Complete genome sequence and comparative genomics of the fish pathogen *Streptococcus iniae* QMA0248

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Abstract

Fish mortality caused by *Streptococcus iniae* is a major economic problem in fish aquaculture in warm and temperate regions globally. There is also risk of zoonotic infection by *S. iniae* through handling of contaminated fish. In this study, we present the complete genome sequence of *S. iniae* strain QMA0248, isolated from farmed barramundi in South Australia. The 2.12 Mb genome of *S. iniae* QMA0248 carries a 32 Kb prophage, a 12 Kb genomic island, and 92 discrete insertion sequence (IS) elements. These include 9 novel IS types that belong mostly to the IS*3* family. Comparative and phylogenetic analysis between *S. iniae* QMA0248 and publicly available complete *S. iniae* genomes revealed discrepancies that are likely due to misassembly in the genomes of isolates ISET0901 and ISNO. We also determined by long-range PCR that a tandem duplication of an rRNA region in the PacBio assembly of QMA0248 was an assembly error. We suggest that a similar rRNA duplication in the PacBio genome of *S. iniae* 89353 may also be a misassembly. Our study not only highlights assembly problems in existing genomes, but provides a high quality reference genome for *S. iniae* QMA0248, including manually curated mobile genetic elements, that will assist future *S. iniae* comparative genomic and evolutionary studies.

Introduction

Streptococcus iniae is a fish pathogen that causes mortality in a wide range of fish species in wild and farmed, marine and freshwater environments, resulting in large economic losses to aquaculture (1, 2). *S. iniae* is also considered an opportunistic human pathogen, causing sporadic infections mostly in the elderly who have more than one underlying health condition such as diabetes mellitus, or

50 chronic rheumatic heart disease (3, 4). *S. iniae* pathogenesis is imparted through
51 a repertoire of virulence factors (VF) including surface proteins, secreted toxins,
52 and capsular polysaccharide (CPS) (4). VFs can be acquired through lateral gene
53 transfer (LGT) of mobile genetic elements (MGE) such as composite transposons,
54 genomic islands (GI) or prophages.
55
56 MGEs are a means by which bacterial pathogens acquire traits that help adapt to
57 changing conditions including vaccination, antibiotics, a new host or environment
58 (5, 6). Indeed, they are considered the main drivers of gene flux in bacteria,
59 contributing to diversity within species (7). Insertion sequence (IS) elements, for
60 instance, are small MGEs (0.7-3.5 Kb) that have an important role in evolution and
61 genome plasticity. IS insertion within bacterial chromosomes or plasmids can
62 result in genetic modifications through insertional inactivation of genes or up-
63 regulation of adjacent intact genes through outward-facing promoter sequences
64 carried by some IS (8, 9). In some cases pairs of IS can mobilize intervening
65 sequence as a composite transposon (8). The mobility of IS elements leads to their
66 expansion or loss within bacterial lineages. Expansion is associated with
67 accumulation of pseudogenes, which is considered an early stage in genome
68 reduction as a mechanism for adaptation (9). Accordingly, to obtain the complete
69 evolutionary picture within bacterial species it is important to study the
70 distribution and abundance of IS elements.
71
72 As yet there is no study that focuses on the diversity and distribution of MGEs in
73 *S. iniae* genomes. In fact, only four complete *S. iniae* genomes were deposited in
74 Genbank at the commencement of the present study, none of which had

75 comprehensive annotations for MGEs: SF1, YSFST01-82, ISET0901, and ISNO. *S.*
76 *iniae* SF1 (accession: CP005941) was cultured from moribund flounder from a fish
77 farm that experienced an epidemic in North China (10). YSFST01-82 (accession:
78 CP010783) is an isolate from a diseased Olive flounder from South Korea (2012)
79 (11). ISET0901 (accession: CP007586) is reported to be a highly virulent strain,
80 which was isolated from diseased Nile tilapia during an outbreak in Israel (2005)
81 (12). The strain ISNO (accession: CP007587) was obtained through selection for
82 resistance of *S. iniae* ISET0901 to novobiocin (13).
83
84 There are currently 25 different IS families in the ISFinder database, but only one
85 IS described for *S. iniae* (IS*Stin1* of the family IS*256*). As is typical for complete
86 bacterial genomes, the annotation of the four complete *S. iniae* genomes available
87 at Genbank are limited to some of the transposase genes associated with IS
88 elements without definition of the IS boundaries. Indeed, the difficulty in
89 annotating IS elements and the lack of reliable automated annotation methods
90 means that only a small subset of complete genomes have accurate IS annotations.
91 Small partial IS elements are often disregarded although they reflect valuable
92 insights on ancestral recombination events. With the increasing availability of
93 long-read sequencing there is a need for a high quality, well-characterized *S. iniae*
94 reference genome that better enables the impact of IS elements on evolution and
95 diversity to be determined.
96
97 In this study, we have completely characterized the genome of *S. iniae* QMA0248.
98 Manual curation of annotations for IS, genomic islands, prophages, and CRISPR
99 was carried out along with a comparison with the four publicly available complete

100 genomes from NCBI (SF1, YSFST01-82, ISET0901, and ISNO). Comparative and
101 phylogenetic analyses revealed discrepancies between the MGE content
102 indicating likely misassembly in the genomes ISNO, and ISET0901. The complete
103 genome of QMA0248 will provide an important scaffold for future phylogenomic
104 studies of the *S. iniae* species.
105

106 **Results and Discussion**

107 **Genomic features of *S. iniae* QMA0248**

108 The genome of *S. iniae* QMA0248 consists of a single circular chromosome of
109 2,116,570 bp with no plasmids (Figure 1 and Table 1). The QMA0248
110 chromosome has an average GC content of 36.8 %, consistent with the other four
111 *S. iniae* genomes (SF1, YSFST01-82, ISET0901, and ISNO) and in common with
112 several other *Streptococcus* spp. such as *S. agalactiae* (35.6%) (14), *S. pneumoniae*
113 (39.7%) (15), and *S. pyogenes* (38.5%) (16). Of note, there is a high degree of
114 strand bias in the genome of *S. iniae* QMA0248 where genes are preferentially
115 oriented in the leading strand, which is typical for Firmicutes (Figure 1). Only
116 seven percent of QMA0248 protein-coding genes are annotated as hypothetical
117 proteins compared with a quarter in SF1 and 11-14% in the other three published
118 genomes: YSFST01-82, ISET0901, and ISNO. There are 68 pseudogenes identified
119 in QMA0248 (GenBank assembly accession: CP022392.1), most due to
120 interruption by IS (Figure 1, Supplementary Table S1). This is approximately five
121 times greater than the number of pseudogenes predicted in SF1. Collectively,
122 these differences between the compared genomes QMA0248, SF1, YSFST01-82,
123 ISET0901, and ISNO are likely to reflect different approaches to annotation. The

124 other 25 pseudogenes are caused by in-frame stop codons or frame-shifts, all
125 supported by additional mapping of Illumina reads of the strain QMA0248 against
126 its PacBio assembly.

127

128 QMA0248 has a single CRISPR locus (Clustered regularly interspaced short
129 palindromic repeats) (Figure 1), which harbors a tandem array of 15 identical 36
130 bp repeats, separated by 14 distinct 30 bp spacers, which is about double the size
131 of the CRISPR array in SF1, ISET0901, and ISNO (Supplementary Figure S1).
132 Upstream of QMA0248 CRISPR are four Cas genes, *csn2*, *cas2*, *cas1*, and *cas9*, which
133 alongside the CRISPR locus provide adaptive immunity against foreign DNA (e.g.
134 phage and plasmids) (17).

135

136 QMA0248 harbors 58 tRNA genes and 15 rRNA loci, consisting of 5S, 16S, and 23S
137 genes, arranged in five loci. In contrast, there is one rRNA operon fewer in SF1,
138 ISET0901, and ISNO than in QMA0248 (Table 1). Furthermore, during the
139 preparation of this manuscript, a PacBio complete genome was published for the
140 strain *S. iniae* 89353 (accession: CP017952) which has an identical rRNA
141 arrangement to QMA0248 except that one rRNA locus encodes a ~7 Kb tandem
142 duplication (i.e. six loci in total) (18). Such intra-species variation in the number
143 of rRNA genes (and tRNA genes) is not uncommon in bacteria (including
144 streptococci) (19, 20), and prompted us to investigate further.

145

146 **Confirmation of rRNA assembly in *S. iniae* QMA0248 genome**

147 PacBio reads for QMA0248 were initially assembled into a large ~2 Mb contig
148 representing most of the chromosome of *S. iniae* QMA0248 and three contigs less
149 than 10 Kb in length. The short contigs appeared to be single read chimeras that
150 were discarded from the final assembly. However, the identification of the tandem
151 rRNA region in *S. iniae* 89353 prompted us to review the assembled short contigs.
152 One of these ~7 Kb discarded contigs encoded an rRNA region (5S, 16S, and 23S
153 genes in tandem with an intervening cluster of tRNA genes). Subsequent
154 reassembly and visualization of mapped raw reads indicated that the additional
155 rRNA contig could be placed in three of the five rRNA operon locations to form a
156 ~13 Kb putative tandem duplication of 5S, 16S and 23 rRNA genes as seen in the
157 89353 genome (18). Closer examination of the read pileup for the ~7 Kb rRNA
158 contig revealed that the tandem duplication was not well supported by
159 overlapping reads (Supplementary Figure S2) suggesting that it may be a chimeric
160 assembly of reads from more than one rRNA locus. To investigate the location of
161 the putative tandem rRNA duplication in *S. iniae* QMA0248 we carried out long-
162 range PCR across each of the five potential rRNA loci in the chromosome. PCR
163 revealed no tandem rRNA duplication in any locus (Supplementary Figure S3).
164 Never-the-less, we cannot rule out that during the culturing step, prior to DNA
165 extraction and genome sequencing, there existed a sub-population of QMA0248
166 cells with the tandem rRNA duplication. This would be consistent with finding
167 only a small number of reads spanning the tandem repeat. Similar doubt exists
168 over the tandem rRNA repeat in 89353, which should also be investigated at the
169 read-level.
170
171 **Characterization of large mobile genetic elements in *S. iniae* QMA0248**

172 Our investigation of the rRNA discrepancies in *S. iniae* SF1, ISET0901, and ISNO
173 also revealed differences in the mobile genetic element (MGE) content within the
174 available complete genomes. The chromosome of QMA0248 has a single ~12 Kb
175 genomic island (GI-Leu) inserted within the tRNA-Leu downstream of a large
176 number of consecutive ribosomal genes (Table 2). GI-Leu encodes an integrase at
177 its 5' end (QMA0248_0125), which is predicted to be responsible for the GI
178 insertion. A ~2.8 Kb region at the 5' end of the GI (87881–90708) is homologous
179 to the fish pathogen *S. parauberis* KCTC 11537, with 90% nucleotide sequence
180 identity, including part of the integrase, plasmid replication gene, and two
181 hypothetical proteins. The island encodes a collagen-binding surface protein (Cna,
182 B-type domain), which is a virulence factor with an LPxTG cell wall anchor motif,
183 a conserved Gram-positive *cocci* pentapeptide (21). An IS*30* family transposon
184 insertion truncates the collagen-binding domain encoded by *cna*, likely leading to
185 loss of functionality in QMA0248. This adhesin has been shown to play an
186 important pathogenic role in *Staphylococcus aureus* by facilitating bacterial cells
187 adherence to host collagen (22). Most of this GI appears to have been deleted in
188 the genomes of SF1, ISET0901, and ISNO only, along with the rRNA operon
189 upstream of it (Figure 3), which explains the difference with QMA0248 in total
190 number of rRNA and tRNA genes (Table 1).
191
192 The genome of *S. iniae* QMA0248 harbors a single ~32 Kb incomplete phage (Phi1)
193 (1991661–2023508), including a 5' integrase gene (QMA0248_1936), inserted
194 upstream of a tRNA-Cys gene (Table 2). A total of 44 genes encoding phage
195 proteins were identified, including genes involved in DNA replication such as DNA
196 polymerase III, tail morphogenesis, as well as host lysis such as holin and lysin, in

197 addition to 24 phage hypothetical proteins. More than half of the genes encoding
198 phage proteins carried by QMA0248 are homologous to proteins encoded by
199 temperate bacteriophage *Streptococcus* PH10 (56.8% according to PHAST) (23).
200 Furthermore, Phi1 in QMA0248 exhibits a remarkable nucleotide sequence
201 identity (99%) to a prophage encoded within the SF1 genome in the same locus,
202 whereas it is entirely absent in YSFST01-82, ISET0901, and ISNO (Figure 3).
203

204 **Characterization of *S. iniae* QMA0248 insertion sequences**

205 Insertion sequences (IS) were analyzed in the *S. iniae* QMA0248 genome using the
206 ISFinder database coupled with manual curation. The analysis revealed 92 IS
207 (Table 3), which is higher than the average number per bacterial genome (n=38)
208 but consistent with the lifestyle of *S. iniae* as a facultative pathogen (24, 25).
209 Furthermore, the number of IS found in *S. iniae* QMA0248 is substantially higher
210 than other Streptococci such as *S. mitis* strain B6 (n=63) but comparable to that of
211 the Gram positive fish pathogen *Lactococcus garvieae* (26, 27). The 92 IS elements
212 belong to 7 different IS families and 20 IS types. These include 9 novel types
213 belonging to IS*3*, IS*30*, IS*1182*, and IS*200*/IS*605* families, which we have submitted
214 to the ISFinder database (IS*Stin2*–IS*Stin10*) (Table 3 and Supplementary Tables
215 S2–4). Around half of all IS copies in QMA0248 belong to these 9 novel types
216 consistent with expansion of *S. iniae* specific IS since speciation (Table 3).
217 Amongst those genes disrupted by IS in QMA0248 is the restriction enzyme
218 component of a type II Restriction Methylation system that probably recognizes
219 "GCNGC" (28). This insertion renders the cognate methyltransferase (MTase)
220 (QMA0248_0516) an orphan and, given the high number of GCNGC sites
221 (3814/Mb in QMA0248) across the genome, suggests a potential role for this

222 MTase in global gene regulation. By comparison, there are 7762/Mb available
223 GATC sites in the *E. coli* K-12 MG1655 genome available for methylation by Dam,
224 the archetypal orphan type II MTase known to play a major role in gene regulation
225 (29). Further discussion of the methylome data generated by PacBio sequencing
226 of QMA0248 is provided as Supplementary Information. . Taken altogether, the *S.*
227 *iniae* genome harbors a large repertoire of IS elements, which may be associated
228 with adaptation to host or environment. Indeed, it is well accepted that IS
229 expansion is an early sign of genome reduction as a mechanism of adaptation to
230 host (25, 30, 31).
231
232 **Phylogenetic and comparative analysis of *S. iniae* QMA0248, SF1, YSFST01-**
233 **82, ISET0901, and ISNO**

234 The core genome of *S. iniae* QMA0248, SF1, YSFST01-82, ISET0901, and ISNO
235 accounts for ~75% of the chromosome. IS were compared between the reference
236 chromosome of QMA0248 and each of the *S. iniae* chromosomes (SF1, YSFST01-
237 82, ISET0901, and ISNO) using Artemis Comparison Tool (ACT) (32). Although IS
238 elements typically result in genomic rearrangements and loss of synteny, this is
239 not seen in *S. iniae.* This lack of rearrangement is reflected by the consistent
240 pattern of GC skew in the genome of *S. iniae* QMA0248 (Figure 1). Eight IS copies
241 out of the 92 detected in QMA0248 are absent in the genomes of SF1, ISET0901
242 and ISNO only (Supplementary Table S2). Other IS elements are unique to the
243 genomes SF1, ISET0901 and ISNO in syntenic positions. An interesting example is
244 an IS*981* (SF1 locus_tag: K710_0799 and K710_0800), inserted in *cas9* gene in the
245 CRISPR/Cas region only in those three genomes (Figure 2). Another example is

246 IS*Stin1*, inserted only in SF1, ISET0901, and ISNO (SF1 locus_tag: K710_0761).
247 Additionally, three IS copies are present in syntenic positions only in QMA0248,
248 ISET0901, and ISNO. Eight insertions are absent in YSFST01-82 only and another
249 seven IS copies are found exclusive to QMA0248 (Supplementary Table S2).

250

251 In addition to the IS differences, the five *S. iniae* genomes have major differences
252 in the content of other mobile genetic elements (MGE) that reflect variations in
253 the length of their respective chromosomes (Table 1). These MGEs include two
254 prophages (Phi1 and Phi2), a genomic island (GI), and two other regions of
255 difference (ROD) (Table 2, Figure 3). This includes a ~28 Kb region that is only
256 found in YSFST01-82 (ROD1), and a ~20 Kb region that is present in four genomes
257 but almost entirely absent from YSFST01-92 (ROD2) (Table 2, Figure 3).

258

259 Most variations in MGEs (including IS) were found to be incongruent with the core
260 SNP phylogeny. For instance, the deleted ~12 Kb GI-Leu and the *cas9* gene
261 disrupted by IS*981* exist only in SF1, ISET0901, and ISNO (Figures 2 and 3), but
262 these three isolates appear on divergent branches, indicating potential
263 independent events (Supplementary Figure S4). To investigate the discrepancies
264 between MGEs and phylogeny we compared multiple phylogenetic trees that were
265 constructed using different methods, including the core genome, core SNP and
266 using different software (Supplementary Figure S4). All phylogenies consistently
267 revealed that *S. iniae* isolates QMA0248 and SF1 cluster together in one clade,
268 whereas ISET0901 and ISNO cluster in another, and all four isolates cluster

269 separately to YSFST01-82, the latter diverging earliest from the root
270 (Supplementary Figure S4).
271
272 **Discrepancies between the *S. iniae* genomes are likely due to misassembly**
273 Taken together, our comparative analyses suggest that one or more of the *S. iniae*
274 genomes under comparison have been misassembled. The genomes ISET0901 and
275 ISNO were both assembled from Illumina data using BioNumerics (Applied Math)
276 and the genome *S. iniae* SF1 as a reference (12, 13). *S. iniae* SF1 was assembled *de*
277 *novo* from a combination of 454 GS FLX+, Illumina MiSeq and Sanger sequencing
278 (12). During the preparation of the present manuscript SF1 was removed from the
279 NCBI RefSeq database (https://www.ncbi.nlm.nih.gov/refseq/). YSFST01-92 was
280 also a hybrid assembly (454 GS FLX Titanium, Opgen optical mapping and Sanger
281 sequencing) but it remains in the RefSeq database. Reference-guided assembly
282 was introduced to enable comparisons between two very closely related isolates.
283 However, this practice can result in the erroneous inclusion of MGEs that exist in
284 the template genome but are absent from the comparison strain. Even with careful
285 curation it is impossible to avoid misplacing repetitive sequences such as IS, as
286 observed here in the case of *cas9* insertion and the other 8 IS copies that are absent
287 in SF1, ISET0901, and ISNO only. Moreover, reference-guided assemblies may
288 result in the loss of novel regions that are only present in the newly sequenced
289 strain, in which case a *de novo* approach is always required (33). Although
290 reference-guided assembly is no longer generally accepted for prokaryote
291 genomes, a number of examples remain available in public repositories such as
292 GenBank. For both ISET0901 and ISNO the assembly strategy is clearly outlined in
293 the comment field of the GenBank file, and the primary publications (12, 13).

294 Never-the-less, the consequences of using such genomes in downstream analyses
295 may not be apparent to all (for example, all three genomes are available in widely
296 used genome databases such as PATRIC (www.patricbrc.org version 3.5.36)(34)).
297 Removal of some early hybrid 454 complete genomes from public repositories like
298 RefSeq should help maintain the quality of available complete genomes. Long-read
299 sequencing data from Pacific Biosciences and Oxford Nanopore bring complete
300 bacterial genomes within reach of most laboratories, but here also significant care
301 is often required to avoid misassembly. Furthermore, as illustrated here and in
302 other studies (35, 36), what appear to be misassemblies may in fact be biologically
303 relevant. Ultimately the onus is on the user of public data to exercise caution when
304 validating the source, assembly strategy, and quality of available complete
305 genomes.
306
307 **Conclusions**
308 We assembled and annotated a high quality complete genome sequence for *S. iniae*
309 QMA0248, including manual curation of 92 insertion sequences. Comparative
310 analysis with publicly available complete genomes *S. iniae* SF1, YSFST01-82,
311 ISET0901, and ISNO revealed discrepancies in the MGE content consistent with
312 errors introduced by reference guided assembly of ISNO and ISET0901. Such
313 problems are not new, but many bacterial genomes assembled in this way remain
314 in public repositories of complete genomes. Our results emphasize the need to
315 critically appraise complete genome assemblies prior to comparative analysis.
316 Despite long-read sequencing becoming the gold standard for complete genome
317 assembly of bacterial isolates, caution is needed to avoid misassembly. To better
318 understand how insertion sequences, genomic islands, and other mobile elements

319 contribute to *S. iniae* diversity, there is a need for larger genomic studies using
320 global collections of *S. iniae* isolates from dissimilar origins. The genome of *S. iniae*
321 QMA0248 represents an important resource for future *S. iniae* comparative
322 genomic and evolutionary studies.
323
324 **Materials and Methods**
325 *Bacterial strain and sequencing*
326 *Streptococcus iniae* strain QMA0248 was isolated from diseased barramundi
327 (*Lates calcarifer*) from a farm in South Australia in 2009 (37). Genomic DNA was
328 prepared from several well-isolated colonies of *S. iniae* QMA0248 grown for 18 h
329 on Todd-Hewitt agar from a master seed stock (non-subcultured) with the
330 Genomic Tip 20 kit (Qiagen). Pre-incubation for 2 h at 37°C of cells suspended in
331 500 µL 50 mM EDTA containing 200 units of mutanolysin and 2 mg lysozyme was
332 found to improve cell lysis prior to following the manufacturers protocol for
333 purification of high molecular weight DNA. The genome of the strain QMA0248
334 was sequenced using 3 SMRT cells on the Pacific Biosciences (PacBio) RS II
335 platform and P4C2 sequencing chemistry, which generated a total of 57,083 reads
336 with an average length of 6,178 bp. Reads were deposited at BioProject
337 PRJNA385746 under accession SRP109617. The genome of the strain QMA0248
338 was also sequenced using Illumina Nextera libraries on HiSeq2000.
339
340 *Genome assembly and modified bases detection*
341 PacBio sequencing reads derived from *S. iniae* QMA0248 genomic DNA were
342 assembled using HGAP (hierarchical genome assembly process) using the PacBio
343 Single Molecule Real Time (SMRT) Portal (V2.3.0) (38), with default settings and

344 minimum seed read length of 4,509 bp. Contiguity was used to visualize the
345 assembly and the overlap between contigs using BLASTn (39, 40). The resulting
346 assembly was used as a reference in the RS Resequencing module of PacBio's
347 SMRT Analysis v2.3.0 to map the raw reads onto the reference genome producing
348 a highly accurate genome consensus. Illumina-sequenced reads of the strain
349 QMA0248 were mapped to the PacBio assembly using Snippy v3.0
350 (https://github.com/tseemann/snippy). To analyze read pileup in the potential
351 rRNA tandem operon, raw reads of QMA0248 were mapped onto the ~7 Kb rRNA
352 contig using BLASR v2.2, as part of the PacBio's SMRT Analysis Suite, and
353 visualized using Artemis (41). Methylated DNA bases were identified in the
354 resulting genome assembly using the RS Modification and Motif Analysis protocol
355 and Motif Finder v1 within the SMRT Analysis suite v2.3.0 using Quality Value
356 (QV) cutoff of 30. DNA Methyltransferases (MTases) were identified using
357 nucleotide comparisons against REBASE (42).
358
359 *PCR to investigate the rRNA tandem duplication*
360 To investigate the presence of a rRNA tandem repeat, long range PCR from unique
361 adjacent region to unique adjacent region was performed using specific primers
362 for each of 5 rRNA regions (Supplementary Table S1), which were designed using
363 Primer3 (43). PCR was done using LongAmp® Taq PCR Kit (NEB) from 40 ng of
364 QMA0248 *S. iniae* genomic DNA as follows: 5 min at 94°C; 30 cycles of (30 sec at
365 94°C, 30 sec at 56°C, and 15 min at 65°C); and 10 min final extension at 65°C. The
366 gel was loaded with 5 µL QUICK-LOAD® 1 Kb Extend DNA Ladder and 1µL of PCR
367 products and run for 90 min at 70V using 0.7% TAE buffer solution and stained
368 with HydraGreen.

369

370 *Genome and mobile genetic elements annotation*

371 Automated genome annotation was done using Prokka v1.11 (Prokaryotic
372 Genome Annotation System) (44) and then manually curated using Artemis (41)
373 and Geneprimp (45). The start codons of known coding sequences (CDS), such as
374 in the capsule and streptolysin S operons, were further adjusted where
375 appropriate using UniProtKB (http://www.uniprot.org/) and Pfam (46). CRISPRs
376 (Clustered Regularly Interspaced Short Palindromic Repeats) were predicted
377 using CRISPR finder tool (http://crispr.u-psud.fr/crispr/) (47). Prophage
378 annotation was done using PHAST (Phage Search Tool)
379 (http://phast.wishartlab.com/) (48). Island Viewer
380 http://www.pathogenomics.sfu.ca/islandviewer/) was used to predict genomic
381 islands (GI) (49). Boundaries of phage and GI regions were manually adjusted to
382 their respective attachment sites. IS Saga (http://www-genome.biotoul.fr/) was
383 used for the initial identification of insertion sequences (IS). Additional manual
384 curation was carried out to confirm the boundaries of complete and partial IS
385 elements using the IS Finder database (http://www-is.biotoul.fr/). IS element
386 matches against the database that were at ≥95% nucleotide identity were
387 assigned the top matching IS name. IS elements of less percent identity are novel
388 and were assigned the names IS*Stin2*–IS*Stin10* by ISFinder. The impact of IS
389 elements on flanking coding sequences was analyzed using Artemis and by
390 searching the amino acid sequence in UniProt KB (http://www.uniprot.org/), and
391 Pfam databases (41, 46). The complete annotated genome sequence was
392 deposited at GenBank under the accession number CP022392.

393

394 *Comparative genomics and phylogenetic analysis*

395 Alignments of the whole-genome or genomic sub-regions, such as the CRISPR and
396 genomic islands, were done using BLASTn implemented in EasyFig v2.1 (40, 50).
397 Detailed analysis of regions of difference and comparison of insertion sequences
398 (IS) were done using Artemis Comparison Tool (ACT) (32). The core genome of
399 the five genomes QMA0248, SF1, YSFST01-82, ISET0901, and ISNO was defined
400 using Roary (51). Phylogenetic trees were constructed using the core genome and
401 the core SNP methods, using multiple programs (see below), using the strain
402 QMA0140 as the out-group (52). The strain *S. iniae* QMA0140 is a dolphin isolate
403 from USA in 1976, and was sequenced using Illumina HiSeq 2000 (See Bioproject
404 PRJNA417543). For quality control, the first 20 bp of each read derived from
405 QMA0140 genomic DNA were hard trimmed using Nesoni v0.132
406 (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) with a
407 minimum length and quality of 70, and 20, respectively. Hard trimmed filtered
408 reads of QMA0140 were assembled using SPAdes v3.9.0 where contigs < 10X
409 coverage and smaller than 100 bp were removed (53). For core genome
410 phylogenies, whole-genome alignment of the five complete genomes and
411 QMA0140 draft assembly was done using Mauve v2.4.0 and Parsnp v1.2 with
412 default parameter settings (54, 55). For the alignment using Mauve, conserved
413 blocks in all 6 genomes longer than 500 bp were selected and concatenated using
414 the stripSubsetLCBs script producing the core genome alignment. For core SNP
415 phylogenies, error-free simulated reads were created using wgsim v0.3.2
416 (https://github.com/lh3/wgsim) and mapped to the reference genome QMA0248
417 along with QMA0140 hard trimmed and filtered raw reads using bowtie v1.0.0
418 (56), where variants were called using Nesoni v0.132

419 (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) using default
420 parameters. Core SNPs were also identified by mapping the reads of the 6
421 genomes to QMA0248 using BWA-MEM v0.7.15 (r1140), implemented in Snippy
422 v3.0 (https://github.com/tseemann/snippy) (57). All trees were produced by
423 RAxML v8.2.9 (58) using the general time-reversible (GTR) and GAMMA
424 distribution model of among-site rate variation with bootstrapping from 1000
425 replicates, and viewed using FigTree v1.4.0
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592 **Table 1: General features of five *S. iniae* complete genomes.**

Strain	QMA0248	SF1	YSFST01-82	ISET0901	ISNO
Accession Number	CP022392	CP005941	CP010783	CP007586	CP007587
Genome Size (bp)	2,116,570	2,149,844	2,086,959	2,070,822	2,070,182
GC Content (%)	36.8	36.7	36.8	36.8	36.8
Total CDS number	1,946	2,125	1,897	1,872	1,865
Total gene number	2,196	2,196	2,029	1,997	1,996
rRNA (5S, 16S, 23S)	15	12	15	12	12
tRNA	58	45	58	45	45
Reference	This study	(12)	(11)	(12)	(13)
Assembly type	PacBio RSII P2C4	454 FLX+/ Illumina MiSeq/ Sanger	454 FLX Titanium/ Opgen/ Sanger	Illumina 1500 HiSeq/ Reference guided assembly	Illumina 1500 HiSeq/ Reference guided assembly

593

594 **Table 2: Large mobile genetic elements (MGE) and regions of difference (ROD) identified in the 5 *S.***
595 ***iniae* genomes analyzed (QMA0248, SF1, YSFST01-82, ISET0901, and ISNO).**

Region	Genomic Island (GI-leu)	ROD1	Prophage 2	ROD2	Prophage 1 (Phi1)
Coordinates[1]	87374-100014	177819-206359 (YSFST01-82)	848479-890501 (SF1)	1767227-1787619	1991661-2023508
Length (Kb)	12.6	28.5	42.0	20.4	31.8
GC content (%)	35.9	37.2	35.3	34.3	37.6
Features	MGE; 13 tRNA and 1 rRNA operon (5S, 16S, and 23S) upstream; integrase; IS*30* and IS*256* family IS elements	Integrase; IS*3* and IS*256* family IS elements	MGE; integrase	IS*3* family IS element	MGE; integrase; 1 tRNA-Cys
No. of CDSs	11	33	63	18	53
Major CDSs	Cro/CI family transcriptional regulator; ECF subfamily RNA polymerase sigma factor; plasmid replication protein; membrane protein	ESAT-6-like protein; two-component sensor histidine kinase; galactose mutarotase; 3 PTS galactitol transporter subunits (IIA, IIC, and IIB)	Phage DNA replication protein; prophage antirepressor, phage capsid and scaffold protein; putative tail protein; holin; endolysin; antigen C; several phage hypothetical proteins	ESAT-6-like protein; O-glycosyl hydrolase; phage infection protein; 3 lipoproteins; protein kinase	DNA helicase; Cro/CI family transcriptional regulator; tail and capsid proteins; holin; lysin; DNA N-4 cytosine methyltransferase; site specific recombinase; several phage hypothetical proteins
Best hit (% identity, % coverage)	*S. parauberis* KCTC 11537 (90, 41)	*S. dygalactiae subsp. equisimilis ATCC 12394* (70, 34)	*S. parauberis* KCTC 11537 (91, 45)	*S. thermophilus* JIM 8232 (81, 13)	Bacteriophage PH10 of *Streptococcus* (71, 34)

596 [1]Coordinates are in QMA0248 GenGenBank annotation (GCA_002220115.1) unless otherwise indicated.

597

598 **Table 3: Summary of all insertion sequences (IS) identified in QMA0248. Partial IS are suffixed by –p.**

IS Family in QMA0248	Number of IS copies	IS types (copy number, mean % amino acid identity)
IS*3*	32	IS*Sag2* (10, 98.6), IS*Sag2*-p (2, 98.7) IS*981* (8, 99.8) IS*Spy1* (1, 86.7), IS*Spy1*-p (1, 90.5) *IS*Stin6* (3, 94.5), *IS*Stin7* (4, 90.0), *IS*Stin5* (2, 69.3), unclassified most similar to *IS*Stin5-p* (1, 73.8)
IS*30*	22	IS*Sag9* (3, 99.6), IS*Sag9*-p (5, 99.6) *IS*Stin4* (2, 93.3), *IS*Stin4*-p (2, 89.6), *IS*Stin9* (9, 81.4), *IS*Stin2* (1, 84.7)
IS*256*	17	IS*Stin1* (16, 91.0), unclassified most similar to IS*Stin1*-p (1, 90.2)
IS*1182*	13	*IS*Stin8* (7, 86.3), *IS*Stin8*-p (2, 89.8), *IS*Stin3* (1, 87.7), *IS*Stin3*-p (2, 87.1), unclassified most similar to *IS*Stin8-p* (1, 70.5)
IS*200*/IS*605*	5	*IS*Stin10* (3, 99), *IS*Stin10*-p (1, 98.6), unclassified most similar to *IS*Stin10* (1, 86.5)
IS*L3*	1	Unclassified most similar to IS*Sth1*-p (1, 77.8)
IS*110*	2	Unclassified most similar to IS*L4* (2, 65.8)

599 ** Novel IS element.*

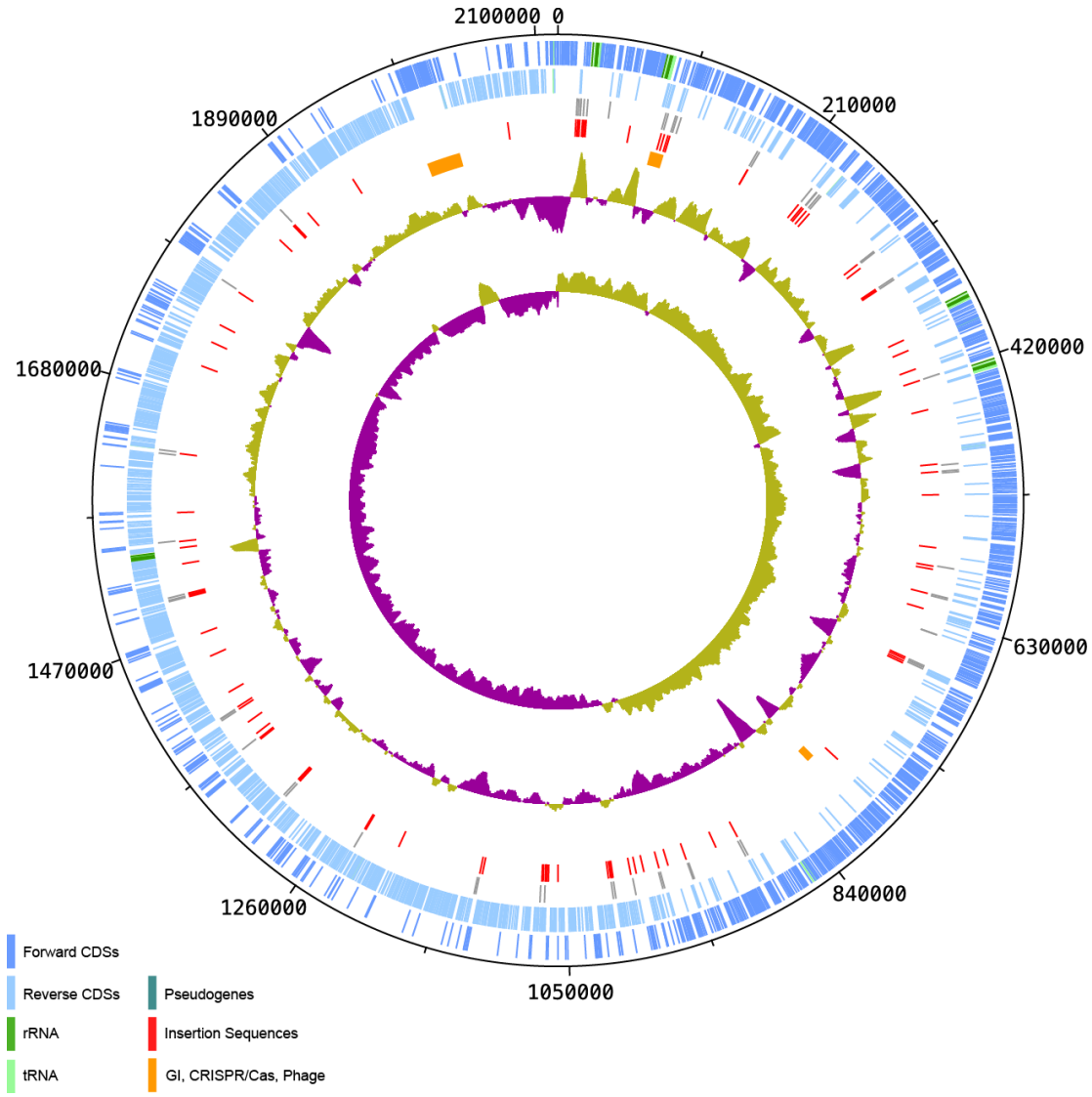
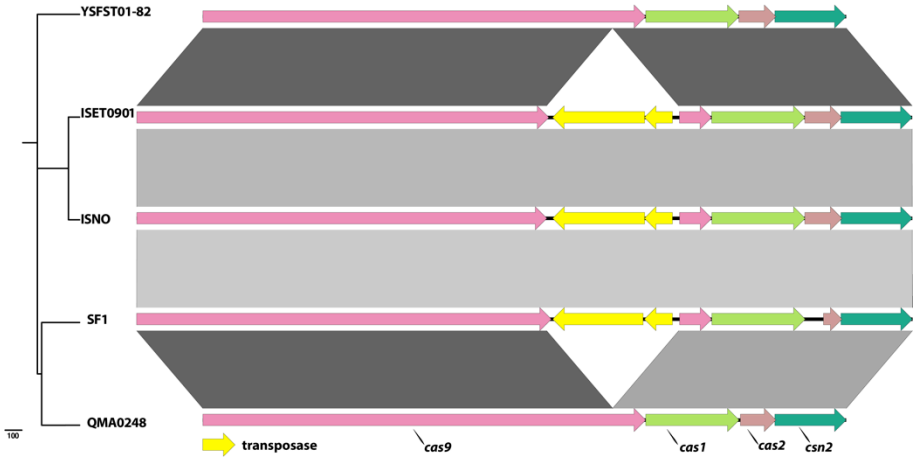


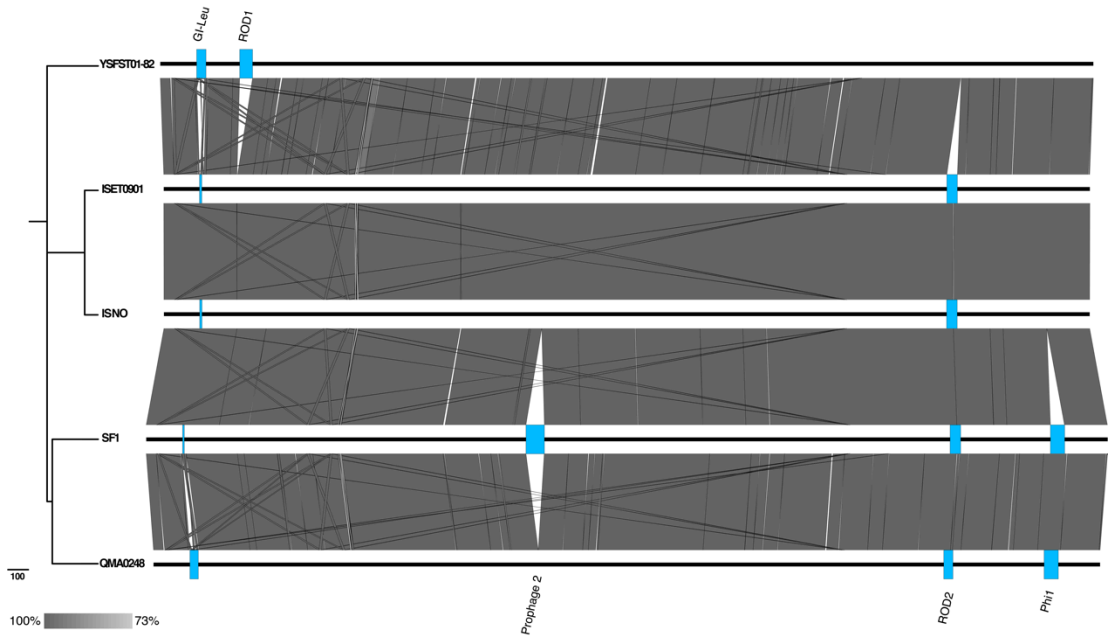
Figure 1: Circular map of the genome *Streptococcus iniae* QMA0248. Genomic features from outer ring to the inner ring are described in the key to the left, where the innermost two rings correspond to the GC skew (inner), and GC plot (outer). CDS: coding sequence. GI: genomic island. The circular map was generated using DNAPlotter (59).

608 

609 **Figure 2: Comparison of the CRISPR/Cas region between QMA0248, SF1,**
610 **YSFST01-82, ISET0901, and ISNO.** Alignment of Cas genes where the genomes
611 are ordered according to their position in the phylogenetic tree (left). The
612 maximum Likelihood (ML) phylogeny was rooted to QMA0140 (not shown) and
613 built using 1,111 SNPs. Arrows correspond to Cas genes, which are labeled at the
614 bottom. Figure was produced using EasyFig (50) using 500 bp as minimum length,
615 90% as minimum identity value, and 0.001 as maximum e-value.

616

617



618

619 **Figure 3: Whole-genome alignment of the 5 genomes QMA0248, SF1,**
620 **YSFST01-82, ISET0901, and ISNO.** The genomes are ordered according to their
621 position in the core SNP-based phylogenetic tree**.** The maximum Likelihood (ML)
622 phylogeny was rooted to QMA0140 (not shown) and built using 1,111 SNPs. The
623 scale bar indicates the number of substitutions represented by branch lengths.
624 BLASTn comparison was produced using EasyFig (50) using 2000 bp as minimum
625 length, 50% as minimum identity value, and 1 *x* 10^{-17} as maximum e-value.

Importance

The present manuscript provides *Streptococcus iniae* PacBio sequenced genome (QMA0248) with complete characterization of its mobile genetic elements (MGE) content including genomic islands, prophages, and insertion sequences (IS). The genome of *S. iniae* QMA0248 harbours 92 IS elements including 9 novel ones belonging mostly to IS*3*, which were submitted to ISFinder database. Here we also present the first analysis for *S. iniae* methylome. We also performed comparative and phylogenetic analysis between *S. iniae* QMA0248 and publicly available complete *S. iniae* genomes, which to our knowledge has not been done for *S. iniae* before. This analysis revealed discrepancies that are likely due to misassembly in the genomes of strains ISET0901 and ISNO.Our study not only highlights assembly problems in existing *S. iniae* genomes, but provides a high quality reference genome for *S. iniae* QMA0248, including manually curated MGEs, that will assist future *S. iniae* comparative genomic and evolutionary studies.