Complete genome for *Actinobacillus pleuropneumoniae* serovar 8 reference strain 405: comparative analysis with draft genomes for different laboratory stock cultures indicates little genetic variation

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**Abstract**

We report here the complete genome sequence of the widely studied *Actinobacillus pleuropneumoniae* serovar 8 reference strain 405, generated using the Pacific Biosciences (PacBio) RS II platform. Furthermore, we compared draft sequences generated by Illumina sequencing of six stocks of this strain, including the same original stock used to generate the PacBio sequence, held in different countries and found little genetic variation, with only three SNPs identified, all within the *degS* gene. However, sequences of two small plasmids, pARD3079 and p405tetH, detected by Illumina sequencing of the draft genomes were not identified in the PacBio sequence of the reference strain.

**DATA SUMMARY**

Raw sequence data were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession numbers PRJEB2343 and PRJEB10244. Individual accession numbers for each sequence are reported in Table 1. The complete closed genome of strain 405, generated by PacBio sequencing, has been deposited in GenBank with accession number CP078508. The complete sequence of the p405tetH plasmid has been deposited in GenBank under accession number MZ436971.

**INTRODUCTION**

The porcine respiratory tract pathogen, *Actinobacillus pleuropneumoniae*, was first described as a *Haemophilus*-like organism, isolated in 1957 from pneumonic lung lesions of pigs in Great Britain [1]. It was subsequently found to be the...
Serovar 8 was first isolated in Ireland and Denmark in 1984, with the Irish strain 405 designated as the reference strain [13]. This serovar has since been found to be the most prevalent in the United Kingdom [14, 15] and Norway [16], and it has also been identified in North and South America [17, 18], Denmark [19], and Belgium. We previously reported the complete genome sequence of a genetically tractable serovar 8 clinical isolate, MIDG2331, from the UK [20]. We further showed that MIDG2331 encodes a 56 kb integrative conjugative element (ICEApll) found in a subset of serovar 8 isolates, and absent in strain 405 [21]. Draft and or complete genome sequences have been previously reported for the reference strains of many A. pleuropneumoniae serovars [22–24], but not strain 405.

Not all of the A. pleuropneumoniae serovar reference strains are available through the common culture collections, such as the American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC), and sets of reference strains have commonly been distributed between research groups around the world. Mistakes in strain identification can lead to genotypic/phenotypic results being ascribed to the wrong strain/serovar. For example, the draft genome sequence deposited as the serovar 1 reference strain Shope 4074 (accession number NZ_AACK01000000) is actually a serovar 5 strain, as indicated by the serovar specific capsule genes present [11]. Similarly, the draft genome sequence deposited as the biovar 2 serovar 13 reference strain N273 (accession number NZ_ADOM00000000) has a capsule locus indicating it is a serovar 7 isolate and has a truncated nadV gene, indicating it is biovar 1 [11]. Although still the same serovar, Gram et al. [25] reported that a Danish field isolate of serovar 8 had been mixed up with reference strain 405 during routine lab use, leading to confusion over the reported genotype of omLA for this strain [19]. Furthermore, changes in reference strain genomes due to genetic drift following multiple passages in the lab have also been reported for various bacterial species, sometimes leading to phenotypic differences [26–29]. Genetic changes can also occur following extended incubation over several days in rich medium, such as when agar stabs are used for shipping bacterial strains between research groups [30].

In this study, we used PacBio sequencing technology that facilitates long DNA reads to generate a complete closed genome for the A. pleuropneumoniae serovar 8 reference strain, 405, using DNA from an aliquot of the original stock [13] held at the National Veterinary Institute, Technical University of Denmark. Additionally, using the Illumina platform, we generated draft genome sequences from this sample, as well as DNA from five other stocks of this strain held in different laboratories around the world in order to determine, using SNP analysis, if any genetic differences exist between them.

**METHODS**

The sources of DNA for the different stocks of A. pleuropneumoniae strain 405 are shown in Table 1. The original stock, held in Denmark and termed 405D, was used for generation of the complete closed reference genome, using the PacBio platform, as well as for generation of a draft genome by Illumina sequencing (see below). Although DNA was prepared from cultures grown from master freezer stocks (stored at −80 °C in 15–25 % glycerol) in each laboratory, it is not known how many passages each stock may have been subjected to prior to acquisition from other groups. There were some differences in media used to grow the culture and methods to prepare the genomic DNA between the labs, as indicated (Table 1).
For draft genome sequences, paired-end libraries were generated from approximately 500 ng of genomic DNA as previously described [31, 32] and sequencing was performed at the Wellcome Sanger Institute (Hinxton, UK) on an Illumina HiSeq 2000 analyzer to obtain paired 75 bp reads. For each draft genome, the sequenced underwent quality assessment using bifrost (https://github.com/ssi-dk/bifrost) and were assembled into contigs using SKESA [33].

PacBio sequence reads were assembled using HGAP v3 [34], as part of the SMRT analysis software v2.3.0 (https://github.com/PacificBiosciences/SMRT-Analysis). When picking the minimum fragment length for assembly, the fold coverage to target was set to 30 and the approximate genome size was set to 3 Mbp. Circulator v1.1.3 [35] was used to circularise the assembly, which was then polished using the PacBio RS_Resequencing protocol and Quiver v1 of the SMRT analysis software v2.3.0.

Assembled sequences were analysed for the presence of acquired antimicrobial resistance genes using ResFinder v4.1 [36], with a threshold of 90 % identity and minimum length of 60 %. The PacBio closed genome was annotated using the National Centre for Biotechnology Information’s (NCBI’s) Prokaryotic Genome Annotation Pipeline (PGAP).

Following removal of duplicated regions using NUCmer, detection of single nucleotide polymorphisms was performed using the Northern Arizona SNP (NASP) pipeline v1.2 [37] using BWA to align Illumina reads from individual draft genomes of the six stock cultures of the A. pleuropneumoniae serovar 8 reference strain 405 against the PacBio closed genome generated for the original stock culture (strain 405D). Positions with less than ten-fold coverage and less than 90 % unambiguous variant calls were excluded.

RESULTS

PacBio sequencing of the 405D sample yielded 105 545 circular consensus reads, with a mean length of ~4300 bp, of which a total of 6017 reads with a mean length of ~8000 bp after error correction were used to obtain a single contig of 2323218 bp with a GC content of 41.1%, which is typical for A. pleuropneumoniae genomes. The genome of strain 405 is syntenic with that of the previously reported complete genome of the serovar 8 clinical strain, MIDG2331 [20]. The only differences consist of phage gene insertions, which vary between the two genomes, and the ICEApII insertion in MIDG2331 [21] which is absent from 405.
Quality control and assembly of the genomes for the six different stock 405 strains, sequenced using the Illumina platform, showed that each draft genome was of high quality, with sequencing depths of >75. For each draft genome, the reads were assembled into 41 or 42 contigs, all with a GC content of 41.0 %. Alignment of the draft genome sequences with the PacBio closed reference genome showed, after removal of duplicated regions, a core of 2208689 bp equivalent to 95.07 % of the reference chromosome across all six samples, with only three SNPs identified, all in the degS gene (Table 2).

Each of the three detected nucleotide changes is associated with a nonconservative substitution in the 343 amino acid (AA) DegS protein. The sequence of the original strain 405 stock culture (both the draft 405D genome and the complete PacBio sequence) encodes a DegS protein with a serine at residue 230, whereas all of the other stock culture genomes show a proline residue at this site that appears to be conserved in all other sequenced A. pleuropneumoniae genomes, including other serovar 8 sequences (WP_005600971, WP_039709089). Additionally, the 405H protein differs at residue 46 (an aspartic acid rather than alanine), and that of 405U differs at residue 216 (a glutamic acid rather than a lysine), from the other A. pleuropneumoniae DegS sequences. Whereas the degS genes in 405C, 405Ch and 405S encode proteins sharing 100 % identity with those from other sequenced serovar 8 isolates of A. pleuropneumoniae (WP_039709089).

ResFinder analysis of the assembled PacBio sequence did not identify any known resistance genes, whereas tet(H) (Y15510) and sul2 (AY034138) were identified on two different small contigs in each of the draft genomes. These appeared as contigs 1 and 2 in all the SKESA assemblies, and were identified as circular contigs of 5470 bp and 4063 bp, respectively. Further analysis of the 4063 bp contig, identical in each draft genome, revealed that sul2 is encoded on a plasmid that shows 99 % ID with pARD3079, previously described for A. pleuropneumoniae [38]. Analysis of the 5470 bp contig, also identical in each draft genome, revealed that the tetracycline resistance gene, tet(H), is encoded along with the tetR regulator and plasmid mobilization genes, mobA and mobC, in a novel plasmid that shares partial identity with some previously described plasmids in various Pasteurellaceae species, including the A. pleuropneumoniae plasmid p9956 [39] and pB1018 from Pasteurella multocida [40]. The novel plasmid in A. pleuropneumoniae strain 405, designated p405tetH, shares 99 % identity across the tet(H)/tetR genes, but less conservation across the mobilization genes (83 % ID), which are found in the opposite orientation in p9956 and pB1018.

### DISCUSSION

Unintentional mutations can occur in bacterial stock cultures that can affect phenotypes under study, possibly leading to contradictory results from different laboratories. When compared to the genome of the Escherichia coli strain MG1655 published by Blattner et al. [41], Freddolino et al. [27] found a set of seven mutations in the genome sequences of different stocks of this strain that had been acquired from the Blattner laboratory in 2003, as well as in stocks of ATCC 700926 acquired on two separate occasions; whereas an older stock (ATCC 47076) contained only a subset of the mutations compared to the published genome. For the Campylobacter jejuni reference strain, NCTC 11168, the sequence has been documented to vary at least 200 times over three decades, significantly affecting its phenotypic properties [29].

It has been noted that mutations can occur during transfer of bacterial strains between laboratories, when bacteria are often incubated for several days in rich medium transport stabs [30], and also during long-term preservation of stocks within culture collections [42]. Furthermore, even limited passages...
in the laboratory can result in mutations [26, 43], which may cause phenotypic changes.

Most *A. pleuropneumoniae* research groups have acquired their reference strain collection from other laboratories, rather than from curated culture collections such as ATCC or NCTC. Given the previous report that a Danish field isolate of *A. pleuropneumoniae* serovar 8 had been mistaken for the reference strain 405 [25], meaning it could be in circulation as the reference strain, we decided to do comparative sequence analysis of different international laboratory stocks of this strain in addition to generating a closed reference genome that will facilitate further functional genomic analysis of this serovar [44].

All of the genomes sequenced in this study had the *omlA* gene sequence previously described for strain 405 (Y12811) and not that of the Danish clinical serovar 8 isolate (U86683) mistakenly used as the reference strain in development of an *omlA*-based diagnostic PCR [19], indicating they are all true stock of the *A. pleuropneumoniae* serovar 8 reference strain 405. Furthermore, the draft genome sequences show little genetic variation between the different stock cultures, with only three SNPs identified, all within the *degS* gene.

**DegS** is a serine protease which functions as a periplasmic stress sensor required for activation of the alternative sigma factor, RpoE, via degradation of the anti-sigma factor, RseA [45]. In *A. pleuropneumoniae*, both *rseA* and *rpoE* were identified as important for survival of the bacterium within the pig during acute infection [46], and RpoE was subsequently shown to be a key regulator of biofilm formation by this bacterium [47]. A *degS* deletion mutant has been generated in *A. pleuropneumoniae*, leading to increased production of outer membrane vesicles [48], but no other phenotypes were tested, and no structure/function analysis has been done for DegS in this bacterium. The DegS protein is best characterised in *E. coli*, with a crystal structure supporting domain analysis for identification of key active site residues [49]. Although the *A. pleuropneumoniae* DegS shares only 49% identity with that of *E. coli*, regions of conservation include the alanine, lysine, and proline residues described above and found in the majority of *A. pleuropneumoniae* DegS sequences. However, none of these residues has been identified as critical for the structure or activity of the *E. coli* protein, and it is unclear if the alternate AAs would have any effect on the *A. pleuropneumoniae* DegS proteolytic activity.

Overall, the results of this study confirm that the strain 405 cultures held in stock collections in various laboratories around the world are all the correct serovar 8 reference strain. Furthermore, despite acquisition from various other laboratories over the years, with uncertain number of passages (possibly on different types of media) between transfers, only three SNPs were detected in the genomes prepared from the different cultures. That all three SNPs affect the *degS* gene, encoding a protease predicted to be involved in activation of RpoE, suggests possible selection for these mutations in response to extracytoplasmic stress(es) encountered during passage/transport/storage, rather than random genetic drift.

Some studies have indicated that PacBio-generated sequences may be less accurate than those generated with the Illumina HiSeq platform, though improvements have been achieved more recently [50, 51]. Our comparison of the Illumina- and PacBio-generated genomes for the original stock 405D culture revealed no SNPs between these sequences, indicating comparable accuracy with both platforms. It has also been previously reported that small plasmid sequences may not be identified using the PacBio platform [52, 53], and this was seen in our results, with sequences for two small plasmids (pARD3079 and p405steH) found in all of the draft genomes, but not the PacBio sequence. It should be noted that the presence of the two plasmids will not affect the use of the strains as serovar controls, but has the potential to introduce variation into other types of studies, e.g. those involving antimicrobial resistance. The availability of the complete closed genome (and associated plasmid sequences) for the serovar 8 reference strain 405 will facilitate functional genomic analysis, as well as further comparative genome studies with other isolates of this increasingly prevalent *A. pleuropneumoniae* serovar.

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