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Metagenomic analysis of dental calculus and tooth roots of the ancient human

Anna Malykhina
Bioinformatics Institute, Saint-Petersburg, Russia

Sidorenko Oksana
Bioinformatics Institute, Saint-Petersburg, Russia

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Abstract

Metagenomic analysis is always a difficult, resource-intensive, but very exciting task, especially when it comes to such an interesting object as the ancient biomolecules from the microbiota preserved in calcified dental plaque (dental calculus) of a person who died more than a thousand years ago. Such kind of a time machine can tell a lot about the nutrition and diseases of ancient people, the evolution of microorganisms, including pathogens, and much more. In this work, we focused on possible mechanisms of pathogen evolution and antibiotic resistance. The red complex pathogens were detected in ancient dental calculus microbiota and *Tannerella forsythia* - one of the members of the red complex triad - was studied in more detail. In particular, we not only confirmed the expected new antibiotic resistance factors in the genome of a modern *Tannerella forsythia*, but also found sequences with homology to antibiotic resistance genes in the genome assembly of its ancient ancestor.

Keywords: Metagenomics, Ancient bacteria, Pathogen evolution, Microbiome, Paleogenomics

Introduction

The microbes that exist in the human body are collectively known as the human microbiota. This amazingly complex and poorly understood group of communities has an enormous impact on humans. Sometimes referred to as our second genome, the genes of microbes that make up the microbiome outnumber human genes by more than 100-fold, with over 3 million bacterial genes in the gut alone [1]. An increasing number of conditions are being examined for correlative and causative associations with the microbiome. Dysbioses in the microbiome have been associated with numerous diseases, including inflammatory bowel disease, multiple sclerosis, diabetes (types 1 and 2), allergies, asthma, autism, and cancer [2]. The fundamental goal of human microbiome research is to measure the structure and dynamics of microbial communities, the relationships between their members, what substances are produced and consumed, the interaction with the host, and differences between healthy hosts and those with disease. The complexity of microbial communities makes studying them challenging. There may be hundreds of different species, and enumerating what organisms are present with standard microbiological techniques is not possible because many organisms have never been grown in culture and may require special, as yet unknown, growth conditions. In addition, the abundance of some microbes can range over orders of magnitude, so deep sampling is required to detect the less-abundant members.

Culture-independent methods of taking a microbial census began about 25 years ago and were based on targeted sequencing of 5S and 16S ribosomal RNA genes, which differ for each species and are a convenient identifier. As this became a tractable research area, next-generation sequencing (NGS) technologies were developed and allowed more extensive analyses, both targeted 16S rRNA gene sequencing and whole-genome shotgun sequencing of microbes in communities (Figure 1) [3] [4]. During primary analysis, shotgun techniques can produce reads from DNA, which are then aligned to reference genomes to identify variants and community population genetics, assembled into contigs to make gene predictions or compared with databases. Alternatively, targeted sequencing such as 16S rRNA gene sequencing can be used to take a community qualification. A bacterial species is hard to define, but an operational taxonomic unit (OTU) is often taken as organisms with 16S rRNA gene sequences having at least 97% identity. A 16S rRNA gene sequence of about 1.5 kilobases has nine short hypervariable regions that distinguish bacterial OTUs. These data are then compared with databases to create tables of taxa and abundance.

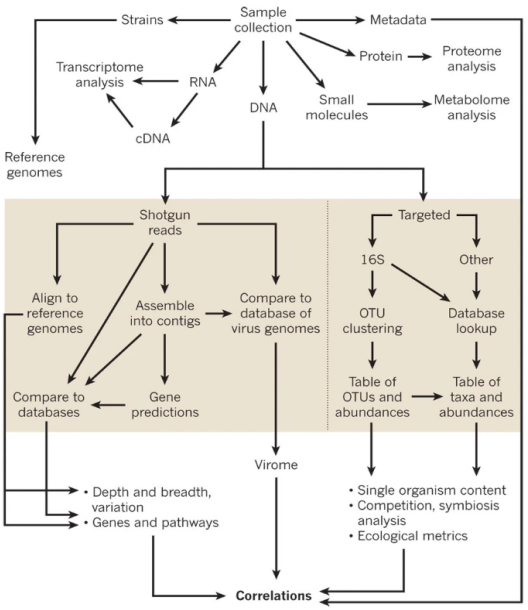


Figure 1: Microbiome analysis workflow.

In this study we have analyzed an oral microbiome of the dental tissues of four adult human skeletons (G12, B17, B61 and B78) with evidence of mild to severe periodontal disease from the medieval monastic site of Dalheim, Germany (c. 950–1200 CE) [5]. Here we have confirmed the presence of the triad of oral anaerobic bacteria, the so-called "red complex" (*Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*) for ancient humans. This triad have historically been considered as the basic infectious organisms associated with periodontitis [6]. Moreover, the ancient *Tannerella forsythia* genome assembly was compared with modern reference revealing the emerge of at least two acquired antibiotic resistance genes in modern bacterium.

Methods

Data acquisition

Archaeological material was obtained from the medieval St. Petri church and convent complex in Dalheim, Germany, and radio-carbon dated to c. 950–1200 CE [5]. We used the samples from ancient dental calculus and from the ancient tooth roots, in particular, the results of sequencing portions of V5 16S ribosomal RNA obtained by an Roche GS Junior 454 Sequencer (single-end reads in FASTQ format with Phred33 encoding). Warinner *et al.* submitted the raw data and sequences to public databases (MG-RAST SERVER) [7]. These data can also be downloaded from
FIGSHARE ONLINE OPEN ACCESS REPOSITORY.

Amplicon sequencing

QIIME 2 (version 2023.2.0) pipeline was used for performing microbiome analysis from raw DNA sequencing data [8]. QIIME 2 plugin 'dada2' version 2023.2.0 (from package 'q2-dada2' version 2023.2.0) was used for filtering chimeric sequences and denoising the data [9]. QIIME 2 plugin 'dada2' version 2023.2.0 and BIOM format were involved in QIIME 2 pipeline for FeatureTable and FeatureData summaries creating [10]. In this case we used OTUs as features. Feature IDs were mapped with BLAST against the NCBI database [11] [12]. VSEARCH, RESCRIPt tools, scikit-learn library (version 0.24.1), pandas package (version 1.5.3) for Python 3.8.15, QIIME 2 plugin 'feature-classifier' version 2023.2.0 (from package 'q2-feature-classifier' version 2023.2.0), SILVA database were involved in QIIME 2 pipeline for comparing the representative sequences with the taxonomy database, visualization building and and plots generating [13] [14] [15] [16] [17] [18] [19] [20]. For the additional statistical, functional, and meta-analysis of microbiome data MicrobiomeAnalyst platform was used [21] [22].

Comparison of the modern *Tannerella forsythia* reference genome with the genome of its ancient ancestor

The reference genome of *Tannerella forsythia 92A2* and annotation were downloaded from NCBI database [12] [23]:
`https://www.ncbi.nlm.nih.gov/nuccore/NC_016610.1`
Reads assembled into contigs after the whole metagenome shotgun sequencing of dental calculus from the individual G12 were downloaded from MG-RAST SERVER.

The BWA-MEM software (version 0.7.17-r1188) was used to index the reference file and then to align the contigs to the reference genome (with default parameters) [24]. The resulting SAM file was compressed with the samtools software (v.1.16.1 (using htslib 1.16)) with the parameters -S (for statistics) and -b (for converting a SAM file to a BAM file) [25]. We also used the samtools flagstat command (with default parameters) to get basic statistics. The resulting BAM file was sorted and indexed with the samtools sort and samtools index commands (with default parameters). The BEDtools software (version 2.30.0) commands bedtools bamtobed (with default parameters) and bedtools intersect with the parameter -v were used to only report those entries in the modern reference genome that have no overlaps with assembly of the ancient genome. Genomic coverage plot was created using BLAST Ring Image Generator (BRIG) based on the default settings [26].

Putative antibiotic resistance factors of the ancient human oral microbiota

Reads assembled into contigs after the whole metagenome shotgun sequencing of dental calculus from the individual G12 were screened for putative antibiotic resistance elements using BLASTN search against the Comprehensive Antibiotic Resistance Database (CARD) [27].

Results

Demultiplexing and quality control

There were an average of about 5139 sequences obtained per sample with the median of 5272. The quality scores were 26, 39 and 40 for the 25th, 50th and 75th percentile respectively for the sequence base position 100; 0, 12 and 22 for the 25th, 50th and 75th percentile respectively for the sequence base position 190 and 12, 12 and 19 for the 25th, 50th and 75th percentile respectively for the sequence base position 310. Generally quality scores were higher for positions 0 - 179 and lower for positions 180 - 340 with a clear border.

An average of 96.4% of the input sequences passed the filter during DADA2 pipeline and there were an average of 93.2% of the non-chimeric input sequences.

FeatureTable and FeatureData summaries

465 features were obtained (total frequency of 43,126). The mean frequency per sample was 4,791 with the median of 4,862 and the mean frequency per feature was 92.8 with the median of 28.

Taxonomic analysis

Comparison of the representative sequences with the taxonomy database showed that the vast majority (more than 95%) of the both ancient dental calculus and tooth roots microbiota is represented by bacterial DNA and only a small part is represented by Archaea.

It can be noted that archaeal phylum Euryarchaeota is presented only in dental calculus. Among bacteria the most dominant phyla were Firmicutes (an average of about 54.7%), Proteobacteria (an average of about 15.7%), Actinobacteria (an average of about 7.6%), Synergistota (an average of about 6.9%), Bacteroidetes (an average of about 6.7%), Chloroflexi (an average of about 1.4%), Fusobacteria (an average of about 1.3%), Euryarchaeota (an average of about 1.2%), Spirochaetota (an average of about 0.8%).

Tooth roots microbiota was highly populated by bacterial phyla Proteobacteria (an average of about 47.8%), Actinobacteria (an average of about 14.7%), Firmicutes (an average of about 13.2%), Bacteroidetes (an average of about 9.4%), Chloroflexi (an average of about 2.4%), Gemmatimonadota (an average of about 2.3%). However, there were few or no representatives of Fusobacteria, Euryarchaeota, Spirochaetota, Synergistota (Figure 2).

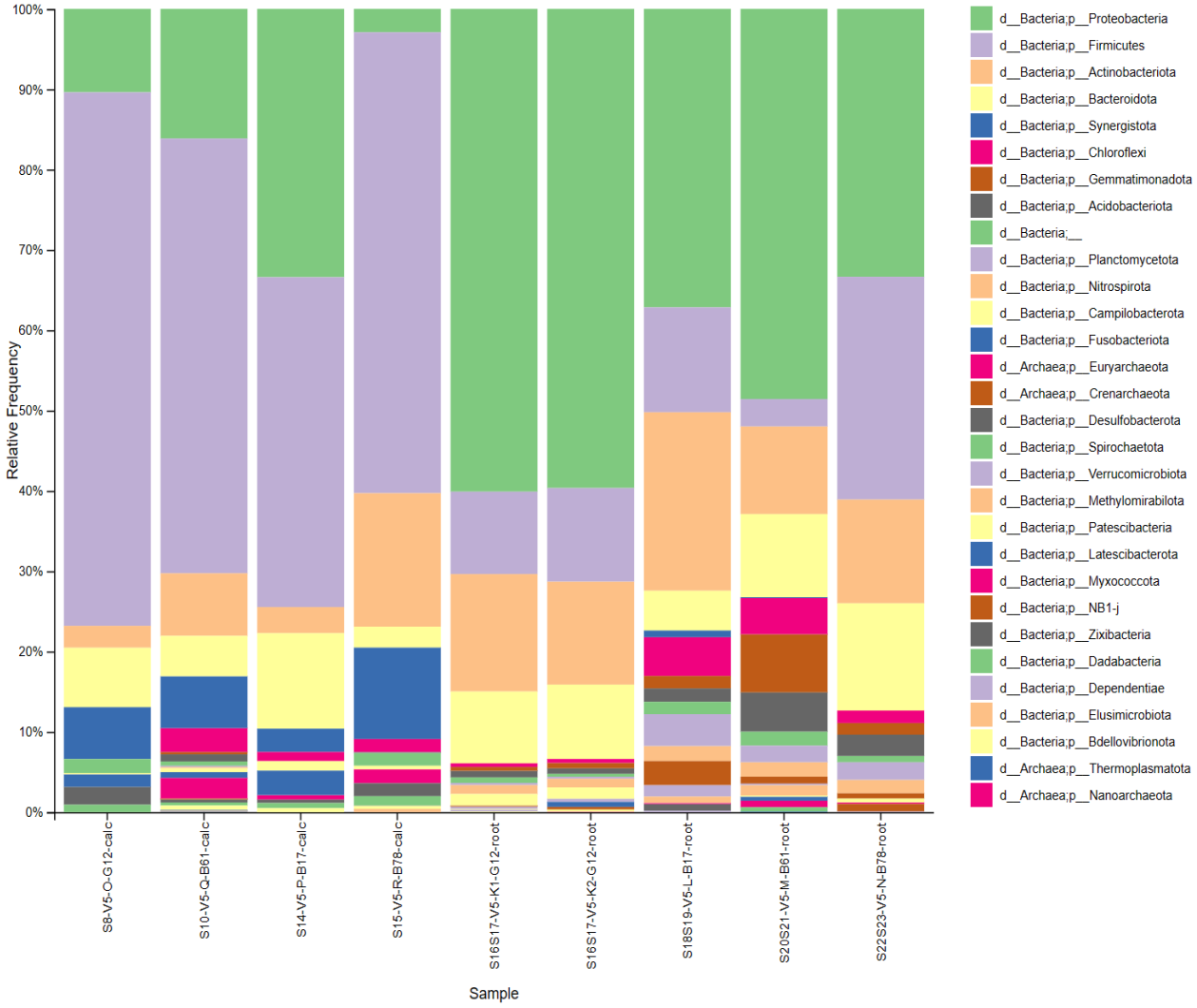


Figure 2: Relative frequency of phyla of the microbiota represented in the ancient dental calculus and tooth roots.

Taxonomic differences between certain phyla and classes of microbial communities of dental calculus and tooth roots are visualized in the Figure 3.

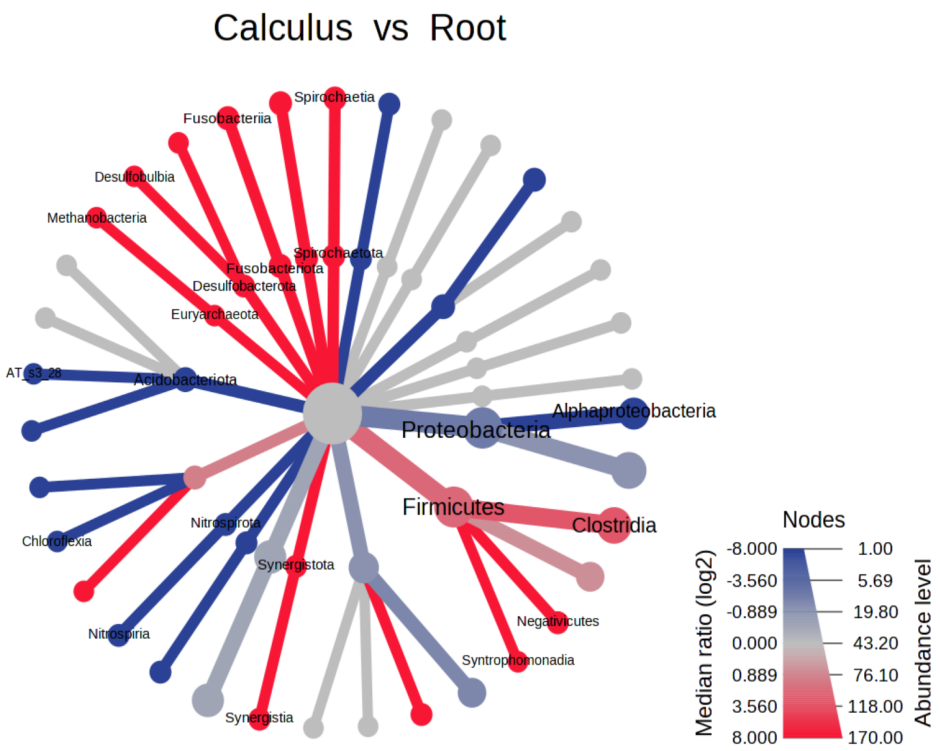


Figure 3: The heat tree analysis leverages the hierarchical structure of taxonomic classifications to quantitatively (using the median abundance) and statistically (using the non-parametric Wilcoxon Rank Sum test) depict taxonomic differences between dental calculus and tooth root microbial communities microbial communities. Wilcoxon p-value cutoff = 0.05.

The red complex

Figure 4 shows the presence of the genus *Treponema* in all four dental calculus samples tested in this study, and the presence of genera *Porphyromonas* and *Tannerella* in three and two samples respectively. We have detected all of the three members of the red complex among the dental calculus microbiota of the samples S8-V5-O-G12 and S10-V5-Q-B61 . In sum, red complex bacteria represent a fairly high percentage of the dental calculus microbiota (an average of about 1.2%).

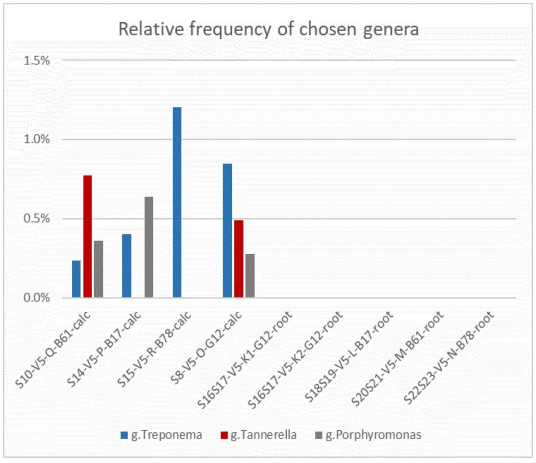


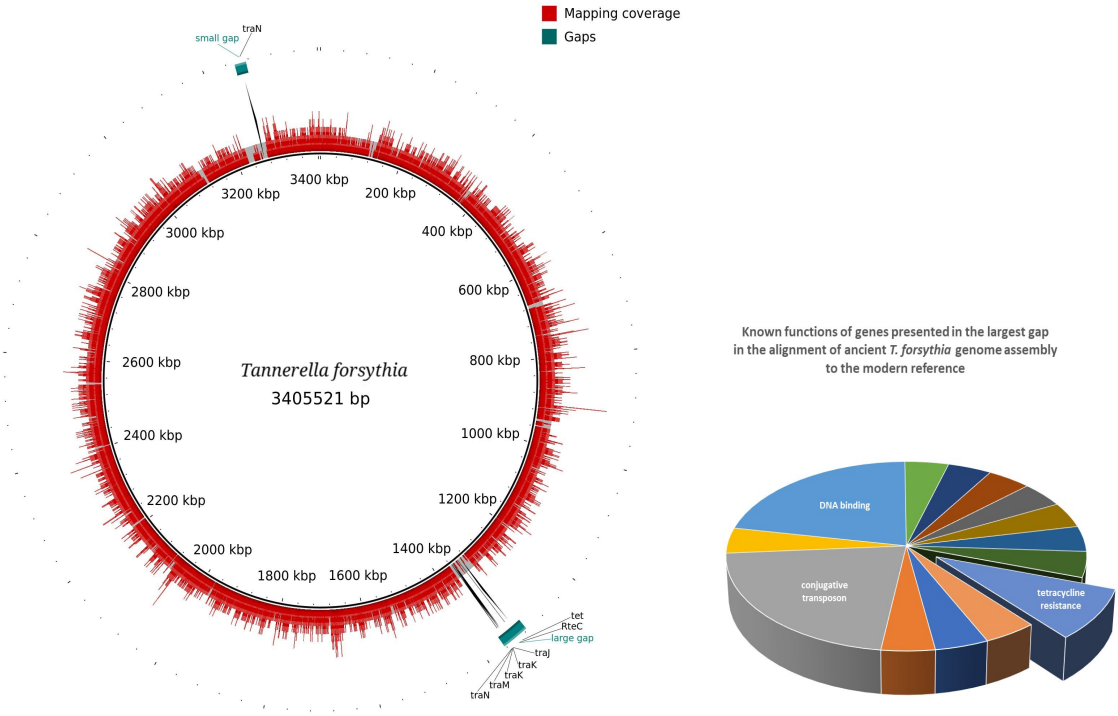
Figure 4: Relative frequency of the red complex representatives in samples.

Antibiotic resistance

Affected by red complex bacteria individual G12 was selected for a dental calculus whole metagenome shotgun sequencing. Using both manual and automatic approaches to screen the assembly for putative antibiotic resistance elements we detected numerous DNA sequences with homology to antibiotic resistance genes, including genes for multidrug efflux pumps (*e.g.* *AxyX*, *AxyY*) and native resistance genes to aminoglycosides (*e.g.* *aadA*, *aphA15*), beta-lactamases (*e.g.* TEM-type, SHV-type, GES-type, KPC-type, OXA family, VIM-type, IMP-type, CMY-type, NDM-type) and macrolides (*e.g.* *mefE*, *erm* genes). We did not study the complete list of obtained genes in the framework of this study, however, it is proposed for further detailed analysis since the exact function of the discovered genes is not yet clear.

Comparison of the modern *Tannerella forsythia* reference genome and sequences of the genome of its ancient ancestor

Reads assembled into contigs after the whole metagenome shotgun sequencing of dental calculus from the individual G12 were aligned to the modern reference genome of *Tannerella forsythia 92A2*. We detected two gaps of interest in the alignment: the largest gap extended in coordinates NC_016610.1:1 314 968-1 362 893 (approximately 48000 bp) and the another gap extended in coordinates NC_016610.1:3 255 020-3 275 306 (approximately 20300 bp) (Figure 5a).



(a) **(b)**

Figure 5: Genomic coverage plot for of the modern *Tannerella forsythia* reference genome and sequences from the individual G12 dental calculus (a). Gene content of the largest gap (b).

There were *traJ, traK, traM, traN, traL, traO* conjugative transposon genes, DNA-binding genes (helix-turn-helix domain-containing, putative DNA binding domain-containing), tetracycline resistance ribosomal protection (BFO_RS05370 locus tag) and RteC domain-containing (BFO_RS05390 locus tag) putative tetracycline resistance genes as part of the gene content of this region (Figure 5b).

The smaller gap was also represented by genes with transposase and conjugal transfer activity.

All these genes were presented only in modern reference genome.

However, a lot of common putative antibiotic resistance genes for sequences from the ancient *Tannerella forsythia* and the modern reference were detected (*e.g. RteC* domain-containing gene (two common loci besides to the mentioned unique reference), *HlyD* family efflux transporter periplasmic adaptor subunit, efflux RND transporter permeases, MATE family efflux transporters, *pbpC* penicillin-binding 1C, etc.).

Discussion

Taxonomic analysis and specific pathogens

The microbial composition that we have identified was generally similar to the modern one at the phyla level we have considered [28] [29].
Since we know there is osteological and proteomic evidence of periodontal disease in at least two of the subjects in study, we can conclude that the individuals S8-V5-O-G12 and S10-V5-Q-B61 had the disease. We can conclude that almost a thousand years ago, the presence of the red complex bacterial triad was associated with periodontal disease, as it is nowadays, despite tremendous advances in living standards, medicine, and personal hygiene.

Antibiotic resistance

A number of similar studies have also detected resistance genes in the remains of ancient people, for example in gut microbiome of pre-Columbian mummies (980 to 1170 CE) [30]. A functional and highly efficient beta-lactamase was reconstructed from the remains of a woman buried in the late-Byzantine era in Troy in a calcified abscess likely the result of *Staphylococcus saprofyticus* infection [31]. Together, these studies highlight that even in the absence of selection from therapeutic antibiotic use, the human microbiome is a reservoir of resistance genes accessible to pathogens [32]. In particular, we become witnesses of how oral microbiome functions as both a source and a reservoir of new antibiotic resistance [5].

Comparison of the modern *Tannerella forsythia* reference genome and sequences of the genome of its ancient ancestor

Tannerella forsythia is a gram-negative anaerobic bacterium that is associated with the development of destructive periodontal disease [33]. Here we made sure that this bacterium has been part of the human oral microbiota for many centuries. Bravo-Lopez *et al.* reported the evidence of ancient *Tannerella forsythia* DNA in dentin and dental calculus samples from archaeological skeletal remains that span from the Pre-Hispanic to the Colonial period in Mexico [34].

We could see that the modern bacterium has acquired at least two additional resistance genes, most likely through horizontal gene transfer, judging by the set of neighboring genes in the large gap. It can be assumed that the acquisition of resistance genes is associated with the development of medicine and clinical selection for increased and more specific resistance.

However, as it was in the general case of all representatives of the microbiota, discussed in the previous section (”Antibiotic resistance”), ancient *Tannerella forsythia* almost a thousand years ago had a lot of DNA sequences with homology to antibiotic resistance genes in its genome. Here we got acquainted with similar evidence from other authors.

Antibiotic resistance and pathogen evolution: alternative hypothesis

Morar *et al.* consider the concept of resistance determinants found in nonpathogenic micro-organisms. The resistance totality in the global microbiota is the antibiotic resistome and includes not only established resistance genes but also genes that have the potential to evolve into resistance elements. Authors hypothesize that they share common ancestry with other functional units known as housekeeping genes [35].

Waglechner *et al.* give an example of this concept. Ribosomal protection proteins (RPPs) that confer resistance to tetracycline by binding the ribosome and dislodging the antibiotic in a GTP-dependent manner [36]. RPPs have homology to GTPase elongation factors (EF) EF-G/EF-2 and EF-Tu/EF-1α, which are themselves related through an ancient gene duplication event [37] [38]. These resistance genes are found in the BGCs of tetracycline-producing *Streptomyces* as well as in pathogens associated with mobile genetic elements. Phylogenetic analysis of RPPs and EF found homologs in all three domains of life and provides evidence that RPPs and EF-G arose through duplication and divergence of an ancestral GTPase prior to the divergence of bacteria from archaea and eukaryotes [39]. This analysis used a strict molecular clock with a midpoint rooted tree, though lacking time calibrations meant that branch lengths could not be converted into dates. Nonetheless, it suggests that the ancestral RPP arose before the appearance of streptomycetes, including tetracycline producers. Whether these RPPs had a role beyond antibiotic resistance or, alternatively, if tetracycline production also occurred prior to *Streptomyces* emergence remains to be seen. In either case, it provides compelling evidence that RPPs arose from a proto-resistance gene derived from GTPase EFs [32].

Within each class of Ser beta-lactamases are families of enzymes that share sequence similarity, some having hundreds of members. This sequence diversity makes beta-lactamases an excellent model for evolutionary studies that have been performed many times over [40] [41] [42] [43]. For example, a Bayesian phylogenetic tree using a molecular clock calibrated to Gram-positive/Proteobacteria divergence 2.2 Ga was performed on Ambler class D OXA beta-lactamases [44]. The analysis suggested that the family arose over 2 Ga and transferred to plasmids hundreds of millions of years ago. This finding questions the commonly held notion that resistance gene mobilization is in response to human antibiotic use, in this case at least [32].

However, deep phylogenetic analysis is needed to reproduce and test such hypotheses. In any case, we could see that robust and well-preserved dental calculus proved to be a reliable reservoir that can preserve ancient biomolecules from the oral microbiota, host tissues and diet for tens of thousands of years [5]. Due to this property, it has become the subject of interest of many researchers in recent years and may hold many more secrets and grounds for scientific controversy to be revealed in the future.

Supplementary

The lab notebook with the detailed pipeline, settings and details can be found at this LINK.

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