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A probable prehistoric case of meningococcal disease from San Francisco Bay: Next generation sequencing of *Neisseria meningitidis* from dental calculus and osteological evidence

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ABSTRACT

Next Generation Sequencing (NGS) of ancient dental calculus samples from a prehistoric site in San Francisco Bay, CA-SCL-919, reveals a wide range of potentially pathogenic bacteria. One older adult woman, in particular, had high levels of *Neisseria meningitidis* and low levels of *Haemophilus influenzae*, species that were not observed in the calculus from three other individuals. Combined with the presence of incipient endocranial lesions and pronounced meningeal grooves, we interpret this as an ancient case of meningococcal disease. This disease afflicts millions around the globe today, but little is known about its (pre)history. With additional sampling, we suggest NGS of calculus offers an exciting new window into the evolutionary history of these bacterial species and their interactions with humans.

1. Introduction

Meningococcal disease is caused by bacteria spread through human contact. Over one million cases are estimated to occur annually worldwide, with higher concentrations in certain geographic regions such as sub-Saharan Africa (Nelson, 2006; Rosenstein et al., 2001). The disease manifests as infection of the meninges and the fluids surrounding them, and is most common after a head or spinal injury, or following an illness that weakens the immune system. In some cases, bacteria also spread to the bloodstream and cause meningococcal sepsis. Symptoms of meningitis initially include fever, headache, and stiffness in the neck, and can progress to include hemorrhagic skin lesions, impaired mental capabilities, seizures, stroke, coma, and death (Schildkamp et al., 1996). Although treatment outcomes are typically positive in developed countries (< 10% mortality), when untreated, the disease is often fatal. For example, a mortality rate of 26% was measured for an outbreak of bacterial meningitis in Gambia in the 1980 s (Greenwood et al., 1987). Once severe symptoms develop, the disease may progress rapidly. In one study of meningococcal disease deaths (n = 43), 73% of patients died within two days of admission to a hospital (Schildkamp et al., 1996). Understanding the history of the disease and its prevalence and geographic distribution in ancient times will help to clarify the origin and spread of meningococcal disease. For example, it is not even known if the disease is New or Old World in origin, or both.

Three respiratory pathobionts, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, are the most common causes of bacterial meningitis, with the former two most common. In one clinical study in the Netherlands, *S. pneumoniae* and *N. meningitidis* accounted for 51% and 37% of episodes, respectively (van de Beek et al., 2004). Humans are the only reservoir for these bacterial species, and both are spread by person-to-person contact, primarily as aerosols (*e.g.*, through sneezing) and oral and nasal secretions (*e.g.*, through kissing or sharing food). Between 5 and 10% of the present-day human population are carriers of these bacterial species but do not develop pathological conditions. Advanced age, smoking, and sinusitis are known risk factors for developing the disease, and research seeks to determine why some carriers develop the disease and others do not. Infection by *N. meningitidis* specifically, is known as meningococcal disease (Rosenstein et al., 2001), and is the focus of this study.

Despite its prevalence and danger today, surprisingly little is known about the (pre)history of meningococcal disease. Reports of symptoms

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consistent with the disease are known from 16th century AD Europe, but the first definitive identification dates to 1805 (Rosenstein et al., 2001). Identifying meningococcal disease from the archaeological record is difficult. Meningitis caused by an infection can stimulate bony growth and leave behind endocranial lesions that may indicate presence of the disease, but only if an individual lives long enough to elicit this response from their immune system (Lewis, 2004; Pathan et al., 2003). Further, other pathological conditions can cause similar endocranial lesions, complicating accurate diagnoses (*e.g.*,Janovic et al., 2015; Lewis 2004).

A small number of osteoarchaeological studies suggest ancient cases of meningitis. Schutkowski et al. (1996) identified an individual from the Late Neolithic in Germany with an unhealed spinal arrow wound that they suspect may have led to bacterial meningitis. Their deduction is based not on the presence of endocranial lesions, but on the hyperextended (opisthotonus) burial position and extended arms, which is typical of death due to meningitis. Likewise, Paredes et al. (2012) document a child dating from the 16th to 19th century from Portugal with endocranial lesions that, after ruling out other causes, they interpret as the byproduct of bacterial meningitis. Other studies are less committal. Schultz et al. (2007) identify extremely high rates (72%; n = 262) of meningeal irritations among infants and children in a 14th century AD skeletal population from Arizona, North America (Grasshopper Pueblo). The authors suggest outbreaks of bacterial meningitis as a possible cause, but acknowledge other factors could result in the same condition.

Next generation sequencing (NGS) of archaeological dental calculus offers a new means to test for ancient meningococcal disease. Because *N. meningitidis* colonize the mucosal cells of the nasopharynx, they are a common component of the mucous and saliva of carriers. As such, these bacteria may become incorporated into dental plaque formations, which in turn may harden into dental calculus and preserve over archaeological time scales. Recent research has shown a wide array of bacterial species preserved in ancient dental calculus (Adler et al., 2013, de la Fuente et al., 2013; Warinner et al., 2014a, b; Weyrich et al. 2015). Detection of *N. meningitidis*, combined with other pathological conditions (*e.g.*, endocranial lesions), may assist in the diagnosis of ancient cases of meningococcal disease. Such information may allow us to trace the prehistory, spread, and evolution of this disease among humans.

2. Background

CA-SCL-919 is an archaeological site in Santa Clara County, California (Fig. 1) that was excavated as mitigation for a new light rail transportation corridor. Twenty-two accelerator mass spectrometry (AMS) radiocarbon dates from the site range between AD 1250 and 1640 (median date), with 18 falling in a narrow window between AD 1515 and 1640. These dates are consistent with temporally diagnostic, geomorphological, and other chronological information from the site, indicating a very Late Holocene but pre-contact period site.

In addition to dense midden and a range of hearth and pit features, 17 primary human interments were discovered. With permission from the state-assigned most-likely-descendant (MLD, co-author KP), stable isotope and ancient DNA studies were conducted to reveal more about this ancient population. For this study, dental calculus from four individuals (see Table 1) was selected for DNA extraction and next-generation sequencing (NGS) to determine bacterial species present in the oral cavity. Each calculus sample was paired with a sediment sample from the burial matrix of that individual, which was also examined using ancient DNA techniques followed by NGS. To our knowledge, these are the first such analyses of a pre-contact population in California.

The first individual (Burial #2) represents an adolescent of undetermined sex with no carious lesions or osteological pathologies that could be attributed to infectious disease, but with a large number of linear enamel hypoplasias (n = 9 on the left lateral maxillary incisor) indicating periods of severe stress prior to death. The second (Burial #4) represents an adult male, approximately 40–45 years of age at death, with a large (18.18 mm x 10.74 mm, 3.8 mm deep) lytic lesion with sharp edges and no signs of healing on the nasal surface of the left palatine, and a large patch of well-healed woven bone on the floor of the right maxillary sinus with random spicules on the medial wall of the sinus, which are probably the result of sinusitis. One tooth with caries was noted for this individual. The third individual (Burial #15) is an adult female aged 35–44 at the time of death. No caries were present, but she displays healing periosteal reaction as patches of woven bone on both tibial shafts, indicative of non-specific infection, perhaps due to minor trauma to her legs.

Finally, the fourth individual, Burial #16, is the main subject of this study. Burial #16 is an old adult female, aged over 50 years (likely 55–65 years) at the time of her death, with extreme dental wear, osteoarthritis, and degenerative joint disease. This woman is one of two individuals buried in the seated position at the site (the other being a child aged 2.5–3.5 years old), the remainder being in a flexed position or cremated. On her endocranial surface, we observed slightly increased porosity with some abnormal fiber bone formation and mildly pronounced meningeal or capillary grooves (Fig. 2). This condition is consistent with the early stages of meningitis.

3. Methods

3.1. DNA extraction – calculus, sediment samples, and controls

We extracted DNA and prepared genomic libraries for sequencing in a dedicated ancient DNA facility at the University of California Santa Cruz Paleogenomics Laboratory, which is physically isolated from other molecular biology research. All extraction and library preparation steps followed protocols for handling ancient DNA to minimize the potential of contamination by exogenous DNA sources (Fulton 2012). We used negative (no sample) extraction controls in both the calculus and sediment procedures.

We removed visible dental calculus from a single tooth from each individual by scraping or prying the calculus off the tooth. To avoid contamination between individuals, we sterilized the scalpel blade between samples via immersion in a mixture of chloroform and methanol for several minutes, and then rinsed the blade with ethanol. We washed the calculus samples with 500 μL EDTA at room temperature for either 15 min, for samples weighing over 5 mg (N = 2), or 5 min for samples weighing less than 5 mg (N = 2). We agitated the samples periodically during the wash step. After washing, we pelleted the calculus via centrifugation and then removed and retained the supernatant. We then ground the concentrated and cleaned calculus to a fine powder using a spatula. We incubated the resulting fine powder for 2 days at room temperature in 1 mL of 0.5 M EDTA/10% proteinase K solution with constant rotation. We then followed the extraction protocol described by Dabney et al. (2013), which was developed specifically to retain the small DNA fragments that tend to characterize ancient samples. Following extraction, we eluted DNA in 40 μL TET.

To investigate whether bacteria from the sediment surrounding the burial site became incorporated into the calculus, we collected sediment samples from beneath the burial site of each processed sample. For these sediment samples, we extracted DNA using the PowerLyzer soil DNA extraction kits (MoBio, Carlsbad, CA) following manufacturer's instructions. We eluted the resulting DNA in a final volume of 60 μL TET.

3.2. Library preparation and sequencing

We prepared barcoded single-indexed Illumina sequencing libraries from all extracts (calculus, sediment, and controls) following Meyer and Kircher (2010; with modifications described by Heintzman et al.,

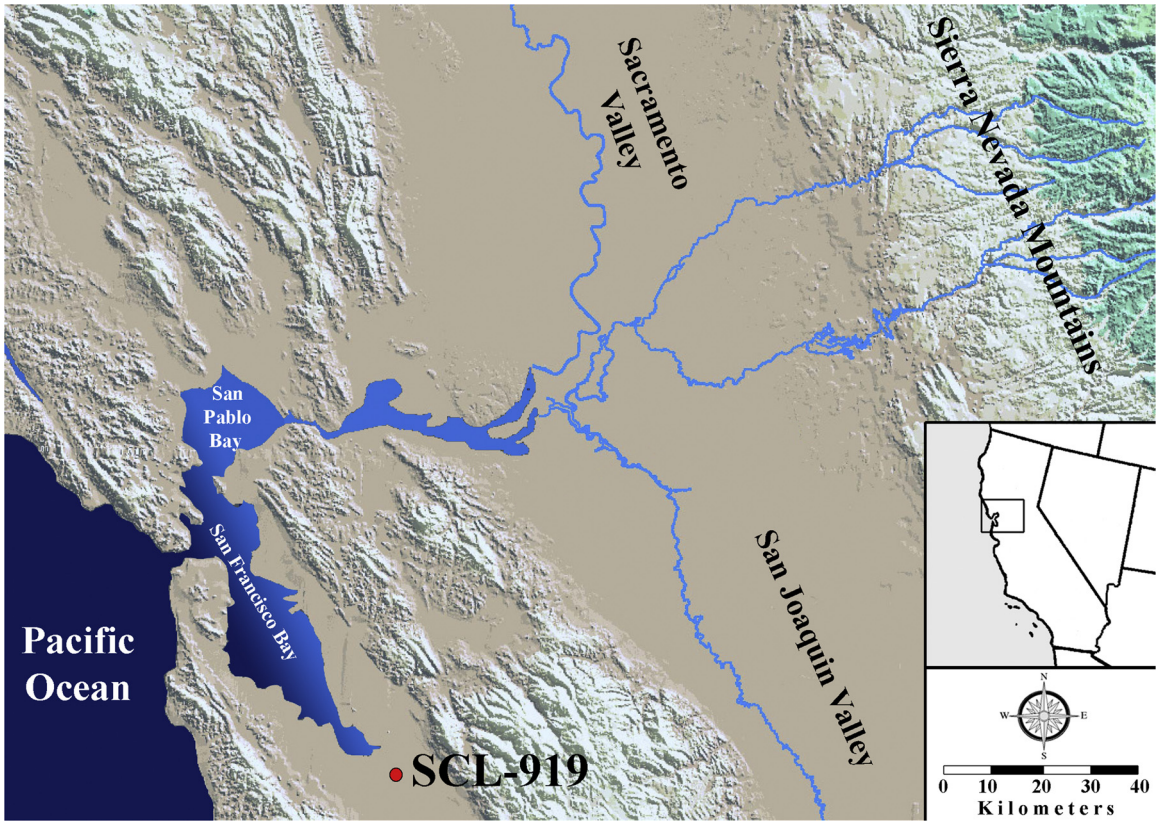

Fig. 1. Map of Central California, showing location of CA-SCL-919 at center-bottom.

2015). We pooled the libraries in equimolar ratios and sequenced them on an Illumina MiSeq using v3 chemistry 150 cycle kits (2 × 75 paired-end sequencing). We sequenced between ~2-7 million reads per sample (Table 2). Because sample RN4 was identified as potentially including meningococcal-disease causing bacteria (see below), this sample was sequenced more deeply than the other three samples (~7 million reads, Table 2).

3.3. Data processing and sequence matching

Following sequencing, we bioinformatically identified libraries via their unique barcodes, and then trimmed and merged reads using SeqPrep (https://github.com/jstjohn/SeqPrep; flags: -o 20, -L 25, -q 15). We removed low complexity reads and duplicated sequences using the DUST approach in PRINSEQ (Schmieder and Edwards, 2011) and a complexity score threshold of 7. The remaining sequences were identified to the most precise taxonomic level possible (given available data in the 'nt' database) using the BLAST algorithm (blastn; flags: -outfmt 6). Any taxon that was present in the data set with fewer than 30 mapped reads was discarded as potential low level barcode switching (Kircher et al., 2012). We visualized the community of species that were identified from the sequences using MEGAN5 (Huson et al., 2007) with the default LCA parameters.

3.4. Bray-Curtis dissimilarity

We used Bray-Curtis dissimilarity values calculated in MEGAN to quantify differences between samples. A value of 0 indicates that two samples have identical composition (*i.e.* share the same taxa) and a value of 1 indicates that two samples do not share any taxa (Bray and Curtis 1957). Each sample was compared to all other samples in a matrix. To compare the communities found in sediment and calculus samples, we used a *t*-test to determine whether the mean Bray-Curtis value was significantly different from 0 (indicating identical composition). Additional *t*-tests of this kind were done to compare control samples to sediment and calculus samples. The Bray-Curtis values were also used to create a PCoA plot in MEGAN using default parameters.

3.5. Phylogenetic analysis

Initial screening of the resulting data identified one of the calculus samples (RN4, Table 2) as potentially including sequence fragments from *Neisseria meningitidis* (genome size ~2.3 million base pairs), a potential pathogen associated with meningococcal disease. To confirm this, we performed two additional analyses. First, we used the Burrows Wheeler Aligner (BWA) (Li and Durbin 2009) and mapDamage (https://github.com/ginolhac/mapDamage) to assess whether the DNA

Table 1
Demographic information on four individuals from CA-SCL-919 included in this study and tooth sampled for calculus.

Burial	Sex	Age at Death	Tooth Calculus	Convent. AMS Date	Calibrated age AD	Pathology notes	Weight of Calculus (mg)	DNA yield (ng/mg)
2	Indet.	10-13	Right M1	no date		Enamel hypoplasias	1.8	276
4	Male	40-45	Right M3	465 ± 33	1465-1635	Sinusitis	6	29.6
15	Female	35-44	Left M3	420 ± 25	1500-1645		< 5	NA
16	Female	50+	Left M1	435 ± 25	1475-1635	Endocranial lesions	49	48.9

Notes: Indet. = Indeterminate.

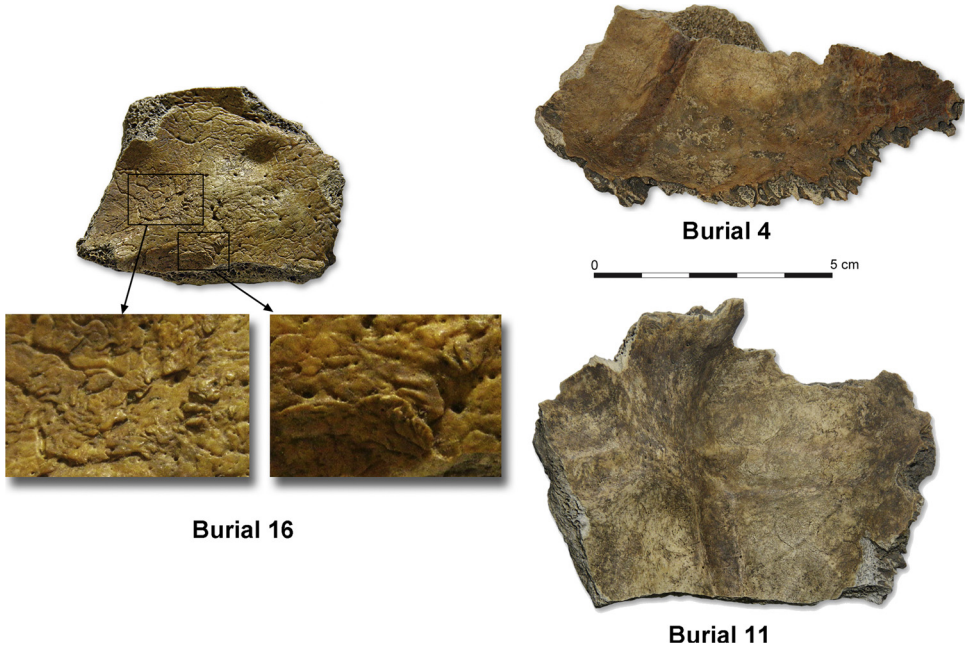

Fig. 2. Endocranial surface of Burial #16 (left) showing bony lesions with enlargements below, relative to endocranial surfaces of two other similarly-aged individuals from the site (Burial #4, top right, and Burial #11, bottom right).

Table 2
Number of raw sequences and the percent of useable sequences which are unique relative to other sequences within the sample. Unique percent is calculated by dividing the number of reads after duplicate removal by the total number of reads.

Burial	Sample	Raw Reads	Unique (%)
2	RN1-Calculus	3454059	0.995
4	RN2-Calculus	2772477	0.992
15	RN3-Calculus	2359209	0.994
16	RN4-Calculus	7357756	0.995
–	Calculus Control	1768314	0.932
2	RN1-Sediment	1813687	0.996
4	RN2-Sediment	1575029	0.996
15	RN3-Sediment	1644108	0.996
16	RN4-Sediment	1551979	0.996
–	Sediment Control	1389677	0.798

sequences aligning to *N. meningitidis* exhibited damage patterns that are typically associated with ancient DNA (Briggs et al., 2007). Compared to RN4, there were not enough reads mapping to *N. meningitides* in the other three calculus samples to create informative damage plots. Next, we used multilocus sequence typing (MLST) to confirm the taxonomic ID of the recovered strain (Maiden et al., 1998). While *Neisseria* species are known to frequently exchange genes (Smith et al., 1999; Feil et al., 1999), several previous studies have found that phylogenies inferred from seven MLST genes accurately distinguish *Neisseria* species (Maiden et al., 1998; Hanage et al., 2005; Hotopp et al., 2006). This approach takes advantage of an online database (http://pubmlst.org/neisseria) that types bacterial strains using seven MLST genes (*abcZ*: putative ABC transporter; *adk*: adenylate kinase; *aroE*: shikimate dehydrogenase; *fumC*: fumarate hydratase; *gdh*: glucose-6-phosphate dehydrogenase; *pdhC*: pyruvate dehydrogenase subunit; *pgm*: phosphoglucomutase). We extracted sequence fragments mapping to these seven genes from our sequencing data using BWA. We aligned these genes to previously published data from 18 *Neisseria* strains (14 *Neisseria meningitidis* strains: NC003112, NC003116, NC008767, NC010120, NC013016, NC017501, NC017505, NC017512 - 017,518; three extant *Neisseria gonorrhoeae* strains: NC002946, NC011035, NC017511; and one extant *Neisseria lactamica* strain: NC014752) using Geneious v9 (http://www.geneious.com; Kearse et al., 2012) and then corrected the alignments by eye and by translating them to proteins. The sequences of the MLST genes obtained from RN4 are incomplete: only 38% of sites from six of the MLST genes were able to be called, and none of the sites from *aroE* were present in the sequence data set. This missing data is unlikely to result in false support for a particular taxonomic assignment, but it may reduce our power to do so.

We then inferred a phylogenetic tree using an RY-encoded alignment. This coding ensures that only transversions are considered as informative sites (ancient DNA damage manifests as transitions, therefore avoiding transitions in the phylogenetic analysis allows us to eliminate bias resulting from ancient DNA damage). Using a concatenated alignment of six of the seven MLST genes we estimated a phylogeny using Bayesian inference using a two-state analogue of the F81 model (the 'restriction model') implemented in *MrBayes* v3.2.0 (Huelsenbeck and Ronquist, 2001). We used a gamma + invariant distribution of rates over sites and partitioned the data into three codon positions, allowing each to evolve at a different rate. We ran two MCMC chains for a total of 1,000,000 iterations, and assessed chain convergence using *Tracer* v1.6 (Rambaut and Drummond, 2013). An effective sample size > 200 was returned for all parameters for all runs, suggesting good model convergence. After the first 10% of states were excluded as burn-in, we estimated a maximum clade consensus tree using *MrBayes*.

To investigate the influence of recombination on our placement of RN4, we constructed phylogenies based on individual MLST gene alignments, using the same methods as we used for the concatenated alignment. We then compared the topologies of these phylogenies to the phylogeny based on the concatenated alignment.

4. Results

Sequencing

Table 2 provides the total number and proportion of unique reads for each sample. Variation in the number of raw reads is due to a combination of pooling error and (for RN4) deeper sequencing following initial screening. As is standard in shotgun sequencing experiments, we sequenced both the samples and the controls. As it is nearly impossible to exclude microbial DNA from NGS experiments, some DNA

Table 3
Most common bacterial species determined using NGS in four dental calculus samples and associated sediments from CA-SCL-919. Numbers indicate percentages (up to 1 significant digit) of total bacteria.

Species	Burial 2 (RN1)		Burial 4 (RN2)		Burial 15 (RN3)		Burial 16 (RN4)	
	Calc	Sed	Calc	Sed	Calc	Sed	Calc	Sed
Tannerella forsythia	22.8	0	9.0	0	14.0	0	1.2	0
Porphyromonas gingivalis	12.6	0	1.1	0	1.5	0	0.3	0
Treponema denticola	1.8	0	1.8	0	1.3	0	0.6	0
Streptococcus gordonii	0	0	0.3	0	0.4	0	13.5	0
Neisseria meningitidis	0	0	0	0	0	0	1.1	0
Veillonella parvula	0	0	0.1	0	0.7	0	2.0	0
Propionibacterium propionicum	11.4	0	12.7	0	4.6	0	18.5	0
Haemophilus influenzae	0	0	0	0	0	0	0.1	0
Streptococcus pneumoniae	0	0	0.1	0	0	0	0.2	0
Streptococcus mitis	0	0	0	0	0	0	0.1	0
Sorangium cellulosum	0	1.3	0.1	0.1	0.1	1.2	0	1.5

was recovered in the controls. However, both the number of reads and the unique proportion are low in the controls, indicating minimal contamination.

MEGAN analysis identified a broad diversity of bacterial taxa in both the calculus and sediment samples (Tables 3, S1). Using the Bray-Curtis dissimilarity index, we found that the microbial metagenomic community within the sediment samples differed significantly from that in the calculus (mean Bray-Curtis = 0.86, t = 111.56, df = 15, p-value = 1.29 e-23). This suggests that DNA recovered from calculus is from the original oral context of the individual and not introduced during or after burial from the sedimentary context. The microbial metagenomic community recovered from the control samples also differs from both the sediment and the calculus, again suggesting a different origin (sediment control: mean Bray-Curtis = 0.93, t = 150.29, df = 3, p-value = 3.25 e-7; calculus control: mean Bray-Curtis = 0.9, t = 57.70, df = 3, p-value = 5.73e-6). The control, sediment and calculus samples form three separate clusters in a PCoA (Fig. 3), also suggesting different metagenomics communities for each cluster. None of the microbiota in Table 3 appear in any of the control samples.

Many of the identified taxa within calculus samples are commensal with humans and common in the present-day human oral microbiome (see supplementary table for a complete list). Some are known to be pathobionts (Table 3), and several are associated with periodontal

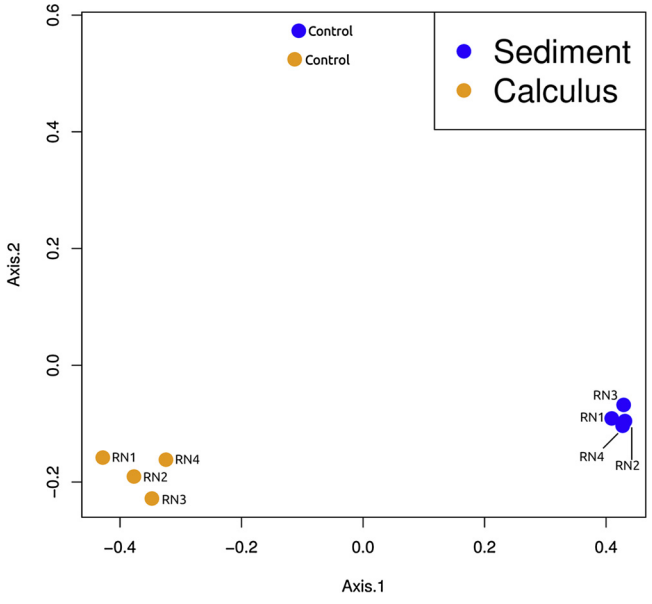

Fig. 3. PCoA of Bray-Curtis distances calculated from the taxonomy profiles from BLAST and MEGAN.

disease, including *Tannerella forsythia, Porphyromonas gingivalis,* and *Treponema denticola*. None of the potentially pathogenic strains listed in Table 3 were identified in the sediment samples or the controls.

One of the calculus samples, RN4 (from Burial #16), contained 9831 sequences identified as *Neisseria meningitidis*. The damage plots (Fig. 4) indicate that the sequences matching *N. meningitidis* contained damage as predicted from ancient DNA (*e.g.* a disproportionate number of C to T and G to A transitions on the forward and reverse strands of the molecules, respectively). This suggests that these sequences are authentically ancient and not recently introduced. The topology of a phylogeny inferred using data from six MLST genes from RN4 and 18 extant *Neisseria* strains matches previous phylogenies of these strains (Bennett et al., 2012) and strongly supports the clustering of the RN4 strain with *N. meningitidis* (Fig. 5). However, we were unable to further determine the placement of RN4 within *N. meningitidis* due to insufficient phylogenetic signal. In 90.3% of the trees in the posterior sample (after removal of burn-in) the sample was found within the diversity of the other *N. meningitidis* strains, and in the other 9.7% of trees, the sample was found basal to the other *N. meningitidis* strains. This lack of support for a particular placement within the *N. meningitidis* clade could result from a large number of missing sites in our sequence of RN4. Despite this lack of support for a particular placement of RN4 within the diversity of modern *N. meningitidis* samples, we find strong support of the grouping of RN4 with modern *N. meningitidis* strains to the exclusion of *N. gonorrhoeae*. Since previous studies have found that *N. meningitidis* and *N. gonorrheae* are sister species (Bennett et al. 2012), this strongly suggests that RN4 was *N. meningitidis*.

While *Neisseria* species are known to undergo frequent recombination, which may complicate species identity, our analyses of individual MLST genes revealed no evidence that our identification of RN4 as *N. meningitidis* was driven by a small portion of the genome. Our identification of RN4 as *N. meningitidis* was further supported by our identification of 33 sequences (corresponding to 877 sites) that mapped to the IS1106 transposase gene, which is specific to *N. meningitidis* and is not found in other *Neisseria* species (Salvatore et al., 2001).

5. Discussion

The presence of early-stage meningeal healing in the form of bony growth on the endocranial surface, as well as an abundance of *N. meningitidis* in the dental calculus of Burial #16, is strongly suggestive of an ancient case of meningococcal disease. This species was not detected in calculus from three other individuals from the site. The proliferation of this species in Burial #16's oral cavity likely represents a more widespread infection that included her meninges. Much recent biomedical research emphasizes the role that microbiota play in our overall health, and demonstrates that changes in the distribution of microbial species is often correlated to development of disease (*e.g.*, Gerritsen et al., 2011;

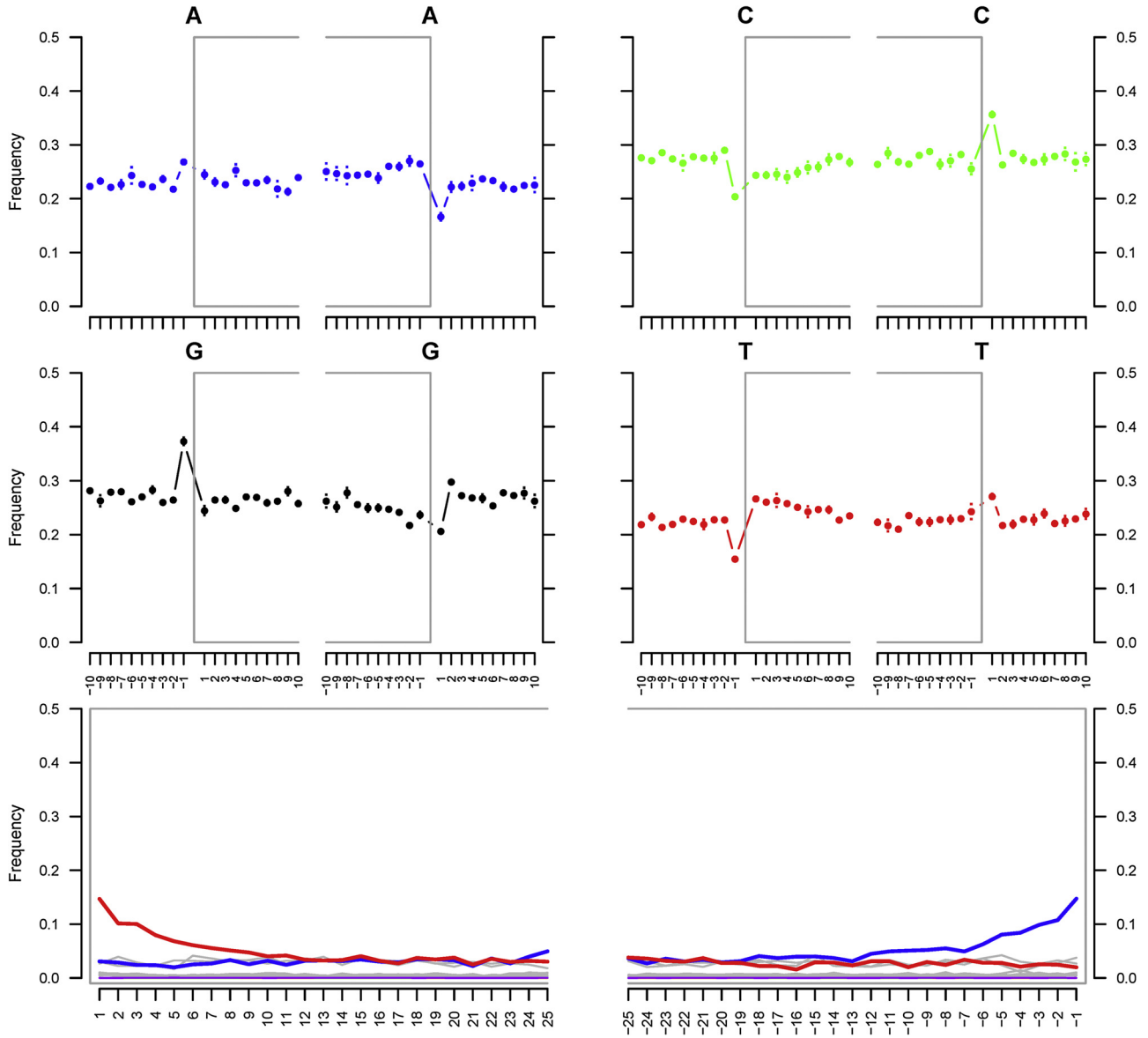

Fig. 4. Fragment misincorporation plot. The top four plots show amino acid frequencies along the length of the sequences. The bottom plot shows the frequencies of fragment misincorporation. The blue and red upticks are indicative of DNA damage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Round and Mazmanian, 2009; Sekirov et al., 2010). We interpret the unusual oral microbiota in Burial #16, relative to three other members of the community, as reflective of her development of meningococcal disease.

We note that *N. meningitidis* is not the only unusual and potentially pathogenic microbe the NGS recorded from the calculus of Burial #16, as *S. pneumonia* and *H. influenzae* were also detected. The latter is common today in the human respiratory tract and oral cavity, but some strains are potentially pathogenic and are associated with upper respiratory infection (Black et al., 1988), and in some cases meningitis (Kerr et al., 1993). If she suffered from a respiratory infection, this may have compromised her immune system, providing the opportunity for *N. meningitidis* to cross from the nasopharanx area and/or oral cavity into the boodstream and cerebrospinal fluid. Fig. 6 (left panel) shows her lower right permanent first molar. As seen, extreme occlusal wear removed all enamel, significant amounts of dentin, and exposed the pulp chamber of the molar. Yet, she continued using this surface for chewing. Such exposure could have provided an entryway for bacteria into the bloodstream. The central panel of Fig. 6 shows her upper right third molar (which has minimal occlusal wear) and adhering supragingival calculus (left side of tooth), while the right panel shows a close-up of calculus removed from the tooth.

Today, old age is an important risk factor in the contraction of meningococcal disease, and we suggest this also contributed to the case in Burial #16. She appears to have suffered for a short amount of time with meningococcal disease. As witnessed by only the beginning stages of endocranial lesions, she appears to have died not long after contracting the disease.

6. Conclusions

The results of this study are significant on several fronts. First, to our knowledge, this is the first documentation of *N. meningitidis*, the bacterial species that causes meningococcal disease, in an ancient sample from the Americas. The antiquity and origin of this disease are poorly documented. Warinner et al. (2014a, b) report the presence of this species in ancient calculus samples from Germany, demonstrating an Old World presence of the bacterial species, though not necessarily

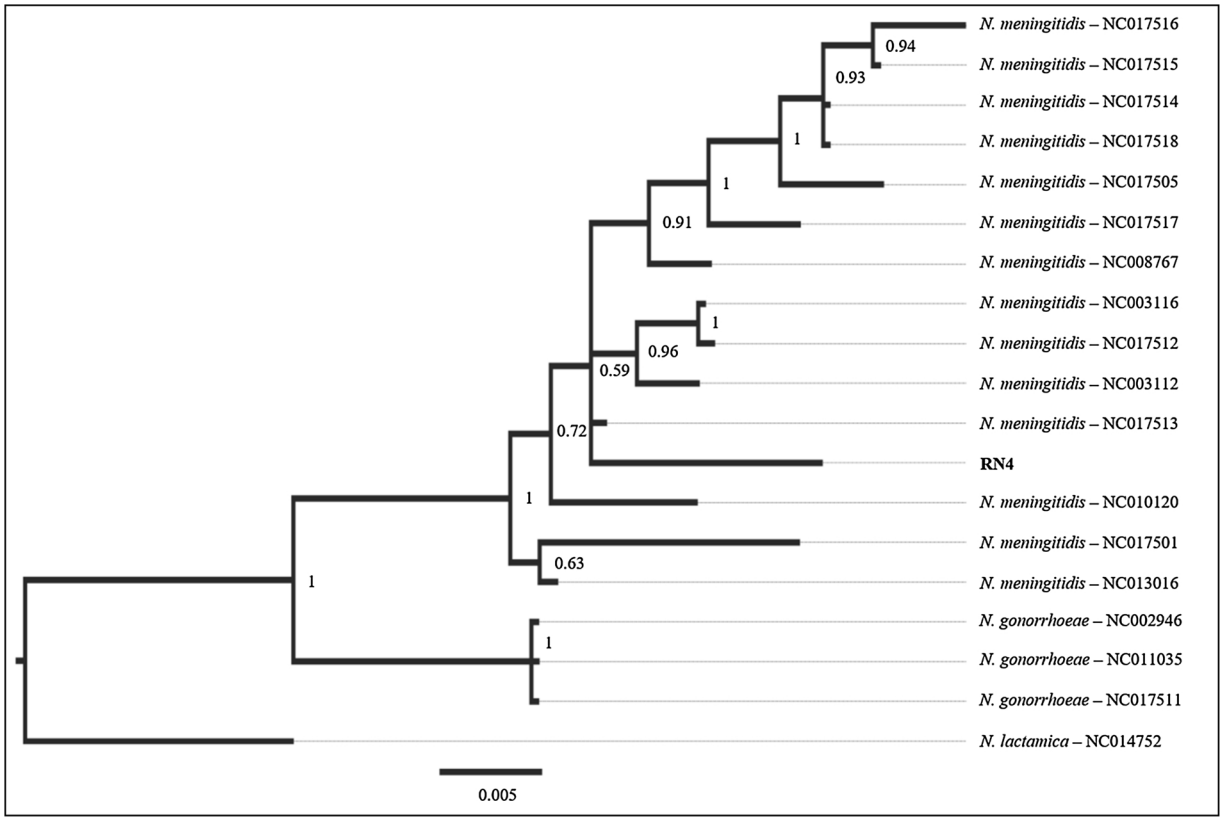

Fig. 5. The maximum clade consensus tree showing the relationship between RN4 (Burial #16) and 18 extant *Neisseria* strains, with nodes with < 50% posterior support collapsed. Bayesian posterior support vales are provided on each node. The phylogeny is rooted using the outgroup, *N. lactamica*. The scale bar shows the rate of transversions.

meningococcal disease. Weyrich et al. (2017) document *N. gonorrhoeae* in dental calculus of Neanderthals from Spain (ca. 48,000 years old), but not *N. meningitidis*.

Despite documenting *N. meningitidis* in Burial #16, it is still unclear whether the disease is indigenous to the New World. Burial #16 dates to the late 15th to early 17th century AD. It is known that various European explorers, including Juan Rodríguez Cabrillo, Francis Drake, and Sebastián Vizcaíno, made landfall in California and had some contact with indigenous peoples in this time frame. Whether they could have spread exogenous *N. meningitidis* to local populations is not known. If so, indigenous populations could then have spread the disease further within local communities through person-to-person contact, eventually into 16th century California populations. Additional sampling of older calculus samples from California and other regions, currently underway, should help resolve this issue.

Second, the results demonstrate the value of ancient DNA and NGS analyses of dental calculus samples to trace the history of pathobionts, and paleopathology more generally. Lacking the genetic data from calculus, this case would have been speculative. The just-forming endocranial lesions were not developed enough to implicate meningitis as a likely cause of death. However, the unusual range of oral microbes for this woman prompted a re-evaluation of the osteological evidence. Combined, we are now able to suggest a specific cause of death for this woman. In the process, we also increase our understanding of health

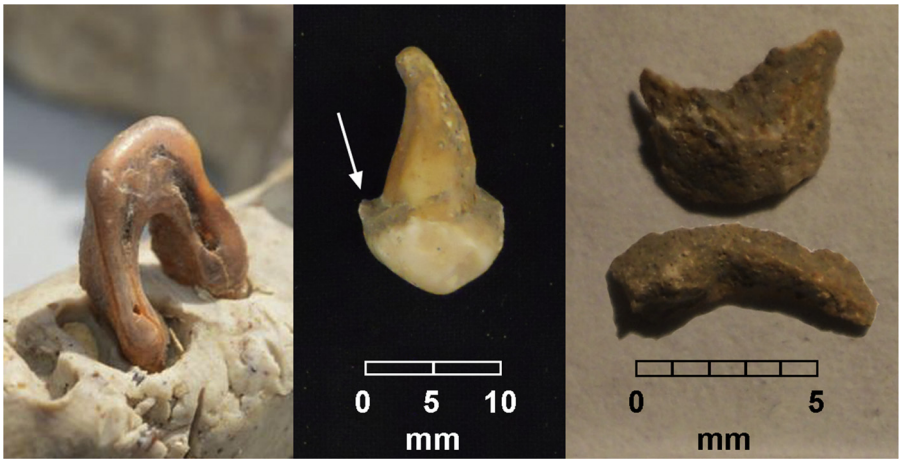

Fig. 6. Exposed pulp cavity of highly worn lower right first molar(left pane; tooth set in clay, note occlusal wear almost to apical root tips), upper right third molar with adhering calculus (center), and removed calculus (right) from Burial #16.

and disease in pre-contact California and help trace part of the history of what is today a global and deadly disease.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijpp.2018.05.001.

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