins, with strong binding affinity for ferric iron [56] (Fig. 3b). Iron availability is scarce at mucosal surfaces due to host iron-sequestering proteins [57]. NRPS-like clusters from family FN0062 are therefore likely involved in the acquisition of iron from the environment and facilitate colonisation of the human oral cavity by *R. mucilaginosa* and *R. dentocariosa* [58]. Six other NRPSs were identified that might produce antimicrobials as judged by growth inhibition of Gram-positive and Gram-negative bacteria by *R. nasimurium in vitro*. However further research would be needed to verify this conclusion. We also report the first endophytic *Rothia* isolate originating from pig tonsil microbiota. *R. endophytica* has been found within healthy plant tissues [13] thus it is possible that the *R. endophytica* strain we isolated from the piglet tonsil swab originated from ingested plant material. Genomes of *Rothia*

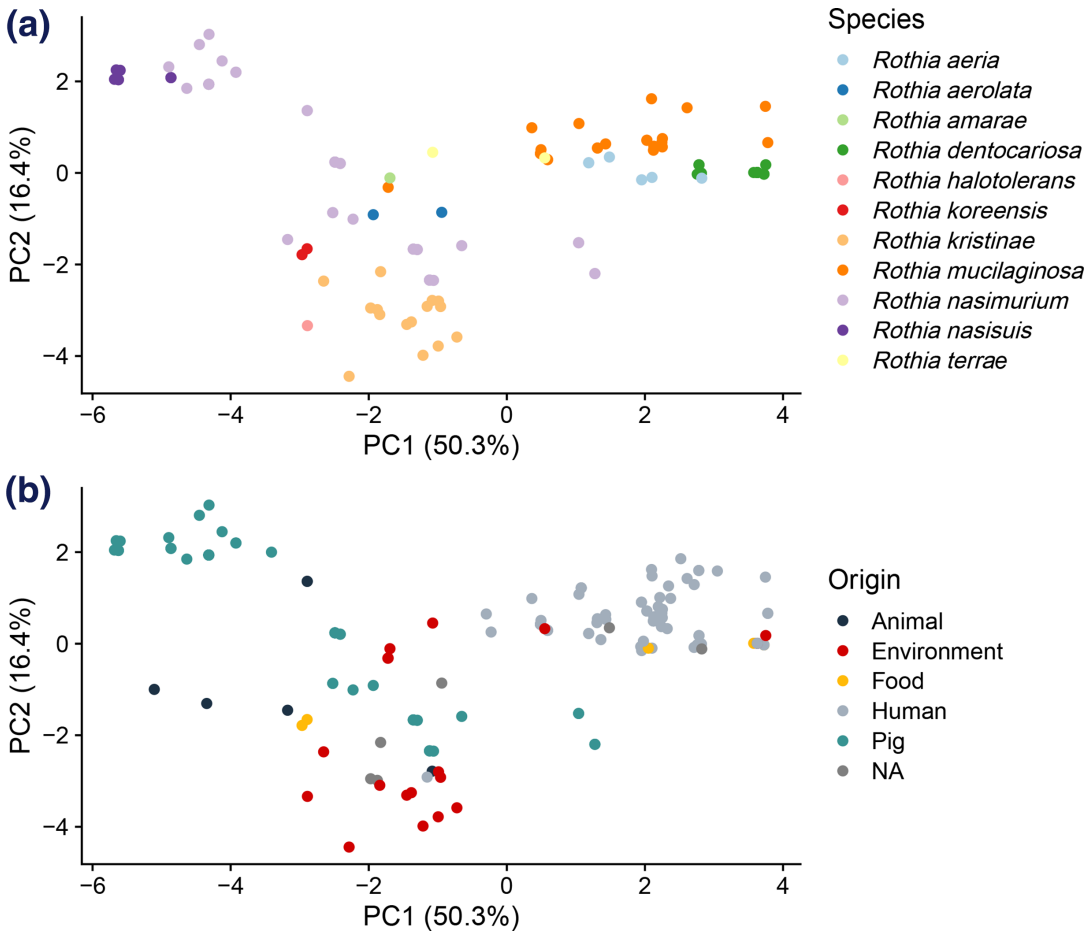


Fig. 6. Principal component analysis (PCA) score plot of carbohydrate active enZyme (CAZy) distribution. The first two axes explain 50.3 and 16.4% of the total variance, respectively. The ecological traits are indicated by (a) taxonomic groups and (b) sampling sites.

species (i.e. *R. nasimurium*, *R. nasisuis* and *R. amarae*) found in pigs encoded multidrug-resistance gene elements (Fig. 2), likely due to the strong selection pressure from the use of antibiotics for treatment of bacterial infections or use as growth promoters in livestock animals prior to the European ban in 2006 [Regulation (EC) No. 1831/2003]. Specific tetracycline resistance genes were found in genomes of *Rothia* strains isolated from human [*tet*(W)] and porcine strains [*tet*(M)]. In the past, the presence/absence of *tet* genes were mainly determined by sampling niche and application of tetracyclines, however, at present, *tet* genes have evolved and occurred more widely dispersed in different environments being present in commensal and pathogenic strains [59]. Erythromycin is an antibiotic used to treat several respiratory infections and resistance genes [*erm*(X)], were present in human (n=27) and some porcine strains (n=7). However, a small number of antibiotic resistant genes were found in the *Rothia* genomes as a reflection of the continuous use in humans and animals on a large scale [60].

As different host organisms and host regions (i.e. tonsil or saliva) can contain different carbohydrates, it is of interest to characterize the carbon-converting enzymatic 'toolbox' of *Rothia* strains sampled from different host organisms and environmental niches. The GH enzymes generally participate in carbohydrate catabolism, suggesting that the enzyme orthologues present in the *Rothia* genomes have the potential to degrade complex carbohydrates originating from mammals, plants, insects, and fungi including mucins, cellulose, membrane glycoproteins, and chitin in acidic environments [61]. The high number of GTs present in *Rothia* genomes are likely involved in carbohydrate biosynthesis, although some GTs have been reported to enzymatically attach carbohydrate moieties to precursors of some naturally occurring antibiotics [62, 63].

GTs identified in *Rothia* genomes can be responsible for the synthesis and assembly of the repetitive units of exopolysaccharides (EPS), which contributes for the biofilm formation, tolerance to desiccation and host antimicrobial peptides, as well as modulation the host immune response by the association with other molecules as glycolipids [64]. The CAZy families GT2 and GT4 are present in all *Rothia* genomes and are involved in the biosynthesis of various (exo)polysaccharides, which are often involved in biofilm formation, and biosynthesis of natural products [65]. Most *Rothia* isolates have a mucoid morphology (Table. S1, available in the

online version of this article) and aggregate in liquid media, which might be directly associated with the EPS production although the morphology of the *colonies* varies at the species level depending on the *in vitro* culture conditions. Exopolysaccharides could also provide alternative carbon sources to support the establishment of a pioneer microbial community in the upper respiratory tract [66]. The genomes of strains from the species *R. dentocariosa* and *R. mucilaginosa,* which colonize the human oral cavity, contained the largest numbers of predicted GT2 genes (Fig. 4). Considering exopolysaccharides are one of the main structural components of bacterial extracellular matrix and biofilm [67], the high amount of GTs may favour the formation of structurally organized biofilm consortia composed by a single taxon or a cluster of taxa interacting with *Rothia* on the oral mucosa and on teeth surfaces [2].

The CAZy families GH33, GH36, GT5, GT35, CBM48 +GH13-11 and CBM48 +GH13-9 (CAZy Group 1) were mostly present in the animal-associated species (*R. nasimurium* and *R. nasisuis*). The GH33 and GH36 families, are mainly present in the porcine isolates and are known to be involved in the utilization of human milk oligosaccharides (HMOs) and O-glycosylated mucins. HMO's shape the microbiota and have beneficial effects in early life [68]. The capacity to catabolize HMOs and glycosylated mucins may be an advantage to pioneer species and drive microbiota diversity [69]. Several CAZy families contain enzymes involved in carbohydrate digestion and hydrolysation. CAZy family GT35 includes glycogen or starch phosphorylase enzymes, family CBM48 includes enzymes participating in carbohydrate binding and glycogen-binding, and GH13 includes enzymes with glycosyl hydrolase activity. Little information is available about the utilization of complex carbohydrates in the oral cavity, but it is well described that the enzymes that release energy from the breakdown of branched substrates and complex carbohydrates can support proliferation and survival of certain species when there other carbon sources are limiting, and by maintaining microbiota diversity, have a positive impact on health [70].

The families GT25 and CBM50 +GH23 contain enzymes with glycosyltransferase activity and are mostly present in the human-associated *Rothia* species. The CAZy family GT25 plays important roles in catalysing utilization of monosaccharides, assembly of glycoconjugates and complex carbohydrates by transferring sugar moieties onto growing liposaccharide chains (PF01755). GT25 enzymes may play a role in utilization of carbohydrates from different sources, and in biofilm formation, while the family complex CBM50 +GH23 is associated with lytic transglycosylase activity related to the peptidoglycan metabolic process. Proteins with this annotation are usually enzymes active in the breakdown of chitin or peptidoglycan into molecules that trigger innate immune responses through recognition by host pattern receptors [71].

The CAZy enzymes GH65, CBM50, and GT20 were only identified in *Rothia* species isolated from environmental niches. The GT20 family contains enzymes annotated with trehalose-phosphatase activity (PF00982) while the GH65 family contains acid trehalases and some phosphorylases that catalyse conversion of trehalose to glucose and glucose-1-phosphate. Trehalose is a common disaccharide used by bacteria, archaea, fungi, invertebrates and others as a carbon source but trehalose also plays an important role in protecting bacteria against stress including desiccation, osmotic stress, oxidation and temperature changes [72]. The CBM50 or LysM domain family members contain a domain of approximately 40–50 amino acid residues that can occur in various carbohydrate-modifying enzymes, for instance, glycoside hydrolase enzymes that play roles in the digestion of complex carbohydrates [73]. The LysM domain is found in carbohydrate-active enzymes, peptidoglycan binding proteins and plant cell surface receptors for fungal chitin oligosaccharides [73, 74]. The LysM domain is also found in proteins correlated with several other biological functions including stress response in plants [75], signalling for specific plant and bacteria interactions [76] and bacterial spore surface development [77, 78]. As these modules are involved in interactions between different organisms, they may play roles during symbiosis and quorum sensing in soil- or plant-associated bacteria during microbiota development. Many of the CAZy enzymes identified in *Rothia* correlate with the environmental or host niche, emphasizing that these enzymes have important roles in microbial ecology and are relevant determinants of which habitats can be colonized and exploited by microbes. *Rothia* species encode CAZy genes for breakdown of chitin in fungal and insect cell walls, mucins and milk oligosaccharides into simple carbohydrates that can be used as nutrients and carbon sources for fermentation by other microbiota members [79]. Such cross-feeding might enable symbiotic interactions between *Rothia* and other microbes and could benefit microbial homeostasis and symbiosis from early life onward.

The presence of genes related to the transport, assimilation and conversion of nitrate to nitrite, denitrification, nitric oxide detoxification and bacterial nitrate reduction enzymes cofactors were identified in several species and highly correlated with the source of isolation (Table S5, available in the online version of this article) . We noted that the human associated species *R. dentocariosa, R. mucilaginosa, R. aeria* and *Rothia* sp. contain most of the genes involved in nitrate and nitrite metabolic pathways as reported previously in ten human-associated oral *Rothia* species isolates [79]. Porcine and other animal associated species as *R. nasimurium* and *R. nasisuis* also carry some of the genes involved in nitrate and nitrite metabolism, suggesting that those species might have similar function(s) in the oral cavity of animals, potentially with consequences for health and disease [32]. We noted that environmental species as *R. kristinae* and *R. koreensis* appeared to lack most of nitrate/nitrite metabolic genes. Nitrate/nitrite metabolic genes await functional characterization. This topic warrants further studies using physiological measurements of nitrate reduction for different *Rothia* species.

In summary, we showed that the NRPS producing the antimicrobial valinomycin was specific to *R. nasisuis* isolates from pig upper respiratory tract and identified novel BGCs encoding NRPSs, PKSs and RiPPs in other *Rothia* species that may produce antimicrobial compounds. According to our analyses, *Rothia* species have an extensive number of CAZy (5040 genes annotated in total), many of which are associated with EPS production and catabolism of carbohydrate sources (possibly including milk oligosaccharides) in different hosts and environments. Members of the *Rothia* genus have the potential to produce novel antimicrobials and may be used as probiotics to shape the microbiota of humans and mammals in early life and provide colonization resistance against pathogens and pathobionts.

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Author contributions
I.M.F.O., Y.K.N., P.v.B. and J.M.W. conceptualized the study. I.M.F.O. and Y.K.N performed the data analysis and interpretation of results under the supervision of P.v.B., M.S., P.S.A and J.M.W. The experiments and strains isolation were performed by I.M.F.O. The manuscript was written by I.M.F.O. and Y.K.N with input from all co-authors. All authors contributed to the article and approved the final manuscript.

Conflicts of interest
The authors declare that they do not have a conflict of interest.

Ethical statement
This study uses samples obtained for diagnostic procedures performed according to the ethical principles and guidelines covered by EU Directive 2010/63/EU.

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