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Note

Method for culturing *Candidatus* Ornithobacterium hominis

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ABSTRACT

Candidatus Ornithobacterium hominis has been detected in nasopharyngeal microbiota sequence data from around the world. This report provides the first description of culture conditions for isolating this bacterium. The availability of an easily reproducible culture method is expected to facilitate deeper understanding of the clinical significance of this species.

Candidatus Ornithobacterium hominis (OH) is a bacterium that has been detected in nasopharyngeal microbiota sequence data from around the world but has never been cultured (Salter et al., 2019; Salter et al., 2017). This bacterium is of growing interest as polymerase chain reaction (PCR)-based studies found that OH was prevalent and persistent in the nasopharynx of a paediatric population at high-risk of respiratory infection (Salter et al., 2019; Salter et al., 2017). Additionally, the closest known relative of OH is *Ornithobacterium rhinotracheale*; a respiratory pathogen of birds (Zahra et al., 2013). These observations prompt research to understand the pathogenic potential and clinical significance of OH. Although genomes can be derived from metagenomic data, OH isolates are needed to deepen understanding of the bacterium's role in human respiratory infections. The aim of this study was to determine culture conditions for recovery of OH isolates.

The study was approved by the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (Approval number: 0785). Culture was performed using biobanked nasopharyngeal swabs that were collected from four Australian children (age 1–2 years) immediately prior to bronchoscopy for investigation for chronic suppurative lung disease (Marsh et al., 2016). The swabs had been stored in skim milk-tryptone-glucose-glycerol broth (STGGB) at −80 °C for up to 10 years and had two freeze-thaw cycles prior to OH culture. These swabs were selected as all were OH-positive by 16S rRNA gene sequencing at 5–55% relative abundance (Marsh et al., 2016).

Ten microlitres of the STGGB swab media was inoculated onto Tryptic Soy Agar with 5% Sheep Blood (TSA), Horse Blood Columbia agar (HBA), Chocolate agar and Brain Heart Infusion agar (BHI). The plates were incubated aerobically, microaerophillically (Campygen, Oxoid) and anaerobically (Anaerogen, Oxoid) at 35 °C for up to five days. Aerobic culture was also performed in the presence of a wet sponge to provide increased humidity (Mayahi et al., 2016). Oxidase testing was done using oxidase test strips (Oxoid). Tributyrin hydrolysis was determined using Catarrhalis discs (Remel). Production of β-lactamase was determined using nitrocefin (Oxoid).

Primary cultures were reviewed for colonies resembling *O. rhinotracheale* (Van Empel and Hafez, 1999). Colonies of oxidase-positive, Gram-negative pleomorphic bacilli were screened using PCR targeting OH-specific regions of the 16S rRNA and *toxA* genes, as described previously (Salter et al., 2019). PCR-positive isolates were confirmed using genome sequencing. Genomes were assembled *de novo* using the Microbial Genome Assembly Pipeline (MGAP) v1.0 (https://github.com/dsarov/MGAP—Microbial-Genome-Assembler-Pipeline) (Chapple et al., 2016). OH identification was confirmed where isolates had > 96% average nucleotide identity (Kim et al., 2014; Richter and Rossello-Mora, 2009) when compared to draft OH genomes OH-22767 (GenBank accession NZ_UNSC00000000.1) and OH-22803 (GenBank accession UNSD00000000.1). Both draft genomes were derived from metagenomic analysis of nasopharyngeal swabs from Thai children (Salter et al., 2019). Isolate genomes were mapped against the draft OH genomes using the Synergised Pipeline for Analysis of Next Generation Sequencing Data in Linux (SPANDx) v3.2.1 (Sarovich and Price, 2014), which wraps Burrows-Wheeler Aligner (Li and Durbin, 2009), Sequence Alignment/Map (SAM) tools (Li et al., 2009), Picard Tools and Genome Analysis Tool Kit (Mckenna et al., 2010). Genomes were aligned using draft OH genome OH-22803 as the reference with an *O. rhinotracheale*

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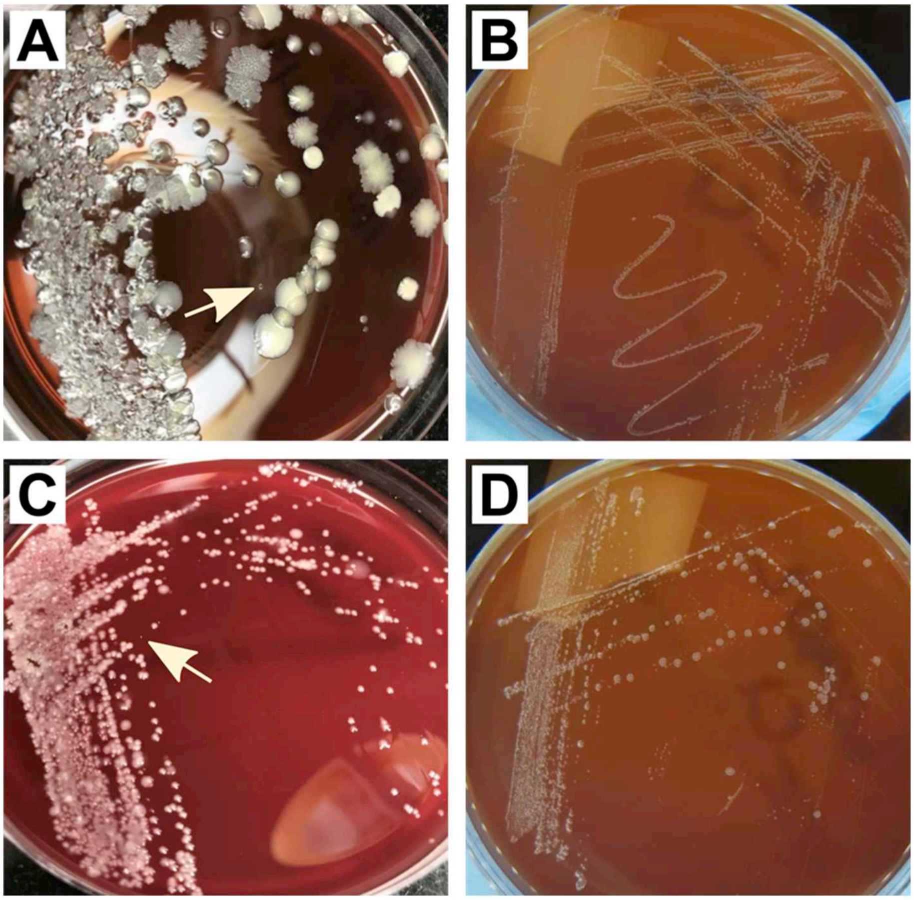

Fig. 1. *Ca.* Ornithobacterium hominis colony morphology.
A) Primary isolation of *Ca.* O. hominis isolate 903C1 on TSA after 120 h aerobic incubation in the presence of a wet sponge. Arrow indicates a *Ca.* O. hominis colony.
B) Purified *Ca.* O. hominis isolate 903C1 after 120 h microaerophillic incubation on TSA. Pure culture of this strain produced a uniform colony size.
C) Primary isolation of *Ca.* O. hominis isolate 902C1 on TSA after 120 h microaerophillic incubation. Arrow indicates a *Ca.* O. hominis colony.
D) Purified *Ca.* O. hominis isolate 902C1 after 120 h microaerophillic incubation on TSA. Pure culture of this isolate producted two colony morphotypes.

genome (ORT-UMN 88, GenBank accession CP006828.1) included as an outgroup. Maximum parsimony phylogenomic trees were generated using Phylogenetic Analysis Using Parsimony (PAUP) v4.0a153 (Swofford, 1998) and visualised using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Bootstrapping was performed in PAUP with 1000 replicates. Lipopolysaccharide comparisons were generated using Easyfig (Sullivan et al., 2011). The OH isolate genomes are available from the Sequence Read Archive (SRA; BioProject number: PRJNA510696).

OH was successfully cultured from all four swabs. Primary isolation was challenging due to substantial overgrowth by other taxa (Fig. 1). Of the conditions tested, optimal primary culture was achieved using TSA incubated in a microaerophilic atmosphere at 35 °C for up to five days. OH also grew on HBA, Chocolate agar and BHI; however, isolates were not consistently recovered from these media. Aerobic growth was possible but required additional humidity (*e.g.* incubating plates in a box containing a wet sponge).

Under microaerophilic conditions, OH colonies were pleomorphic, glistening, grey and concave. Colonies ranged in size from 1 to 3 mm after 48–120 h incubation. All isolates were pleomorphic Gram-negative bacilli. Consistent with the phenotype predicted by the draft genomes (Salter et al., 2019), OH isolates were oxidase-positive, catalase-negative and all produced β-lactamase. All isolates also hydrolysed tributyrin. Some pure isolates produced two colony morphologies (Fig. 1D). This phenotype is suggestive of small-cell variants (Zahra et al., 2013) as both colony types were positive by OH 16S rRNA and *toxA* PCR.

The OH isolate genomes had average nucleotide identity of 97.86–98.23% with draft genomes OH-22803 and OH-22767, indicating that they are members of the same species. Phylogenomic analysis demonstrating the high similarity between the Australian isolates and draft OH genomes from Thailand is shown in Fig. 2. All isolate genomes contained distinct lipopolysaccharide (LPS) biosynthesis clusters which differed to those of the draft genomes (Fig. 3). β-lactamase production was associated with mobile genetic elements that were different in each isolate and occurred at different loci. All isolates also had genes encoding efflux pumps associated with multi-drug resistance.

In summary, following identification of OH *in silico*, we now report culture conditions for its propagation. Of the conditions tested, optimal growth was achieved using TSA with incubation for up to five days in a microaerophilic atmosphere; conditions which are not part of standard culture used to recover respiratory pathogens from nasopharyngeal swabs (Satzke et al., 2013). Primary isolation was challenging due to extensive overgrowth by other flora. We recommend OH-specific PCRs (Salter et al., 2019) are used to confirm isolate identity. The OH colonial morphology was similar to that reported previously for *O. rhinotracheale* (Van Empel and Hafez, 1999), including growth of multiple colony morphologies suggestive of small-cell variants (Zahra et al., 2013). The significance of this observation is unknown; however, small-cell variants of other bacteria (*e.g. Staphylococcus aureus*) have been associated with poorer clinical outcomes in patients with respiratory disease (Wolter et al., 2013). Association of β-lactamase genes with multiple mobile genetic elements indicates that OH β-lactam resistance has been acquired through several independent events. Heterogeneity among the LPS cluster is suggestive of multiple capsular types, consistent with observations from earlier DNA-based studies (Salter et al., 2019; Salter et al., 2017). The availability of an easily reproducible culture method is expected to facilitate deeper understanding of the clinical significance of OH.

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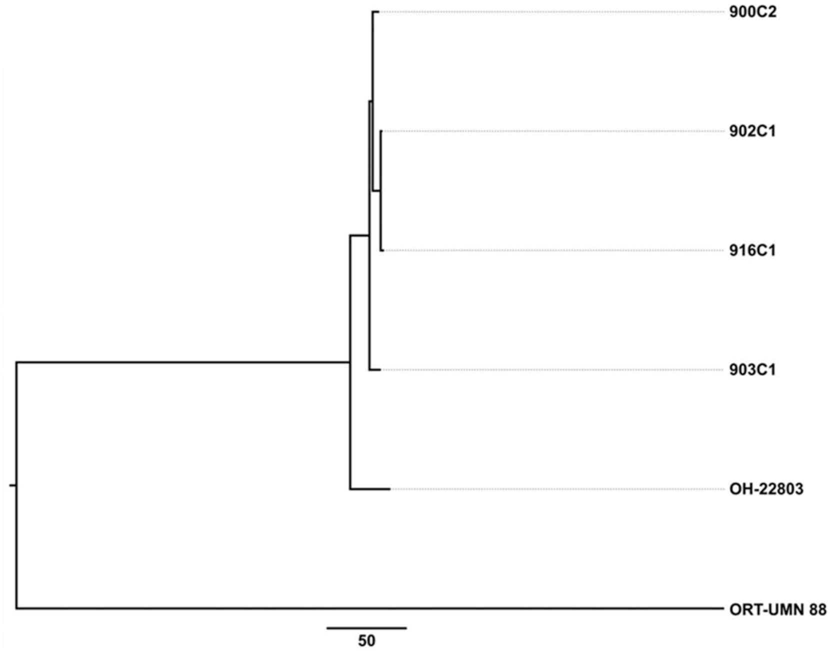

Fig. 2. Phylogenomic analysis of the *Ca.* Ornithobacterium hominis isolates.
A midpoint-rooted maximum parsimony tree was constructed based on 764 biallelic single nucleotide polymorphisms (SNPs) orthologous to the four Australian *Ca.* O. hominis isolates (900C2, 902C1, 903C1 and 916C1); two previously reported draft *Ca.* O. hominis genomes from Thailand (OH-22767 and OH-22803); and an *O. rhinotracheale* outgroup (ORT-UMN 88). Bar indicates a distance of 50 SNPs.

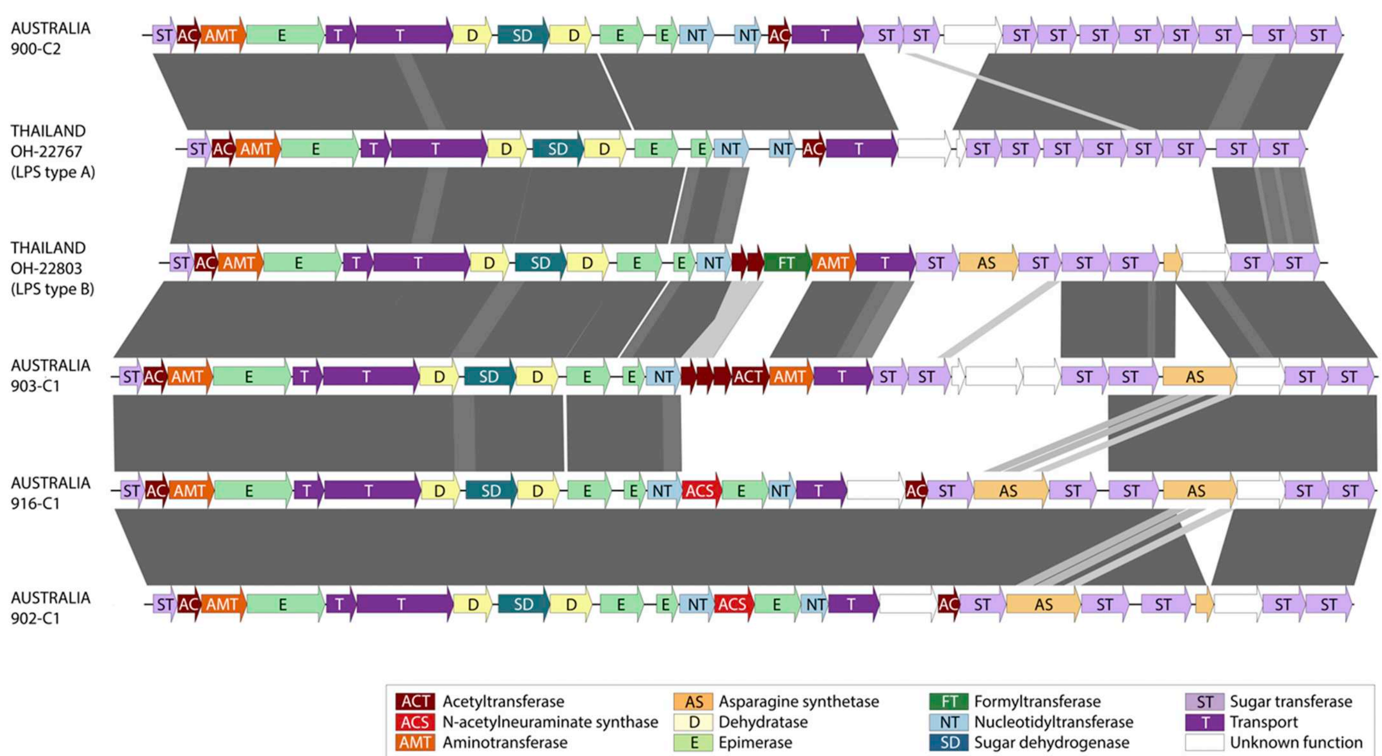

Fig. 3. Comparison of *Ca.* Ornithobacterium hominis lipopolysaccharide biosynthesis loci.
A tblastx alignment of the lipopolysaccharide biosynthesis clusters in the four Australian OH isolates compared to draft genomes OH-22767 and OH-22803 derived from Thailand (2).

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