A high quality reference genome for the fish pathogen *Streptococcus iniae*

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Key words: SMRT sequencing, Insertion Sequence, reference guided assembly, misassembly, mobile genetic elements

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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. 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 YSFST01-82 (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
274 Taken together, our comparative analyses suggest that one or more of the *S. iniae*
275 genomes under comparison have been misassembled. The genomes ISET0901 and
276 ISNO were both assembled from Illumina data using BioNumerics (Applied Math)
277 and the genome *S. iniae* SF1 as a reference (12, 13). *S. iniae* SF1 was assembled *de*
278 *novo* from a combination of 454 GS FLX+, Illumina MiSeq and Sanger sequencing
279 (12). During the preparation of the present manuscript SF1 was removed from the
280 NCBI RefSeq database. YSFST01-82 was also a hybrid assembly (454 GS FLX
281 Titanium, Opgen optical mapping and Sanger sequencing) but it remains in the
282 RefSeq database and is the designated genome for *S. iniae* at NCBI
283 (https://www.ncbi.nlm.nih.gov/genome/?term=streptococcus+iniae; accessed
284 23rd Jan 2020).
285
286 We have no reason to suspect that the YSFST01-82 genome assembly is inferior to
287 that of QMA0248, but adopting the latter as an alternative representative *S. iniae*
288 genome is justified for investigators wishing to take advantage of a manually
289 curated set of MGEs. In contrast we strongly recommend not using ISET0901 or
290 ISNO in future comparative studies of *S. iniae* genomes. Reference-guided
291 assembly was introduced to enable comparisons between two very closely related
292 isolates. However, this practice can result in the erroneous inclusion of MGEs that
293 exist in the template genome but are absent from the comparison strain. Even with

294 careful curation it is impossible to avoid misplacing repetitive sequences such as
295 IS, as observed here in the case of *cas9* insertion and the other 8 IS copies that are
296 absent in SF1, ISET0901, and ISNO only. Moreover, reference-guided assemblies
297 may result in the loss of novel regions that are only present in the newly
298 sequenced strain, in which case a *de novo* approach is always required (33).
299 Although reference-guided assembly is no longer generally accepted for
300 prokaryote genomes, a number of examples remain available in public
301 repositories such as GenBank. For both ISET0901 and ISNO the assembly strategy
302 is clearly outlined in the comment field of the GenBank file, and the primary
303 publications (12, 13). Never-the-less, the consequences of using such genomes in
304 downstream analyses may not be apparent to all (for example, all three genomes
305 are available in widely used genome databases such as PATRIC
306 (www.patricbrc.org version 3.5.36)(34)).
307
308 Removal of some early hybrid 454 complete genomes from public repositories like
309 RefSeq should help maintain the quality of available complete genomes. Long-read
310 sequencing data from Pacific Biosciences and Oxford Nanopore bring complete
311 bacterial genomes within reach of most laboratories, but here also significant care
312 is often required to avoid misassembly. Furthermore, as illustrated here and in
313 other studies (35, 36), what appear to be misassemblies may in fact be biologically
314 relevant. Ultimately the onus is on the user of public data to exercise caution when
315 validating the source, assembly strategy, and quality of available complete
316 genomes.
317
318

319 **Conclusions**

320

321 We assembled and annotated a high quality complete genome sequence for *S. iniae*
322 QMA0248, including manual curation of 92 insertion sequences. Comparative
323 analysis with publicly available complete genomes *S. iniae* SF1, YSFST01-82,
324 ISET0901, and ISNO revealed discrepancies in the MGE content consistent with
325 errors introduced by reference guided assembly of ISNO and ISET0901. Such
326 problems are not new, but many bacterial genomes assembled in this way remain
327 in public repositories of complete genomes. Our results emphasize the need to
328 critically appraise complete genome assemblies prior to comparative analysis.
329 Despite long-read sequencing becoming the gold standard for complete genome
330 assembly of bacterial isolates, caution is needed to avoid misassembly. To better
331 understand how insertion sequences, genomic islands, and other mobile elements
332 contribute to *S. iniae* diversity, there is a need for larger genomic studies using
333 global collections of *S. iniae* isolates from dissimilar origins. The genome of *S. iniae*
334 QMA0248 represents an important resource for future *S. iniae* comparative
335 genomic and evolutionary studies.

336

337 **Materials and Methods**

338 *Bacterial strain and sequencing*

339 *Streptococcus iniae* strain QMA0248 was isolated from diseased barramundi
340 (*Lates calcarifer*) from a farm in South Australia in 2009 (37). Genomic DNA was
341 prepared from several well-isolated colonies of *S. iniae* QMA0248 grown for 18 h
342 on Todd-Hewitt agar from a master seed stock (non-subcultured) with the
343 Genomic Tip 20 kit (Qiagen). Pre-incubation for 2 h at 37°C of cells suspended in

344 500 µL 50 mM EDTA containing 200 units of mutanolysin and 2 mg lysozyme was
345 found to improve cell lysis prior to following the manufacturers protocol for
346 purification of high molecular weight DNA. The genome of the strain QMA0248
347 was sequenced using 3 SMRT cells on the Pacific Biosciences (PacBio) RS II
348 platform and P4C2 sequencing chemistry, which generated a total of 57,083 reads
349 with an average length of 6,178 bp. Reads were deposited at BioProject
350 PRJNA385746 under accession SRP109617. The genome of the strain QMA0248
351 was also sequenced using Illumina Nextera libraries on HiSeq2000.
352
353 *Genome assembly and modified bases detection*
354 PacBio sequencing reads derived from *S. iniae* QMA0248 genomic DNA were
355 assembled using HGAP (hierarchical genome assembly process) using the PacBio
356 Single Molecule Real Time (SMRT) Portal (V2.3.0) (38), with default settings and
357 minimum seed read length of 4,509 bp. Contiguity was used to visualize the
358 assembly and the overlap between contigs using BLASTn (39, 40). The resulting
359 assembly was used as a reference in the RS Resequencing module of PacBio's
360 SMRT Analysis v2.3.0 to map the raw reads onto the reference genome producing
361 a highly accurate genome consensus. Illumina-sequenced reads of the strain
362 QMA0248 were mapped to the PacBio assembly using Snippy v3.0
363 (https://github.com/tseemann/snippy). To analyze read pileup in the potential
364 rRNA tandem operon, raw reads of QMA0248 were mapped onto the ~7 Kb rRNA
365 contig using BLASR v2.2, as part of the PacBio's SMRT Analysis Suite, and
366 visualized using Artemis (41). Methylated DNA bases were identified in the
367 resulting genome assembly using the RS Modification and Motif Analysis protocol
368 and Motif Finder v1 within the SMRT Analysis suite v2.3.0 using Quality Value

369 (QV) cutoff of 30. DNA Methyltransferases (MTases) were identified using
370 nucleotide comparisons against REBASE (42).
371
372 *PCR to investigate the rRNA tandem duplication*
373 To investigate the presence of a rRNA tandem repeat, long range PCR from unique
374 adjacent region to unique adjacent region was performed using specific primers
375 for each of 5 rRNA regions (Supplementary Table S6), which were designed using
376 Primer3 (43). PCR was done using LongAmp® Taq PCR Kit (NEB) from 40 ng of
377 QMA0248 *S. iniae* genomic DNA as follows: 5 min at 94°C; 30 cycles of (30 sec at
378 94°C, 30 sec at 56°C, and 15 min at 65°C); and 10 min final extension at 65°C. The
379 gel was loaded with 5 µL QUICK-LOAD® 1 Kb Extend DNA Ladder and 1µL of PCR
380 products and run for 90 min at 70V using 0.7% TAE buffer solution and stained
381 with HydraGreen.
382
383 *Genome and mobile genetic elements annotation*
384 Automated genome annotation was done using Prokka v1.11 (Prokaryotic
385 Genome Annotation System) (44) and then manually curated using Artemis (41)
386 and Geneprimp (45). The start codons of known coding sequences (CDS), such as
387 in the capsule and streptolysin S operons, were further adjusted where
388 appropriate using UniProtKB (http://www.uniprot.org/) and Pfam (46). CRISPRs
389 (Clustered Regularly Interspaced Short Palindromic Repeats) were predicted
390 using CRISPR finder tool (http://crispr.u-psud.fr/crispr/) (47). Prophage
391 annotation was done using PHAST (Phage Search Tool)
392 (http://phast.wishartlab.com/) (48). Island Viewer
393 http://www.pathogenomics.sfu.ca/islandviewer/) was used to predict genomic

394 islands (GI) (49). Boundaries of phage and GI regions were manually adjusted to
395 their respective attachment sites. IS Saga (http://www-genome.biotoul.fr/) was
396 used for the initial identification of insertion sequences (IS). Additional manual
397 curation was carried out to confirm the boundaries of complete and partial IS
398 elements using the IS Finder database (http://www-is.biotoul.fr/). IS element
399 matches against the database that were at ≥95% nucleotide identity were
400 assigned the top matching IS name. IS elements of less percent identity are novel
401 and were assigned the names IS*Stin2*–IS*Stin10* by ISFinder. The impact of IS
402 elements on flanking coding sequences was analyzed using Artemis and by
403 searching the amino acid sequence in UniProt KB (http://www.uniprot.org/), and
404 Pfam databases (41, 46). The complete annotated genome sequence was
405 deposited at GenBank under the accession number CP022392.
406
407 *Comparative genomics and phylogenetic analysis*
408 Alignments of the whole-genome or genomic sub-regions, such as the CRISPR and
409 genomic islands, were done using BLASTn implemented in EasyFig v2.1 (40, 50).
410 Detailed analysis of regions of difference and comparison of insertion sequences
411 (IS) were done using Artemis Comparison Tool (ACT) (32). The core genome of
412 the five genomes QMA0248, SF1, YSFST01-82, ISET0901, and ISNO was defined
413 using Roary (51). Phylogenetic trees were constructed using the core genome and
414 the core SNP methods, using multiple programs (see below), using the strain
415 QMA0140 as the out-group (52). The strain *S. iniae* QMA0140 is a dolphin isolate
416 from USA in 1976, and was sequenced using Illumina HiSeq 2000 (See Bioproject
417 PRJNA417543). For quality control, the first 20 bp of each read derived from
418 QMA0140 genomic DNA were hard trimmed using Nesoni v0.132

419 (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) with a
420 minimum length and quality of 70, and 20, respectively. Hard trimmed filtered
421 reads of QMA0140 were assembled using SPAdes v3.9.0 where contigs < 10X
422 coverage and smaller than 100 bp were removed (53). For core genome
423 phylogenies, whole-genome alignment of the five complete genomes and
424 QMA0140 draft assembly was done using Mauve v2.4.0 and Parsnp v1.2 with
425 default parameter settings (54, 55). For the alignment using Mauve, conserved
426 blocks in all 6 genomes longer than 500 bp were selected and concatenated using
427 the stripSubsetLCBs script producing the core genome alignment. For core SNP
428 phylogenies, error-free simulated reads were created using wgsim v0.3.2
429 (https://github.com/lh3/wgsim) and mapped to the reference genome QMA0248
430 along with QMA0140 hard trimmed and filtered raw reads using bowtie v1.0.0
431 (56), where variants were called using Nesoni v0.132
432 (https://github.com/Victorian-Bioinformatics-Consortium/nesoni) using default
433 parameters. Core SNPs were also identified by mapping the reads of the 6
434 genomes to QMA0248 using BWA-MEM v0.7.15 (r1140), implemented in Snippy
435 v3.0 (https://github.com/tseemann/snippy) (57). All trees were produced by
436 RAxML v8.2.9 (58) using the general time-reversible (GTR) and GAMMA
437 distribution model of among-site rate variation with bootstrapping from 1000
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439 (http://tree.bio.ed.ac.uk/software/figtree).
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604

605 **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

606

607 **Table 2: Large mobile genetic elements (MGE) and regions of difference (ROD) identified in the 5 *S.***
608 ***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)

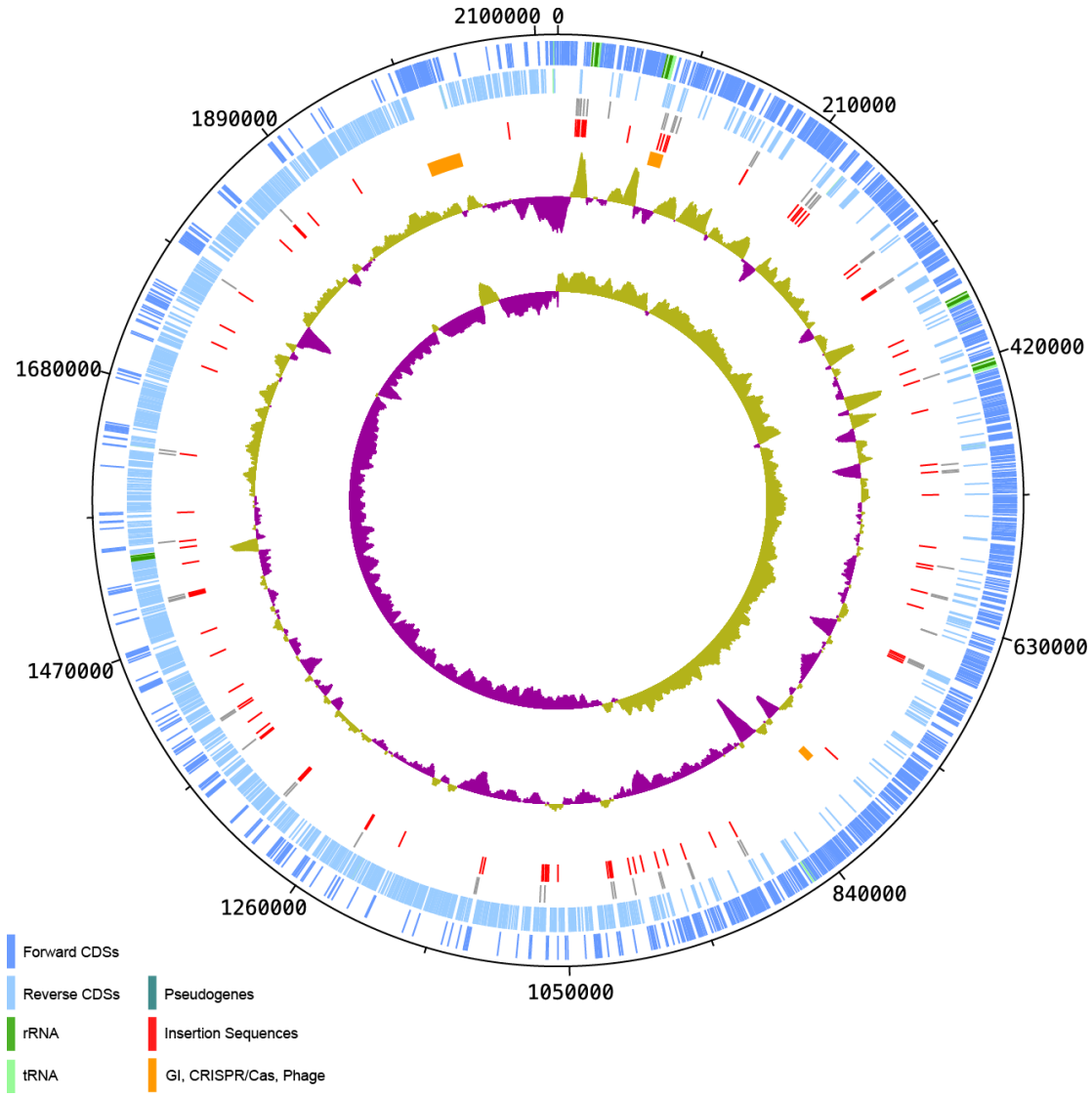
609 [1]Coordinates are in QMA0248 GenGenBank annotation (GCA_002220115.1) unless otherwise indicated.

610

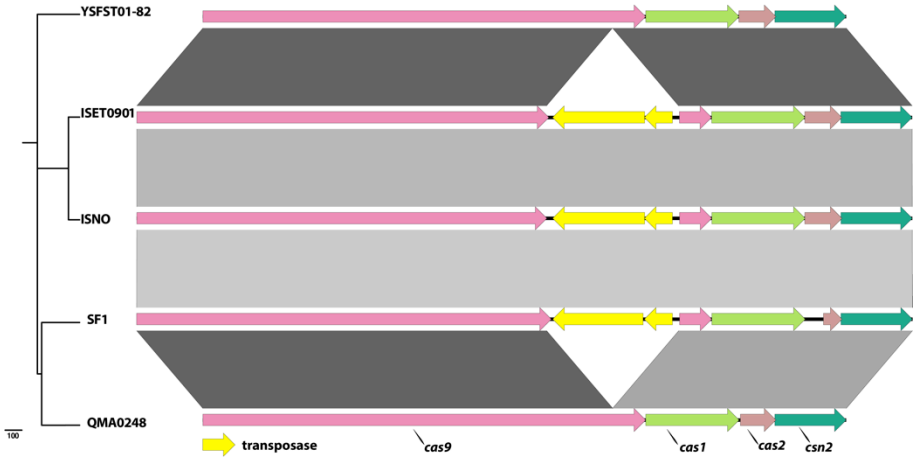
611 **Table 3: Summary of all insertion sequences (IS) identified in QMA0248. Partial IS are suffixed by –p.**

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)

612 ** Novel IS element.*



613
614
615 **Figure 1: Circular map of the genome *Streptococcus iniae* QMA0248.** Genomic
616 features from outer ring to the inner ring are described in the key to the left, where
617 the innermost two rings correspond to the GC skew (inner), and GC plot (outer).
618 CDS: coding sequence. GI: genomic island. The circular map was generated using
619 DNAPlotter (59).
620

621 

622 **Figure 2: Comparison of the CRISPR/Cas region between QMA0248, SF1,**
623 **YSFST01-82, ISET0901, and ISNO.** Alignment of Cas genes where the genomes
624 are ordered according to their position in the phylogenetic tree (left). The
625 maximum Likelihood (ML) phylogeny was rooted to QMA0140 (not shown) and
626 built using 1,111 SNPs. Arrows correspond to Cas genes, which are labeled at the
627 bottom. Figure was produced using EasyFig (50) using 500 bp as minimum length,
628 90% as minimum identity value, and 0.001 as maximum e-value.

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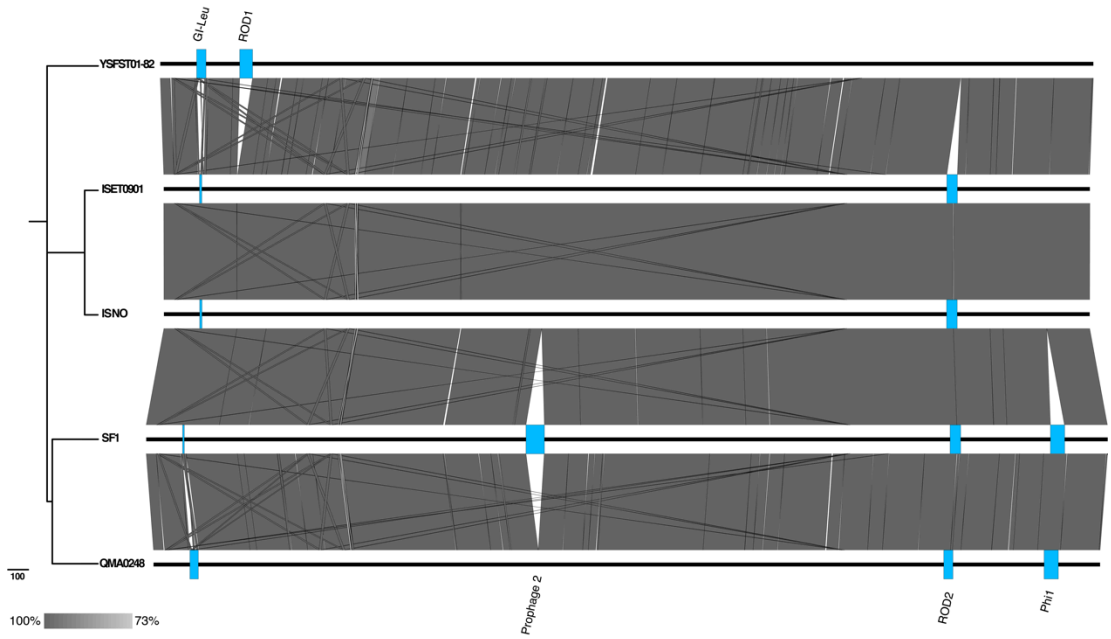
630



631

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

Supplementary Information

A high quality reference genome for the fish pathogen *Streptococcus iniae*

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S. iniae QMA0248 methylome

DNA methylation guides numerous critical processes including defence against foreign DNA, DNA replication and repair, gene expression and virulence. Analysis of PacBio sequence data enabled the detection of genome-wide DNA methylation. Three DNA methyltransferases (MTases) were encoded in the QMA0248 genome. On the basis of homology to known MTases, QMA0248_0514 (annotated as M.Sin248ORF514P in REBASE) likely targets the GCNGC motif and QMA0248_1949 (annotated as M.Sin248ORF1949P in REBASE) likely targets GCCHR (1). QMA0248_0505 (annotated as M.Sin248ORF0505P in REBASE) is encoded ~5kb upstream of QMA0248_0514 but has no close functional homologs and thus has an unknown recognition sequence (1). A small fraction of GCNGC and GCCHR motifs were methylated in QMA0248 (<3%). Collectively these two motifs account for 21,421 sites across the chromosome (or 1 every 100 bp) suggesting a potential role for methylation in the regulation of gene expression in QMA0248. This figure is roughly equivalent to the frequency of Dam GATC sites in *Escherichia coli*. Further work is required to determine if the activity detected here is biologically meaningful.

5-methylcytosine DNA methyltransferase QMA0248_0514

A putative Type II 5-methylcytosine (m5C) DNA MTase (QMA0248_0514, annotated as M.Sin248ORF514P in REBASE) likely targets the GCNGC motif (1). Uncharacterised homologs with 99% amino acid identity are found in 8 other available *S. iniae* complete or draft genomes including SF1, YSFST01-82, ISET0901, ISNO and 89353 (1). In most cases restriction enzymes predicted to recognise GCNGC are predicted to be encoded nearby, or immediately adjacent to

the respective MTase gene. Notably, in QMA0248 the adjacent restriction enzyme (QMA0248_0515) is a pseudogene that has been truncated by an IS*981*. In *S. iniae* KCTC 11634BP, the orthologous gene in its draft quality 454 genome was truncated the same point by a contig break (2), suggesting that in both QMA0248 and KCTC 11634BP the MTase does not function as part of a restriction-modification system. The GCNGC motif is found 8074 times in the QMA0248 genome suggesting that methylation activity could have wide-ranging regulatory consequences.

The closest homologs to QMA0248_0514 for which MTase activity has been determined is the M.CmaLM2II enzyme from *Clostridium mangenotii* LM2 and the M.LmoJ3I enzyme from *Listeria monocytogenes* J3115. Despite sharing modest overall amino acid similarity to QMA0248_0514 (59% and 45%, respectively), regions of high amino acid identity within their predicted target recognition domains (34/34 for M.CmaLM2II and 32/24 for M.LmoJ3I) support the prediction that QMA0248_0514 would also methylate the 2nd cytosine of the GCNGC motif. Detection of m5C using PacBio data is normally unreliable (3). As expected, methylation was detected at only a small fraction of GCNGC sites in the QMA0248 genome and the consensus motif determined by the PacBioSMRT-Portal software includes additional bases that are probably artefactual (e.g. GCNGCAGC) (Supplementary Table S5). Further experimental work (such as using Tet1 pre-treatment to enhance detection of m5C with PacBio sequencing, or Oxford Nanopore sequencing) is needed to determine the true extent of cytosine methylation in the *S. iniae* genome and its role in gene regulation.

N4-methylcytosine DNA methyltransferase QMA0248_1949

The second *S. iniae* QMA0248 MTase (QMA0248_1949, known as M.Sin248ORF1949P in REBASE) shares 95% amino acid identity with the Orphan (gamma) N4-methylcytosine (m4C) DNA MTase M.NgoDCXV (gb|AJ004687.2) from *Neisseria gonorrhoeae*, which specifically targets the GCCHR motif. M.NgoDCXV homologs are remarkably rare and confined to a few streptococcal species (including *S. iniaie* SF1). No specific methylation of GCCHR was detected in the QMA0248 genome but 126 motifs that partially overlapped with GCCHR showed evidence of methylation (Supplementary Table S5). The GCCHR motif is present in 13,347 locations in the QMA0248 genome so this represents only a fraction of available sites. The m4C modification is normally detectable from PacBio sequence data, therefore further work is required to determine if QMA0248_1949 is expressed and functional.

Putative Type II N4-methylcytosine or N6-methyladenine DNA methyltransferase QMA0248_0505

The third MTase in *S. iniae* QMA0248 (QMA0248_0505, known as M.Sin248ORF0505P in REBASE) is encoded ~5kb upstream of QMA0248_0514 and shares a similar strain distribution. There are no close homologs of QMA0248_0505 for which a recognition site has been determined. Accordingly it has been annotated by REBASE as a putative Type II N4-cytosine or N6-adenine DNA methyltransferase of unknown recognition sequence (1).

Supplementary Figures

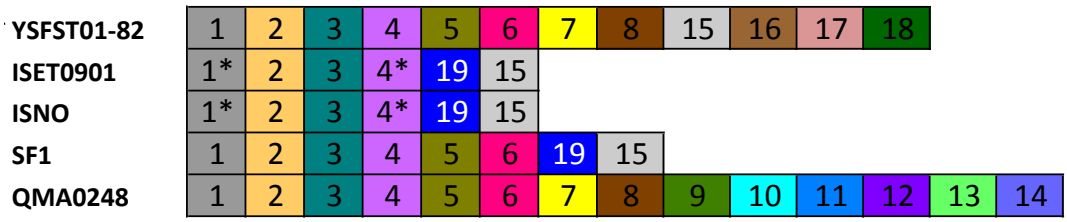

Figure S1: Graphical representation of spacers in CRISPR between YSFST01-82, ISET0901, ISNO, SF1, and QMA0248. Each box represents a spacer, where the same colour and number indicate identical spacers. *: A spacer with an additional direct repeat sequence.

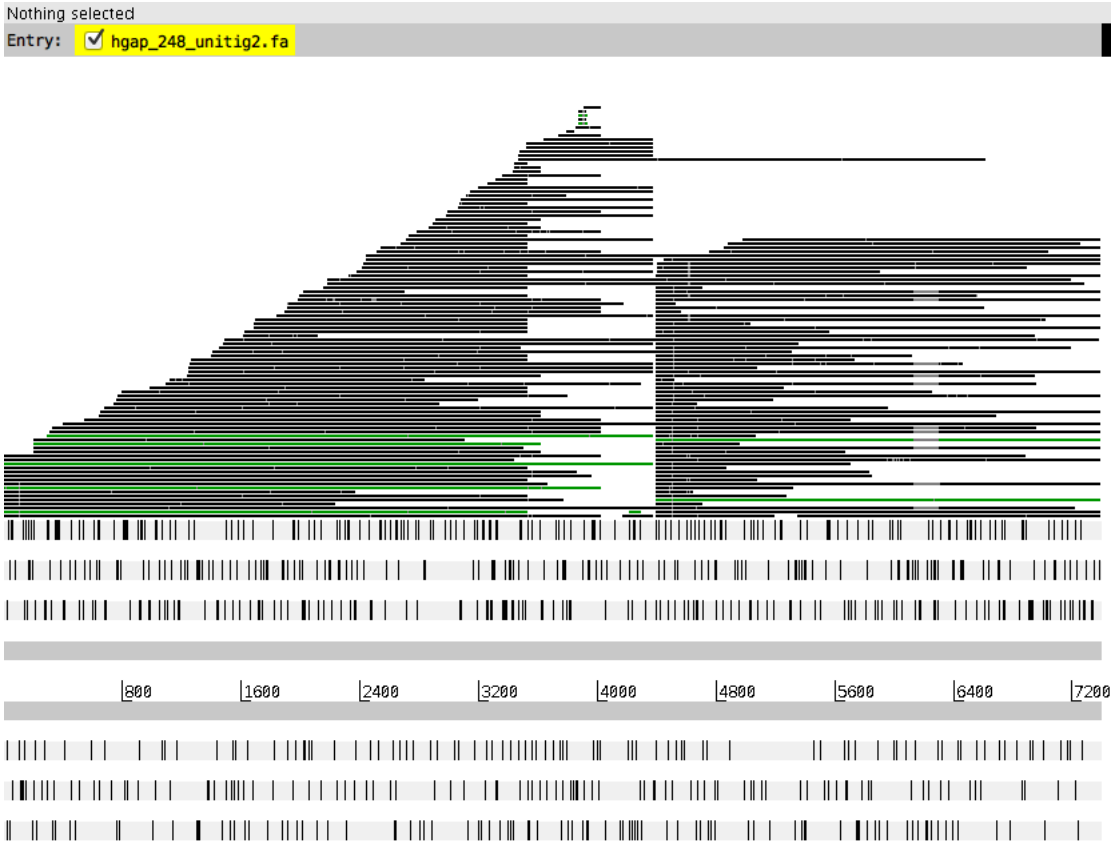

Figure S2: PacBio raw reads pileup of *Streptococcus iniae* QMA0248 on the spurious 7.2 Kb rRNA contig. Alignment of reads is visualised using BamView implemented in Artemis. The lower panel corresponds to the forward and reverse strands of the 7.2 Kb contig, where vertical black lines indicate stop codons. Horizontal lines on the upper panel correspond to the aligned reads, where Artemis colours duplicate reads that span the same region in green black.

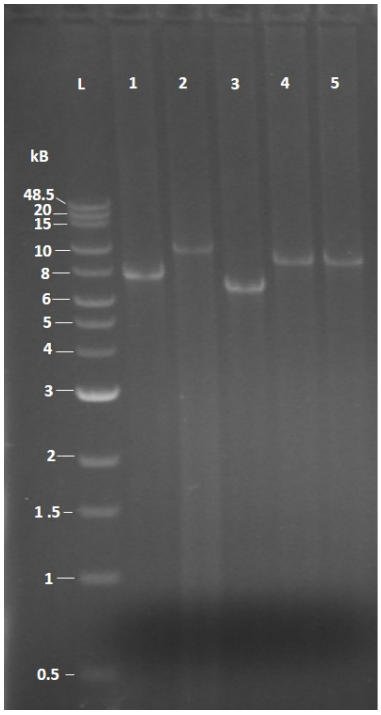

Figure S3: Long-range PCR across rRNA operons. Gel electrophoresis of long range PCR products across five rRNA operons of *S. iniae* QMA0248 (lanes 1-5 in operon-wise order as listed in Table 4). Primers are listed in Table 4. "L" denotes 1 Kb Extend DNA Ladder (NEB).

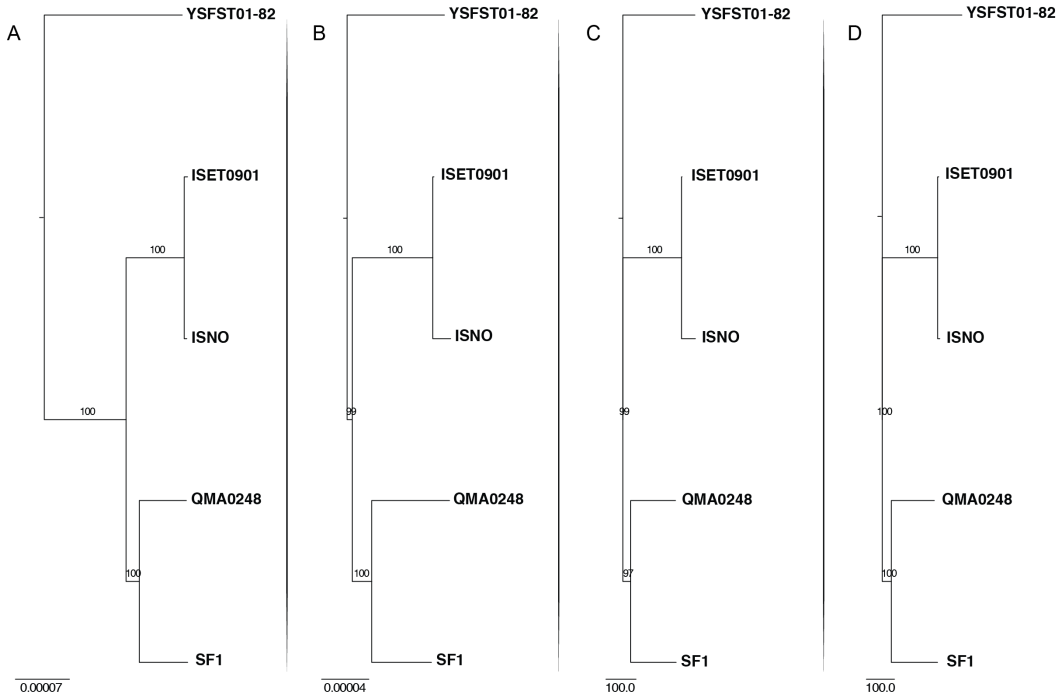

Figure S4: Alternative tree-building methods. Phylogenetic trees constructed for *S. iniae* based on core genome using Mauve (A) and Parsnp (B), and core SNP using Nesoni (C) and Snippy (D). Maximum likelihood (ML) phylograms were built using RAxML where bootstrap support values are shown on branches. Trees are rooted using QMA0140 (not shown). Branch lengths correspond to the number of substitutions (C and D), and the number of substitutions per site (A and B). ML phylograms in A and B were built from 1,928,667 bp and 1,856,820 bp core genome alignment, respectively, whereas C and D were built using 1,241 and 1,116 substitutions, respectively.

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