Shallow MinION sequencing to assist *de novo* assembly of the *Streptococcus agalactiae* genome

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26 **Abstract**

27 Despite the reduced read length, the so-called Next-Generation Sequencing (NGS) of
 28 second-generation has allowed rapid and complete genome characterization of many
 29 species. MinION (Oxford Nanopore Technologies), a portable third-generation NGS
 30 device, enables sequencing of long DNA fragments at low cost. Here we used a low-
 31 coverage MinION sequencing in combination with short-read NGS to improve genome
 32 assembly. We tested this possibility by using MinION R9.0 with Rapid 1D kit and
 33 MiSeq with >300X paired-end 300 bp reads (Illumina, Inc.) for the genome assembly of
 34 a *Streptococcus agalactiae* clinical isolate (2.2 Mb). With as few as 1,171 MinION
 35 reads that covered the genome at 2.4X (the longest read being 186 Kb long), the hybrid
 36 assembly combining MinION and Illumina reads increased the N50 by 4.9-fold
 37 compared to the assembly using Illumina data alone. Almost 50% of the genome was
 38 represented into a single contig (1.02 Mb). Besides, this allowed the full reconstruction
 39 of mobile elements, including a plasmid, and improved gene annotation. Taken
 40 together, our results support that shallow MinION sequencing combined with high-
 41 throughput second-generation NGS constitutes a cost-efficient strategy for the assembly
 42 of whole genomes.

43 **Key words:** Hybrid assembly; Nanopore; artefactual reads

44

45 **Background**

46 Next-Generation Sequencing (NGS) technologies have allowed sequencing and
 47 analysis Group B *Streptococcus* (GBS) genomes from different environments and
 48 organisms[1–6]. The presence of prophages and mobile elements in GBS was
 49 associated with the bacterial adaptation and ability to cause infections, likely enhancing
 50 their diversity and spread[7–10]. In fact, the estimates suggest that roughly 50% of
 51 *Streptococcus agalactiae* genome is grouped into islands of pathogenicity, where
 52 virulence genes and mobile elements are present[11]. *S. agalactiae* genome shows a
 53 complex organization and rearrangements, where the presence of a conserved backbone
 54 and 69 variable regions have been observed[12]. These observations justify the
 55 necessity of using *de novo* assembled genomes for better comparative bacterial studies
 56 and adequate tracking of mobile elements. However, the assembly of bacterial genomes
 57 based on short-read NGS technologies (so called second-generation) usually produce
 58 discontinuous sequences[13,14], often requiring the assistance from algorithms,
 59 alternative library approaches or sequencing technologies to provide sequence
 60 continuity.

61

62 Third-generation NGS technologies allow obtaining longer DNA reads from
 63 PCR-free DNA libraries in real time, constituting a solution for resolving repeats[15]
 64 and obtaining complete assembled genomes. In this context, the portability and reduced
 65 costs of sequencing with the MinION device (Oxford Nanopore Technologies) has
 66 facilitated the adoption of this technology in many distinct applications[16]. Besides its
 67 utility for the analysis of structural variants[17,18], SNP determination[19], cytosine
 68 methylation[20], transcriptomics[21], and in-field experiments in extreme
 69 environments[22], MinION has widely used to sequence small whole genomes from

70 virus (10.8 kb)[23], mitochondrial DNA (16 kb)[24], and bacteria (4.6 Mb)[25],
 71 allowing their assembly into single contigs when depth of coverages were above 20-
 72 40X[26]. Nevertheless, this technology continues to be challenged by its high error rate
 73 (>10%)[27], particularly in homopolymeric regions[28], and the generation of reads that
 74 fail to align against the target[29,30]. Because of this, hybrid assemblies leveraging the
 75 per-base quality of high-coverage (>200X) second-generation data with the bridging
 76 capacity of >10X depth-of-coverage third-generation data is nowadays perceived as the
 77 optimal choice for obtaining accurate assemblies[24,27,31–35]. Given the large
 78 variability in sequence throughput offered by this technology well below product
 79 specifications, here we tested the benefit of shallow MinION sequencing data in
 80 assisting the hybrid genome assembly of a *S. agalactiae* clinical isolate (genome size of
 81 2.2 Mb).

83 **Methods**

84 Bacterial isolate, growing conditions and validation

85 We have previously examined the IMESag-rpsI mobile element distribution in
 86 240 whole genomes of Group B *Streptococcus* isolates. The *S. agalactiae* HRC strain
 87 was subjected to a deeper examination to assist the characterization of the mobile
 88 element in that study[36]. *S. agalactiae* HRC is a serotype V representative strain
 89 belonging to the tetracycline-resistance CC1 lineage Tn916-1[37], which was isolated in
 90 2009 from a 60-year-old woman with abdominal sepsis at the Hospital Ramón y Cajal
 91 (Madrid, Spain)[36].

93 The bacterial isolate was cultured in TSB (Tryptic Soy Broth, Becton
 94 Dickinson) liquid medium at 37°C for 24 h. DNA extraction was performed with the

95 GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) and was quantified on a Qubit
96 3.0 fluorometer using the dsDNA BR assay kit (Thermo Fisher Scientific). Its identity
97 was first validated by 16S rRNA gene sequencing as follows. DNA was amplified by
98 PCR with 30 cycles of 95°C for 20 s, 50°C for 30 s, and 72°C for 15 s, using the
99 HotStarTaq master mix kit (QIAGEN), and the primers 1391R (5'-
100 GACGGGCGGTGWGTRCA-3') and 27F (5'-AGRGTTYGATYMTGGCTCAG-3').
101 Amplified material was purified with ExoSAP-IT (Thermo Fisher Scientific), subject to
102 Sanger sequencing with BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher
103 Scientific), and products purified with DyeEx 2.0 Spin kit (QIAGEN), following the
104 manufacturer's recommendations. Sequencing products were resolved and basecalled in
105 a 3500 Genetic Analyzer (Thermo Fisher Scientific). A simple BLAST[38] search on
106 the sequences obtained verified that the bacterial isolate had a 99% identity to *S.*
107 *agalactiae*.

108

109 Illumina sequencing

110 DNA from *S. agalactiae* HRC (1 ng) was used to generate sequencing libraries
111 with the Nextera XT kit (Illumina, Inc.) following the manufacturer's recommendations.
112 A MiSeq instrument and a MiSeq Reagent kit V3 (Illumina, Inc.) was used for 300 base
113 paired-end sequencing along with a spike-in of 20% of the PhiX Control v3 (Illumina,
114 Inc.). Library construction and the sequencing experiment were performed at the
115 Instituto Tecnológico y de Energías Renovables (ITER).

116

117 MinION sequencing

118 Rapid Sequencing kit (SQK-RAD001) (Oxford Nanopore Technologies) was
119 used for library preparation starting from 200 ng of *S. agalactiae* HRC purified DNA.

120 Sequencing with MinION used SpotOn Flow Cell Mk I R9 Version (Oxford Nanopore
121 Technologies) and followed manufacturer's recommendations, except that the
122 experiment was left running only for 22 h.

123

124 Bioinformatics and statistical procedures

125 MiSeq Reporter Software v1.18.54 (Illumina, Inc.) was used to convert
126 intensities to basecalls and generate the FASTQ file. MinION basecalling was
127 performed with MinKNOW GUI 1.1.21 software (Oxford Nanopore Technologies) and
128 all records were processed with poRe v0.21[39] to extract the obtained reads in FASTQ
129 file format. Unicycler v0.4.1[40] was used with default parameters for hybrid *de novo*
130 assembly of *S. agalactiae* HRC genome, combining Illumina and Nanopore datasets,
131 which involved multiple rounds of short-read polishing. As a reference for comparisons,
132 Unicycler was also used to assemble the Illumina data alone. Assembly comparisons
133 were assessed with QUAST v4.0[41] using the *S. agalactiae* SS1 genome
134 (NZ_CP010867.1) as the reference. The assembled contigs were visualized with
135 Bandage v0.8.1[42]. Annotation of genes and elements in the contigs was done with
136 IonGAP[43]. Mauve v2.4.0[44] was used to identify the insertion sites of the *S.*
137 *agalactiae* HRC mobile elements in the assembly against the reference
138 (NZ_CP010867.1).

139

140 In order to further explore raw MinION reads, per-read GC content distribution
141 was first assessed to identify outliers (>3 SD from the mean). The existence of
142 artefactual reads in the output[29] was evaluated through comparisons against the
143 reconstructed *S. agalactiae* HRC genome, including the two characteristic elements
144 identified. BLAST v2.6.0[38] alignments were used to isolate unaligned artefactual

reads. Bio.SeqUtils library of Biopython release 1.70 was then used to assess their differences in length (bp), GC content (GC%) and mean quality (Q) score against the reads aligning to the *S. agalactiae* HRC assembled genome. A non-parametric Mann-Whitney U-test was used to assess the significance in the comparisons. To test the possibility that particular pores were generating more artefacts than expected, as has been suggested elsewhere[29], unaligned reads were mapped back to the 512 nanopores of the Flow Cell and significance was tested assuming a Poisson distribution. Finally, to evaluate their impact in *de novo* assembly of *S. agalactiae* HRC genome, we then excluded all unaligned reads from the dataset, and used Unicycler under the same conditions to reassess the assemblies.

155

156 **Results**

157 Summary of sequencing results

Sequencing with Illumina resulted in 2,975,356 paired-end reads of 300 bp with Phred>30, which translates into 354X depth-of-coverage of *S. agalactiae* genome. In comparison, the MinION run proceeded with a total of just 276 active pores, providing 1,171 reads with a mean length of 4.5 kb and a Q score average of 10.8, and ranging from 3.8 to 25.4. While the largest proportion of reads accumulated in the 1.86-9.0 kb range (the N50 of the output was 9.2 kb) the output included reads as long as 186 kb (which represents almost 10% of the *S. agalactiae* HRC genome). The total throughput (5.2 Mb) theoretically translates into a 2.4X depth-of-coverage of the bacterial genome, which is well below product specifications, despite all initial quality controls of the platform were passed. Finally, the per-read GC content average of reads was 44.3% (ranging from 10.3% to 95.9%), noticeably larger than that of the reference sequence (35.5%; NZ_CP010867.1).

170 Assembly improvements by using MinION data

171 The Illumina data assembly generated 23 contigs (>500 bp) with a N50 of
 172 147,211 bp, and the largest contig being 525,288 bp long. The length of the assembly
 173 from the Illumina data alone was very close to the *S. agalactiae* reference, accounting
 174 for a genome size of 2,144,732 bp, a GC content of 35.3%, and an aligning length
 175 against the reference of 2,049,610 bp (97.9% of consensus identity) (Table 1). In
 176 comparison, although the hybrid assembly including also combining the Illumina and
 177 MinION reads did not allow to obtain a fully resolved bacterial genome, mostly because
 178 of a major unresolved structure containing repetitive elements, rRNA operon copies and
 179 tRNAs (Fig 1), it reduced the number of contigs to less than a half (8 with >500 bp),
 180 having a N50 of 714,293 bp and the largest contig being 1,018,988 bp long. The hybrid
 181 assembly generated a total length of 2,159,060 bp, with 2,062,825 bp aligning against
 182 the reference (98.5% of consensus identity) (Table 1). Both assemblies also evidenced a
 183 small plasmid (2,491 bp) as an independent replicon, which perfectly aligned with the
 184 *Streptococcus oralis tigurinus* 2426 plasmid pST2426 (ASXA01000016.1), along with
 185 two other mobile elements detected in previously studies[36] (Fig 1). In terms of gene
 186 annotation, the hybrid assembly also outperformed the Illumina only assembly (2,153
 187 vs. 2,135 genes, respectively) when compared to the reference (2,195 genes) (Table 1).
 188 Taken together, the small amount of reads provided by MinION (0.04% of total reads
 189 obtained for the bacterial isolate) allowed to increase the N50 of the genome assembly
 190 by 4.9-fold, to cover almost 50% of the bacterial genome in a single contig, and to
 191 improve the annotation of predicted genes in the assembly.

192

193 Characterization of unaligned MinION reads and impact on the assembly

194 A closer inspection of the MinION run records evidenced the existence of reads
195 with large regions of limited sequence diversity (end to end) that were largely
196 compatible with artifacts. Just by exploring the GC content of the MinION output, we
197 detected 27 reads with large deviations (>3 SD from the mean) corresponding to GC
198 contents below 7.2% or above 77.8%. The reads involved had a mean size of 716 bp,
199 but were as large as 4.2 kb. However, in order to better identify and characterize the
200 reads that were most likely artefactual, we used BLAST at 85% identity threshold to
201 align all reads against the assembled genome. This allowed to identify 68 unaligned
202 reads with a mean size of 2.0 kb (but as large as 21.6 kb) and a mean Q score of 6.7 (but
203 as high as 22.9) (Fig 2). Only three of these unaligned reads (0.25% of total) aligned to
204 sequences from other species or synthetic constructs according to a BLAST search
205 (Table S1), most probably corresponding to experimental contaminants. Surprisingly,
206 MinKNOW software only classified 20 of the 68 as failed based on internal
207 manufacturer's specifications. Unaligned reads differed significantly from reads that
208 aligned for the GC content (74.2 vs. 42.5%; $p=2.5e^{-37}$), Q score (6.7 vs. 11.1; $p=2.2e^{-21}$), and mean read length (2.0 vs. 4.6 kb; $p=1.3e^{-11}$) (Fig 2). Strikingly, from the 276
209 active pores of the run, unaligned reads were obtained from 45, and two pores in
210 particular experienced a significant accumulation of them (28.9%, $p<0.019$) (S1 Fig).
211 Finally, a re-assessment of the hybrid assembly evidenced identical results with or
212 without excluding unaligned reads, supporting that Unicycler was robust to their
213 presence in the input. For simplicity, these results are not shown.

215

216 Discussion

217 Here we demonstrate that the use of very low amount of MinION reads
218 combined with short-read sequencing data has the capacity to increase the continuity of

assembly contigs and plasmid isolation without manual intervention, having the potential to fully resolve bacterial genomes. This was illustrated with the assembly of *S. agalactiae* HRC genome based on as few as 2.4X MinION depth-of-coverage data, paving the way for obtaining cost-effective continuum whole-genome sequences as the nanopore technology throughput increases. While other species may have alternative requirements, to the best of our knowledge, this is the first study assessing the benefits of using MinION reads to assist the complete assembly of microbial genomes with coverages well below 10X (usually assisted with >14X)[14,23,33,34]. Although we were unable to fully resolve the assembly, our results are quite reassuring considering the small number of MinION reads used in the experiment (0.04% of total reads), allowing to obtain almost the 50% of the bacterial genome in a single contig. Besides, we were able to detect and fully reconstruct a plasmid, previously attained only with higher depth-of-coverage and longer MinION reads[45].

232

High quality noise reads generated by MinION have been described recently in the literature[29], consisting on reads that did not map to the target, lab contaminants, or spike-in sequences. Many of those appeared to be artefacts of the technology that were associated with specific active pores[29], to which some authors have previously attributed an insufficient read quality[30]. In this study, MinION generated around 5% of reads of variable quality that did not map to the target. We found that despite a few of them had partial identity with bacterial sequences that may indicate minor experimental contaminations as has been shown by others[15,46], most of them consisted in low-complexity sequences from end to end. We demonstrate that a number of them were generated from specific active pores during our experiment. Based on these evidences, we speculate that most of these reads are artefacts produced during the sequencing

244 process and that they are most likely independent from the basecaller being used. In this
245 study, we demonstrate that they did not negatively affect the hybrid assembly process in
246 the conditions assessed. However, their impact in other projected applications (such as
247 shotgun metagenomics or whole-genome sequencing of eukaryotes) or algorithms other
248 than Unicycler remain unknown. Solutions to reduce or identify and filter out these
249 reads will need to be implemented.

250

251 With sufficient MinION yield providing enough depth-of-coverage, the standard
252 analysis is based on long-read and error-tolerant assemblers such as Canu[47]. Because
253 of the high error rate of this technology at the moment (10-20%)[27,46,48], and the
254 problems resolving the homopolymers[28], the assembly process from MinION data
255 alone typically necessitates of a number of error correction steps to produce high quality
256 genome sequences[49]. Most importantly, this assembly process is computationally
257 intensive[49,50]. Theoretically, MinION can produce many thousands of reads and a
258 few dozen Gb per run[49]. However, in practice MinION outputs are highly variable
259 translating sometimes in a few thousand reads (3,400 in Benitez-Paez et al[51]) and Mb
260 scale outputs. In our hands, regular experiments are roughly in the scale of 100 Mb per
261 run. This is because the number of active pores changes during the experiment (between
262 105 and 426 in Greninger et al[46]), below theoretical maximum in distinct runs, and
263 the effective number of useful reads represents a fraction of the output[15,46]. Some
264 studies have reported that a large proportion of the output (>50%) have failed to align
265 against the target[30,52]. Besides, sudden crashes of the MinION software
266 controller[14] or the computer connection failures[53] have been reported during a
267 sequencing experiment. In addition, unofficial information provided by users in The
268 Nanopore Community report extremely low occupancy of pores (<10%) during the

sequencing experiments with Rapid 1D kits, which have been suggested to be related to the kit production itself. These numbers are comparable to our observations in this study and all these issues have obvious consequences in the throughput of a MinION experiment. Therefore, although not the standard, the limited yield obtained by the MinION sequencing in this study is not uncommon. In this context, a hybrid assembly with a very shallow coverage of MinION data constitutes an advantage over the approaches based on MinION data alone for obtaining cost-effective high-quality continuous bacterial genomes. Besides being computationally faster, this hybrid option reduces the burden of generating the thousands of long reads that are necessary to obtain high quality bacterial genome assemblies with MinION data alone (>20X)[26].

279

280 **Conclusions**

We demonstrate that the combination of a small proportion of long reads with high-coverage short-read data constitutes a promising strategy to generate high quality, highly contiguous assemblies, here allowing to nearly complete the *S. agalactiae* HRC genome. Besides, we detected MinION reads consisting of low-complexity sequences compatible with artefacts of the technology but that did not affect the assembly.

286

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295

296 **Authors' contributions**

297 T.H.B.: performed experiments, data analysis, interpretation, and manuscript drafting;
298 H.R.P., A.D.U., R.G.M., J.L.S.: performed experiments and data analysis; F.L.D.:
299 performed experiments, study conception, interpretation, revising the manuscript
300 critically; C.F.: Study conception and design, data analysis, interpretation, revising the
301 manuscript critically and conceived the project.

302 The authors declare that there are no competing interests.

303

304 **Data availability**

305 All raw reads from MinION and Illumina are available from the SRA database
306 (accession number(s) SRP141332, SRP141319).

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308

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462 **Fig 1.** Schematic representation of the *S. agalactiae* HRC hybrid assembly. The
 463 flanking sequences of the two characteristics elements of this isolate are indicated at the
 464 bottom: 1) A conjugative element attached to the Tn916 transposon located in the
 465 largest contig (1,018,988 bp); 2) A prophage located in the third largest contig (224,154
 466 bp). Most copies of the rRNA (16S, 23S, 5S) and tRNA genes were positioned within
 467 the largest unresolved assembly structure.

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470 **Fig 2.** Distribution of MinION reads by quality (Q) scores and length (bp). Aligned
 471 reads are represented in grey, whereas the 68 unaligned reads are overlaid in black. For
 472 simplicity, the plot was capped to represent reads <60 kb length and Q scores <15.

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Table 1. Summary of the hybrid and Illumina-only assemblies of *S. agalactiae* HRC genome.

Parameters	Illumina	Hybrid
Total length assembled (bp)	2,144,732	2,159,060
Contigs (≥ 500 bp)	23	8
Largest contig (bp)	525,288	1,018,988
N50	147,211	714,293
GC content (%)	35.3	35.4
Genome fraction (%)*	97.9	98.5
Total aligned length (bp)*	2,049,610	2,062,825
Largest alignment (bp)*	524,861	1,018,010
Mismatches (#)*	151	193
Indels (#)*	33	77
Genes**	2135	2153
Coding DNA sequences**	2104	2117
tRNAs**	31	36

*Against the reference sequence: NZ_CP010867.1.

**For comparison, IonGAP predicted 2,195 genes, 2,115 coding DNA sequences, and 80 tRNAs in the reference.

1 ...CAATGACACATCATGTCGAGACGGTAGCACTTTTGTCCAA...TGGACAATGATGAACAAGTTGAGTGTGTTGCTCTGCTTGT...

2 ...GCATTACACTTTTAGAAATCAAGGATAGTAAATTTCTTT...TAATTTTAGTAACCTTGATTGTGGTTAGTGTAGAGCCTT...