DATA NOTE

The use of Oxford Nanopore native barcoding for complete genome assembly

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Abstract

Background: The Oxford Nanopore Technologies MinION(TM) is a mobile DNA sequencer that can produce long read sequences with a short turn-around time. Here we report the first demonstration of single contig genome assembly using Oxford Nanopore native barcoding when applied to a multiplexed library of 12 samples and combined with existing Illumina short read data. This paves the way for the closure of multiple bacterial genomes from a single MinION(TM) sequencing run, given the availability of existing short read data. The strain we used, MHO_001, represents the important community-acquired methicillin-resistant Staphylococcus aureus lineage USA300.

Findings: Using a hybrid assembly of existing short read and barcoded long read sequences from multiplexed data, we completed a genome of the S. aureus USA300 strain MHO_001. The long read data represented only ~5% to 10% of an average MinION(TM) run (~7x genomic coverage), but, using standard tools, this was sufficient to complete the circular chromosome of S. aureus strain MHO_001 (2.86 Mb) and two complete plasmids (27 Kb and 3 Kb). Minor differences were noted when compared to USA300 reference genome, USA300_FPR3757, including the translocation, loss, and gain of mobile genetic elements.

Conclusion: Here we demonstrate that MinION(TM) reads, multiplexed using native barcoding, can be used in combination with short read data to fully complete a bacterial genome. The ability to complete multiple genomes, for which short read data is already available, from a single MinION(TM) run is set to impact our understanding of accessory genome content, plasmid diversity, and genome rearrangements.

Keywords: Whole genome sequencing; Staphylococcus aureus; MinION; long read; hybrid assembly; bacterial genomics; multiplexing; native barcoding

Introduction

The spread of methicillin-resistant Staphylococcus aureus represents a significant burden in both the health-care setting and the community. The USA300 clone is a particular cause for concern, being responsible for an increasing number of skin and soft-tissue infections within the community, particularly in North America [1]. The advent of new sequencing technologies is set to inform on novel intervention and surveillance strategies, although important technical limitations remain.
short read data provide an excellent means to assay the variation within the core genome, which is useful for reconstructing hospital outbreaks, it is usually not possible to infer genome rearrangements or to fully assemble mobile genetic elements such as plasmids from these data. Closure of bacterial genomes has been demonstrated on Escherichia coli using the Oxford Nanopore Technologies (ONT) MinION(TM) reads alone and on a range of bacteria including Bacteroides fragilis, Acinetobacter baylyi, and Francisella spp. using a hybrid approach combining error-prone long reads with low error rate short reads [2–5]. Here we demonstrate that it is also possible to generate complete genomes using multiplexed reads from a single MinION(TM) run in combination with matched Illumina short reads. We used a strain of S. aureus of the USA300 lineage as an example.

**Methods**

**MinION(TM) library construction and sequencing**

S. aureus strain MHO.001 was recovered in 2015 from asymptomatic nasal carriage via a standard nasal swab of a healthy individual with informed consent. DNA from an overnight culture was extracted using the QiaGen Genomic Tip 500/G Kit, following the manufacturer’s instructions, except lysozyme was replaced with lysostaphin to a final concentration of 200 µg/ml. Sequencing library preparation was carried out with Nanopore Genomic Sequencing Kit SQK-MAP006 (ONT, UK) and a PCR-free ‘native barcoding’ kit provided by ONT. The NEBNext Ultra II End Repair/DA Tailing kit (E7546S, NEB) was used to prepare 1000 ng of sheared genomic DNA (1000 ng DNA in 50 µl nuclease free water, 7 µl of Ultra II End-Prep Buffer, 3 µl Ultra II End-Prep Enzyme Mix in a total volume of 60 µl). The reaction was incubated for 5 minutes at 20 °C and heat inactivated for 5 minutes at 65 °C. The DNA was purified using a 1:1 volume of Agencourt AMPure XP beads (A63880, Beckman Coulter) according to manufacturer's instructions and eluted in 31 µl of nuclease free water. Blunt/TA Ligase Master Mix (M0367S, NEB) was used to ligate native barcode adapters to 22.5 µl of 500 ng end prepared DNA for 10 minutes at room temperature. The barcoded DNA was purified using a 1:1 volume of Amplicon XP beads and eluted in 26 µl nuclease free water. Twelve barcoded samples from diverse sources including other bacterial samples were pooled, 58 ng of each sample was added to give 700 ng of pooled library DNA. Hairpin adapters were ligated using 10 µl Native Barcoding Adapter Mix, 50 µl Blunt/TA Ligase Master Mix, and 2 µl Native Barcoding Hairpin Adapter added to 38 µl of the pooled library DNA to give a final reaction volume of 100 µl. The reaction mixture was incubated for 10 minutes at room temperature before the addition of 1 µl of HP tether and a further 10 minutes incubation. The final reaction was cleaned using prewashed Dynabeads MyOne Streptavidin C1 beads (65001; Thermo Fisher Scientific). DNA concentrations at each step were measured using a Qubit Fluorometer. Then 6 µl of the pooled, barcoded library was mixed with 65 µl nuclease free water, 75 µl 2x Running Buffer, and 4 µl Fuel Mix (SQK-MAP006, ONT) and immediately loaded onto a MinION(TM) Flow Cell Mk I R7.3 on a MinION(TM) Mk1 controlled by MinKNOW version 0.50.2.15 software (ONT). Base calling was performed using Metrichor ONT Sequencing Workflow Software v1.19.0 with the Basecall,Barcoding workflow (ONT). The additional DNA samples included in the pooled library were a diverse assemblage of bacterial and eukaryotic DNA samples provided by attendees during the PoreCamp Workshop 2015 at the University of Birmingham. The additional pooled library samples are being prepared for separate publication. Details on the PoreCamp Workshop and associated publications can be found at [http://porecamp.github.io/](http://porecamp.github.io/). MinION reads were deposited in the European Nucleotide Archive under study accession PRJEB14152.

**Illumina library construction and sequencing**

An overnight culture was grown on TSB agar from a 15% glycerol stock maintained at ~80 °C. An aliquot of the culture was added to tubes containing DNA beads and library preparation was carried out by MicrobesNG, University of Birmingham ([http://microbesng.uk/](http://microbesng.uk/)). A single 250-bp paired end library was constructed and sequenced on both MiSeq and HiSeq Illumina platforms. The reads from both sequencing runs were combined before downstream analysis. The sequenced strain is stored in the MicrobesNG indexed repository as strain 2998-174. Reads were deposited in the European Nucleotide Archive under study accession PRJEB14152.

**Assembly, annotation, and analysis**

The full informatics analysis and associated data are available as a step-by-step walk-through at [https://github.com/SionBayliss/MHO_analysis](https://github.com/SionBayliss/MHO_analysis). Illumina reads were trimmed using Trimmomatic-0.33 [6]. Reads were trimmed to a minimum read quality of Q15. Reads <30 bp in length were excluded and sequencing adapters were removed. MinION(TM) 2D reads were filtered into pass and fail reads by the Metrichore basecaller; hereafter, these two categories of reads will be referred to as “2D pass” and “2D fail” reads, following the terminology adopted by the manufacturer and used in Karlsson et al. and Ip et al. [4, 7]. These are equivalent to the “high quality” and “low quality” read groups from Oikonomopoulous et al. [8]. MinION(TM) 1D reads were not used for this analysis. The 2D fail reads, those which did not pass the basecaller quality threshold, were demultiplexed using an in-house script (FilterBarcodes.pl). The twelve 40-bp barcodes used for library construction were compared in a moving 40-bp window to the sequence in the first and last 150 bp of each read. The barcode requiring the fewest insertions, deletions, or substitutions to be permuted into a sequence in the beginning or end of a read, with a maximum cut-off of 14 permutations, was considered a match. Each read could be assigned to only one individual sample; in the case of a tie the reads were discarded. Sequence preceding or following the presence of a barcode at the beginning or end of a read, respectively, were trimmed as adapter sequence. After quality trimming, 439,480 paired short reads, 1324 2D pass reads, and 1499 demultiplexed 2D fail reads (2823 total) nanopore long reads were passed as input files to SPAdes v3.6.1 using the –nanopore, –cov-cutoff 5, and –careful –options [3]. nanopore long reads were deposited in the European Nucleotide Archive under study accession PRJEB14152.

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Table 1. Table summarizing the BLASR analysis of semultiplexed 2D pass and fail nanopore long reads assigned to sample MHO_001. Reads were aligned to the assembled MHO_001 reference genome using BLASR with default parameters. Only the alignment with the highest percentage similarity was considered for each read. The average alignment length was calculated from the length of the top BLASR alignment relative to the length of the input read.

| Pass | Fail |
|------|------|
| # Reads | 1324 | 1499 |
| # BLASR hits (% # reads) | 1320 (99.70) | 1292 (86.19) |
| Mean alignment length (%) | 96.79 | 92.90 |
| Mean similarity (%) | 85.87 | 77.76 |
| # Hits <75% read length (%) | 11 (0.83) | 93 (7.20) |
| # Hits ≥75% read length (%) | 1309 (99.17) | 1199 (92.80) |

Results and discussion

A hybrid assembly using a low coverage of MinION(TM) reads (6–8×) combined with moderate coverage Illumina reads (~50×) was used to generate a complete genome. The assembly resolved regions of the genome that were problematic for short read assembly alone, such as chromosomal rRNA operons. The generation of a complete genome from only ~5% of the possible current yield of a MinION(TM) run using a multiplexed library should represent a cost-effective means to complete multiple genomes during a single MinION(TM) sequencing run, although the approach also requires matching short read Illumina data. Larger or more complex bacterial genomes may require higher coverage read data alongside additional bioinformatics analyses to generate comparably polished, complete genomes.

By demultiplexing the 2D fail reads, we were able to double the number of nanopore reads for assembly from 1324 to 2823 reads. The nanopore reads were aligned to the complete MHO_001 genome using BLASR (Fig. 1, Table 1). 1320/1324 (99.7%) 2D pass reads demultiplexed by Metrichor aligned to the assembly with an average percentage similarity of 85.9% and a mean alignment length of 96.8% of the input read. 1292/1499 (99.7%) 2D fail reads demultiplexed by in-house scripts aligned to the assembly with an average percentage similarity of 77.76% and a mean alignment length of 92.9%. The fail reads in which we failed to find a barcode contained 722/9501 (7.6%) reads that aligned to the MHO_001 genome. In summary, a considerable amount of useful information was contained within the demultiplexed 2D fail reads without which we would have been unable...
to produce a complete genome. We can conclude that we were able to correctly identify the ONT barcodes in ~85% of the 2D fail reads used for assembly.

The chromosome showed minor differences to the USA300 reference genome USA300_FPR3757 including 155 SNP differences and the loss and gain of mobile genetic elements (Fig. 2). To provide an independent confirmation of the 155 SNP differences identified by MAUVE between aligned regions of MHO_001 and USA300_FPR3757, the short reads were mapped to USA300_FPR3757 and variants were called using strict parameters. Of the 155 MAUVE SNPs, 41 (26.5%) were present in repeat regions and excluded from the comparison. Of the remaining 114 SNPs, 111 (97.4%) were supported by short read mapping to USA300_FPR3757. The remaining 3 SNPs (2.6%) were unsupported. No indels were identified by short read mapping to MHO_001 by either GATK/samtools or pindel. In summary, of the 114 SNPs identified by MAUVE that could be robustly investigated by short read mapping, 111 (97.4%) were confirmed using low error rate short reads. Furthermore, the long and short read coverage support at the edge of each of the large structural variants in MHO_001 was 8 to 10x for nanopore reads, with the exception of the 3’ edge of the transposed 13,356-bp insertion sequence, which had a read coverage of 3x compared to the genomic average of 6.8x coverage. The edge of each structural variant was supported by ~25 short reads.

There was minor sequence dissimilarity, including a small deletion, in ribosomal RNA operons. This could either reflect evolutionary changes in these highly conserved sequences or minor misassembly; these regions are typically difficult to assemble. MHO_001 lacked Staphylococcal pathogenicity island 5 (SAPIS), a 13,960-bp exotoxin encoding transposon observed at position 881,852 in the reference. MHO_001 also lacked the prophage phiSA3USA, which harbours the important virulence factor staphylokinase. As the integration site of this phage (the hlb gene) is intact, it is possible that MHO_001 has never acquired this phage. MHO_001 contained a 42,297-bp tyrosine recombinase bacteriophage integrated at position 867,385. This bacteriophage contained a beta-lactamase and a putative Panton-Valentine-like leucocidin and several hypothetical genes. The position of an insertion sequence containing ftsK translocase differs between MHO_001 and the reference genome, consistent with a translocation event (USA300_FPR3757:1,630,720-1,644,076 to MHO_001:679,522-692,877). The location of this element in MHO_001 truncates a gene of unknown function. There is a short 1282-bp deletion of a gene encoding an exotoxin at position 448,767 in MHO_001. MHO_001 also has an extended tRNA cluster at 554,826 containing 7 additional tRNAs (val, thr, lys, gly, leu, arg, pro) relative to USA300_FPR3757, representing either gene expansion or reduction of this gene cluster in USA300_FPR3757.

A BLAST search revealed that the two smaller contigs were identical to previously sequenced plasmids associated with USA300 [20]. The larger of the plasmids contained an N-type replication system (repA) with a pSK1 type plasmid partitioning system. It encoded a host of resistance genotypes including macrolide (mac), erythromycin (ery), cadmium (cadX and cadD), streptomycin (sta), aminoglycoside (aad), neomycin and kanamycin (aph) resistance genes. In addition to this, the plasmid contained a Tn552-like transposon containing a beta-lactam resistance (bin, blaI, blalR1, blaZ) operon and a sin recombinase. The smaller of the two plasmids encoded three hypothetical proteins and a replicase. Both plasmids have been previously observed to occur concurrently in the same host.

There was a discrepancy observed between the coverage of short and long reads of plasmidic and chromosomal contigs (Fig. 2, top and middle panels). The average chromosomal coverage was 49.6x (7.0 SD) with short read data and 6.8x (2.6 SD) with nanopore reads. The average short read coverage of plasmids A and B was 78.4 (8.9 SD) and 730.2 (85.4 SD), respectively. This represents a coverage increase of 1.5- and 150-fold relative to the chromosome. The opposite trend was observed with long reads; plasmids A and B had an average coverage of 4.0 (2.0 SD) and 2.9 (1.7 SD), respectively, which represents a 40% and 60% decrease in coverage relative of the chromosome. In addition to this the smaller of the two plasmids was only intermittently covered by nanopore reads. The reduced number of mappable nanopore reads was likely due to the fragment size
selection steps during library preparation. The inherent problems of aligning long error-prone reads to reference sequences may also have contributed. It is thus important that future studies attempting to reconstruct plasmids or studying plasmid diversity consider the impact of size selection on downstream analysis or to prepare multiple DNA libraries with differential size selection as previously discussed by Koren and Phillippy [21]. However, the clear benefit of hybrid sequencing is that it allows for the generation of larger assemblies with less uncertainties than by using a single sequencing technology preferentially over another.

Additional files
Supplementary Table 1. Table summarizing the BLASR analysis of demultiplexed nontarget sample 2D nanopore long reads and 2D fail reads in which no barcode was detected. Reads were aligned to the assembled MHO_001 reference genome using BLASR with default parameters. Only the alignment with the highest percentage similarity was considered for each read. The average alignment length was calculated from the length of the top BLASR alignment relative to the length of the input read.

Supplementary Table 2. Spreadsheet summarizing the comparison between SNPs called by MAUVE alignment of assemblies created using long and short reads and SNPs called via mapping short reads to USA300_FPR3757.

Supplementary Figure 1. MAUVE alignment of the overlapping region included in the circularized single chromosomal contig aligned to USA300_FPR3757.

Supplementary Figure 2. MAUVE alignment of the overlapping region not included in the circularized single chromosomal contig aligned to USA300_FPR3757.

Supplementary Figure 3. CLUSTAL visualization of the MUSCLE alignment between the two overlapping regions at the edge of the single chromosomal contig.

Supplementary Figure 4. Tablet visualization of the nanopore long reads that span the overlapping regions at the edge of the circularized single chromosomal contig.

Supplementary Analysis. PCR and Sanger sequencing analysis of large structural variants SAPI5 and tRNA expansion.

Competing interests
No competing interests.

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Author contributions
SB and VH were responsible for the conception and design of study and data acquisition. SB performed the analysis and interpretation of data and manuscript drafting. MY carried out the supplementary analysis. HAT and EF revised the manuscript critically for important intellectual content. SB and EF approved the version of the manuscript to be published.

Data availability
The dataset supporting the conclusions of this article is available in the European Nucleotide Archive repository under project number PRJEB14152. Further supporting data is also available from the GigaScience GigaDB repository [22].

Availability and requirements
- Project name: MHO_001 hybrid read assembly and analysis
- Project home page: https://github.com/SionBayliss/MHO_analysis
- Operating system: Unix
- Programming language: R, perl
- Other requirements: Dependencies include Samtools (> = 1.18), Trimmomatic, SPAdes v3.6.1, BWA (0.7.5a-r405), BioPerl, MAUVE, BLASR, prokka, Tablet/Artemis
- License: GNU GPL v3

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