Pathogenic strains of *Shewanella putrefaciens* contain plasmids that are absent in the probiotic strain Pdp11

Marta Domínguez Maqueda [Corresp., 1], **Olivia Pérez Gómez** [1], **Ana Grande Pérez** [2, 3], **Consuelo Esteve** [4], **Pedro Seoane** [5, 6], **Silvana Tapia Paniagua** [1], **Maria Carmen Balebona** [1], **Miguel Ángel Moriñigo** [1]

[1] Departamento de Microbiología, Universidad de Málaga, Málaga, Spain

[2] Área de Genética, Universidad de Málaga, Málaga, Spain

[3] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora"-Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Universidad de Málaga, Málaga, Spain

[4] Departmento de Microbiología y Ecología, Universidad de Valencia, Valencia, Spain

[5] Centro de Investigación Biomédica en Red de Enfermedades Raras, CIBERER, Madrid, Spain

[6] Departmento de Biología Molecular y Bioquímica, Universidad de Málaga, Málaga, Spain

Corresponding Author: Marta Domínguez Maqueda
Email address: martadm@uma.es

Shewanella putrefaciens Pdp11 is a strain described as a probiotic for use in aquaculture. However, *S. putrefaciens* includes strains reported to be pathogenic or saprophytic to fish. Although the probiotic trait has been related to the presence of a group of genes in its genome, the existence of plasmids that could determine the probiotic or pathogenic character of this bacterium is unknown. In the present work, we searched for plasmids in several strains of *S. putrefaciens* that differ in their pathogenic and probiotic character. Under the different conditions tested, plasmids were only found in two of the five pathogenic strains, but not in the probiotic strain nor in the two saprophytic strains tested. Using a workflow integrating Sanger and Illumina reads, the complete consensus sequences of the plasmids were obtained. Plasmids differed in one ORF and encoded a putative replication initiator protein of the repB family, as well as proteins related to plasmid stability and a toxin-antitoxin system. Phylogenetic analysis showed some similarity to functional repB proteins of other *Shewanella* species. The implication of these plasmids in the probiotic or pathogenic nature of *S. putrefaciens* is discussed.

Pathogenic strains of *Shewanella putrefaciens* contain plasmids that are absent in the probiotic strain Pdp11

Domínguez-Maqueda Marta[1], Pérez-Gómez Olivia[1], Grande-Pérez Ana[2,3], Esteve Consuelo[4], Seoane Pedro[5,6], Tapia-Paniagua Silvana[1], Balebona María Carmen[1], Moriñigo Miguel Ángel[1]

[1] Departamento de Microbiología, Universidad de Málaga, Málaga, Spain
[2] Área de Genética, Universidad de Málaga, Málaga, Spain
[3] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora"-Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Campus de Teatinos, 29071 Malaga, Spain.
[4] Departamento de Microbiología y Ecología, Universidad de Valencia, Valencia, Spain
[5] Departamento de Biología Molecular y Bioquímica, Universidad de Málaga, Málaga, Spain
[6] CIBER de Enfermedades Raras, CIBERER, Madrid, Spain

Corresponding Author: Marta Domínguez-Maqueda[1]
Email address: martadm@uma.es

Abstract

Shewanella putrefaciens Pdp11 is a strain described as a probiotic for use in aquaculture. However, *S. putrefaciens* includes strains reported to be pathogenic or saprophytic to fish. Although the probiotic trait has been related to the presence of a group of genes in its genome, the existence of plasmids that could determine the probiotic or pathogenic character of this bacterium is unknown. In the present work, we searched for plasmids in several strains of *S. putrefaciens* that differ in their pathogenic and probiotic character. Under the different conditions tested, plasmids were only found in two of the five pathogenic strains, but not in the probiotic strain nor in the two saprophytic strains tested. Using a workflow integrating Sanger and Illumina reads, the complete consensus sequences of the plasmids were obtained. Plasmids differed in one ORF and encoded a putative replication initiator protein of the repB family, as well as proteins related to plasmid stability and a toxin-antitoxin system. Phylogenetic analysis showed some similarity to functional repB proteins of other *Shewanella* species. The implication of these plasmids in the probiotic or pathogenic nature of *S. putrefaciens* is discussed.

Introduction

Probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts (Sharifuzzaman and Austin 2017). Nowadays, their use is increasingly frequent in various sectors such as veterinary, food biotechnology, human health (Sonnenborn and Schulze 2009), or the growing agro-industrial sector (FAO 2020), which includes the aquaculture industry. Probiotics are widely used in aquaculture species with the aim of increasing the health and productivity of farmed fish as an important source of animal protein (Assefa and Abunna 2018). Several probiotics have been characterized and applied in fish, and some of them are of host origin (Seegers, Bui, and de Vos 2021). Such is the case of *Shewanella putrefaciens* Pdp11 strain isolated from skin mucosa of healthy gilthead seabream (*Sparus aurata* L.) (Chabrillón et al. 2005; Díaz-Rosales et al. 2009) that has been described as a probiotic for farmed fish species such as *Solea senegalensis* and *Sparus aurata* (Sáenz de Rodrigáñez et al. 2009; García de La Banda et al. 2010; Tapia-Paniagua et al. 2010; Lobo et al. 2014; Cordero et al. 2015). However, *Shewanella putrefaciens* has been associated with diseases in common carp (*Cyprinus carpio L.*) (Paździor

2016), rainbow trout (*Oncorhynchus mykiss*) (Paździor, Pękala-Safińska, and Wasyl 2019) and eel *(Anguilla anguilla* L.) (Esteve, Merchán, and Alcaide 2017). Therefore, different strains from the same species can be pathogenic or beneficial, as has been shown for *Escherichia coli* (Sonnenborn and Schulze 2009), *Bacillus cereus* (Cui et al. 2019) or *Vibrio proteolyticus* (Bowden, Bricknell, and Preziosi 2018; Medina, Moriñigo, and Arijo 2020), among others. In order to consider a microorganism as a good candidate for probiotic, each specific strain must be thoroughly analysed (Santos et al. 2020). A good probiotic must meet a series of essential requirements such as the ability to compete, adhere, persist and survive in the conditions of the intestinal tract (Ghattargi et al. 2018). At the same time, probiotics should show absence of virulence factors and multidrug resistance, as undesirable pathogenic traits may appear due to different factors, such as intensive mixed breeding (Marcos-López et al. 2010), mutation and recombination via horizontal gene transfer (Wijegoonawardane et al. 2009), among others. Conventionally, phenotypic, molecular and bioinformatic methods are used to identify genes of interest (virulence/resistance, beneficial…) (Quainoo et al. 2017) that may reside either in the bacterial genome or in plasmids or both. Thus, the presence of a group of 15 genes, mostly related to probiotic traits was specifically found in the genome of the probiotic strain *S. putrefaciens* Pdp11, but not in the pathogenic strains SH4, SH6, SH9, SH12 and SH16, nor in the saprophytic strains SdM1 and SdM2 (Seoane et al. 2019). Plasmids normally include variable repertoires of 'accessory genes', such as those coding for antibiotic resistance and virulence factors. They also include 'backbone' loci, largely conserved within plasmid families (Orlek et al. 2017), such as those involved in key plasmid specific functions (e.g., replication, stable inheritance, mobility) (Conlan et al. 2014; Giess et al. 2016) . Therefore, plasmid characterization could help to discern whether a microorganism is pathogenic or beneficial (Santos et al. 2020).

In the present work, we searched for plasmids in several strains of *S. putrefaciens* that differed in their pathogenic or probiotic character. Plasmids were only found in two of the five pathogenic strains and not in the probiotic or the two saprophytic strains tested. Using a workflow integrating Sanger and Illumina sequences, the complete consensus sequences of these plasmids were obtained. Sequence analysis showed that the plasmids encoded a putative replication initiator protein of the repB family, and proteins related to plasmid stability and to a toxin-antitoxin system. Phylogenetic analysis showed similarity with other *Shewanella* species in functional repB proteins.

The implication of these plasmids in the probiotic or pathogenic nature of *S. putrefaciens* is discussed.

Materials & Methods

Shewanella putrefaciens strains SH4, SH6, SH9, SH12 and SH16 isolated from diseased eels were kindly provided by Dra. Esteve C. from the University of Valencia (Valencia, Spain) (Esteve, Merchán, and Alcaide 2017). Two saprophytic isolates (SdM1 and SdM2) of *S. putrefaciens* were obtained from environmental sources (Seoane et al. 2019) and one probiotic strain, *S. putrefaciens* Pdp11 CECT 7627, was isolated from the skin of healthy *Sparus aurata* L. (Díaz-Rosales et al. 2009b).

All *S. putrefaciens* strains were grown on tryptic soy agar plates (Oxoid Ltd., Basingstoke, UK) supplemented with NaCl (1.5%) (TSAs) for 24 h at 23 ºC. Then, one or two colonies of each strain were picked and grown in 10 mL tubes of tryptic soy broth (Oxoid Ltd., Basingstoke, UK) added with NaCl (1.5%) (TSBs) for 24 h at 23 ºC on shaking at 80 rpm (ELMI DOS-20M Digital Orbital Shaker, USA). Given the possibility that, if present, plasmids could be integrated into the bacterial chromosome, strains were cultured under different growth conditions (temperature, incubation time, growth medium and freeze-thaw) to favour their excision. For this, one or two colonies of each strain were picked and cultured in 10 mL tubes of TSBs and minimal (M9) media, and incubated at 23ºC or 4ºC for 24h and 48 h on shaking at 80 rpm. Cultures inoculated in parallel with TSBs medium containing glycerol (20 %) were subjected to a freeze-thaw cycle (Pesaro et al. 2003) for 24 h at -80 ºC prior to incubation.

Plasmid DNA isolation

One or two colonies of the *S. putrefaciens* strains grown under different growth conditions were picked from the pure culture and grown in 10 mL of TSBs (Oxoid Ltd., Basingstoke, UK) for 24 h at 23 ºC under agitaton at 80 rpm (ELMI DOS-20M Digital Orbital Shaker). As a positive control, *Escherichia coli* V157, a strain harboring seven plasmids (Macrina et al. 1978), was grown on Luria-Bertani agar plates (LB) (Becton, Dickson and Company, Le Pont de Claix, France) for 24 h at 37 ºC. The cultures were centrifuged at 8000 x g for 5 min and the pellet was used for plasmid DNA (pDNA) isolation using the GeneJet Plasmid Miniprep kit (Thermo Fisher, USA) following the manufacturer's protocol. Plasmid DNA integrity was checked by agarose gel (0.8 %, w/v)

electrophoresis in the presence of RedSafe nucleic acid staining solution (InTRON Biotechnology). The 0.2-10 kb Hyperladder molecular weight marker (Bioline, USA) was used to check plasmid size. The pDNA was stored at -20 ºC for further processing.

Plasmid DNA amplification and sequencing

Rolling circle amplification (RCA) was performed using the TempliPhi 100 amplification kit (GE Healthcare, USA) for each isolated plasmid following the manufacturer's instructions. As a positive control for the reaction, pUC19 was used. DNA from each RCA-amplified plasmid was digested with EcoRI and EcoRV (Takara, USA), separately. They were then ligated employing T4 DNA ligase (Thermo Fisher Scientific, USA) to pBluescript SK (+) (pBSK S+) (Addgene, UK) previously treated with shrimp phosphatase alkaline (New England Biolabs, EEUU). Plasmid DNA was transformed into $CaCl_2$-treated *E. coli* DH5α competent cells, as described by Sambrook et al. 1989. Recombinant bacteria were selected using plates containing ampicillin (100 μg/ml) and X-gal (20 μg/ml), and colonies resuspended in 50 μl of DEPC-Water (Sigma) and tested by PCR amplification. Reaction mixture contained 2 μl of bacterial suspension, 62,5 U of Taq Accustart II Trough Mix (Boimerieux, France), 20 pmol of M13R primer 5'-CAGGAAACAGCTATGAC-3' and 20 pmol of M13F primer 5'-TGTAAAACGACGGCCAGT-3' in a final volume of 10 μL. The PCR program was: (1) 94 ºC 3 min, (2) 94 ºC 30 s, (3) 50 ºC 30 s, (4) 72 ºC 1 min/kb. Steps (2) to (4) were repeated during 35 cycles. Amplicons were checked by agarose electrophoresis gel as above. PCR products were sent to Macrogen (Madrid, Spain) for Sanger sequencing.

Plasmid DNA sequence assembly

Plasmids were assembled *de novo* using a workflow integrating Sanger and Illumina reads (Chevreux 2005; Bankevich et al. 2012). Plasmid-specific Illumina sequences used here were obtained by Seoane et al. 2019 using a sequencing library with the Nextera protocol for 2 x 300 bp PE (raw reads available in BioProject PRJNA510237).

First, the Sanger plasmid sequences were pre-processed with BBDuk (Bushnell 2015; Kechin et al. 2017) a tool developed for quality filtering and adapter trimming using k-mers matching, where k-mers are substrings of length "k" contained in the original nucleotide sequence. To remove the sequence of the pBSK S+ vector, different values of the two clean-up parameters k and hdist were

applied, where “k” is the length of the k-mers and “hdist” is the “hamming distance” between two k-mers. We used a k=20 and hdist=1 for pSH12 and a k=22 and hdist=2 for pSH4 (Fig. 1A). Two sets of vector-free Sanger reads were generated when quality trimming with BBDuk was applied using “trimq” parameter. This parameter is the minimum average quality required in a sequence window, if the average is below the threshold, the nucleotides are trimmed from the read. For each plasmid, a low (10) and a high (17) stringency quality values were applied. The low stringency threshold generate longer reads that are used to capture Illumina reads from the BioProject PRJNA510237, denoted as Capture read set (Fig. 1B). With the high strigency threshold, a high quality read set for the final assembly is generated, denoted as Assembly read set (Fig. 1C).

Next, Illumina plasmid reads were captured with the sanger Capture read set using bowtie 2 (Langmead and Salzberg 2012; Langmead et al. 2017). These reads were identified and subsequently extracted with Samtools (Li et al. 2009) by selecting pairs of Illumina reads in which at least one member had aligned to the Capture read set (Fig. 1D). Then, Sanger-Illumina hybrid assembly was performed with two assemblers, Mira (Chevreux 2005) and Spades (Bankevich et al. 2012) using default parameter values. The aforementioned Illumina reads and the Assembly read set, obtaining two possible and complete pDNA assemblies per strain (Fig. 1E). The two sequences per strain were recircularized by the MARS method (Ayad and Pissis 2017) (Fig. 1F) to make possible the next step. The plasmid sequences of each strain, they were aligned by the Clustal method (Sievers and Higgins 2014) of the Seaview program (Galtier, Gouy, and Gautier 1996) (Fig. 1G), leading to a unique plasmid consensus sequence per strain.

Assembly validation

Alignment of the sequence set of SH4 and SH12 plasmids using Mira and Spades programs revealed the presence of unreliable regions in both plasmids. Validation was carried out by PCR and primers were designed for each non-validated plasmid sequence using Primer3 (Untergasser et al. 2012). The composition of the reaction mixture was done as above, except that 10 pmol of the primers pSH4_R 5'<CGGATTGAATGGCTGGCTGGACTG>3 (reverse) and pSH4_F 5'<ATACCAAACGCCCACAGA>3' (direct) were used to validate the assembly of pSH4, and of the primers pSH12_R 5'<GGCTCCACCCTTACCCAAAAAA>3' (reverse) and pSH12_F <5'GCGAGCCCCTCCATGATTTT>3' (direct) to validate the assembly of pSH12. The PCR program was: (1) 94ºC 3 min, (2) 94ºC 30 s, (3) 66ºC 30 s, (4) 72ºC 1 min/kb, with 35 repeat cycles

of steps (2) through (4). PCR products were checked by agarose gel electrophoresis using the 100 bp molecular weight marker Hyperladder (Bioline, USA). Finally, the plasmid assemblies of strains SH4 and SH12 were mapped back to the Sanger assembly read set sequence, the Illumina sequences captured from Sanger capture read set sequence and the PCR product obtained from validation.

Plasmid annotation and functional characterization

Once complete consensus sequences were obtained for each plasmid, sequences were searched for on the PLSDB web server (Galata et al. 2019), a resource containing numerous plasmid records collected from the NCBI nucleotide database. Next, gene prediction was performed for annotation of bacterial operons and open reading frames (ORFs) using Softberry Software (http://www.softberry.com/berry.phtml) including the fgenesB tool (Solovyev et al. 2011) with "Bacterial generic" as the closest organism; the Prokaryotic GeneMark. Hmm version 2 program (Besemer, Lomsadze, and Borodovsky 2001) with "*Shewanella putrefaciens* CN_32" as the selected species. Consensus sequences were also analysed with ORF finder at NCBI website (https://www.ncbi.nlm.nih.gov/orffinder/) with the search parameters: 150 nucleotides of minimal ORF length, genetic code "Bacterial, Archaeal and Plant Plastid", and "ATG and alternative initiation codons" as ORF start codon to be used. For each of the identified ORFs, their amino acid sequence was obtained and queried in blastp (NCBI), to obtain clues about the ORF functions (Altschul et al. 1990; Gish and States 1993). PHYRE 2 version 2.0, was also used for subsequent protein prediction and modelling (Kelley et al. 2015). Finally, Dfast (Tanizawa, Fujisawa, and Nakamura 2018) was used for ORF protein identification and functional annotation. Plasmid maps were drawn with pDRAW32 version 1.1.146 (AcaClone software) (https://www.acaclone.com/).

Plasmid to genome comparison

To detect the degree of similarity between the genomes of the SH4 and SH12 strains and their respective plasmids, a comparison between them was carried out using Sibelia (Minkin et al. 2013). The complete putative pDNA sequences and their corresponding ORFs were also searched by blastn against the probiotic genome and all non-probiotic strains (NPS) genomes previously described by Seoane et al. 2019.

Phylogenetic study

The repB protein sequences of pSH12 and pSH4 were searched in Blast (NCBI). The repB protein sequences of plasmids from different bacterial species were downloaded in FASTA format in a single export file, and alignment was performed by the Clustal method (Sievers and Higgins 2014). A phylogenetic tree was constructed by PhyML employing the Maximum Likelihood method (AWF Edwards 1964) using Seaview software (Galtier, Gouy, and Gautier 1996).

Ethical statement

To conduct the research, we used bacterial strain *Shewanella putrefaciens* (strains SH4, SH6, SH9, SH12, SH16, SdM1, SdM2 and Pdp11), which is not considered a human or animal sample. Therefore, no ethics approval was needed, and no informed consent was required.

Results

Identification of plasmids

S. putrefaciens strains SH4, SH6, SH12, SH16, SH19, Pdp11, SdM1 and SdM2 were analysed to confirm the presence or absence of plasmids under different growth conditions (Table S1). After growth under optimal conditions, only SH4 and SH12 strains were found to contain one plasmid around 3 kb in size (Fig. 2). The same results were obtained when other growth conditions were used (Table S1). *E. coli* V157 strain, used as a positive control, showed all seven plasmids previously described in the literature (Macrina et al. 1978).

Plasmid assembly and sequence validation

The consensus sequences of pSH4 and pSH12 obtained using Mira-Spade workflow from Sanger and Illumina reads showed a size of 3003 bp for pSH4 and 2990 bp for pSH12. However, unvalidated regions were detected in both plasmids (Fig. 3) because the Capture read set sequence did not sufficiently capture the Illumina sequences in pSH4 (Fig. 3A) and pSH12 (Fig. 3B), rendering these regions unreliable. Moreover, the Assembly read set sequence did not support this region in the alignment of pSH4 (Fig. 3A), unlike in pSH12 (Fig. 3B). From the PCR product obtained, a new alignment using Sanger, Illumina and PCR product sequences was carried out for each plasmid and mapped. After this, the unvalidated region of pSH4 was supported by a total of 22 Illumina and 1 Sanger sequences as was the PCR amplicon, while that of pSH12 was invalid.

Thus, fully assembled circular sequences showed 3003 bp in pSH4 but 2960 bp in pSH12, 43 pb less due to the absence of that unreliable region (Fig. 3B). The GC content of both plasmids was similar, 42.69 % (pSH4) and 43.04 % (pSH12). The raw sequence information from Seaview program and agarose gel of PCR amplicons are showed in Fig. S1.

ORF analysis of pSH4 and pSH12

No hits sequences of the plasmids were available on the PLSDB web server (Galata et al. 2019), so a comprehensive ORF analysis was carried out. Analysis using the ORF finder revealed 10 possible ORFs in pSH4 and 9 in pSH12. However, after removing nested predictions (i.e. overlapping ORFs) a total of 7 ORFs remained in pSH4 and 6 in pSH12 (Table S2). The gene prediction tools, fgenesB, GeneMark and ORF finder, confirmed that only 4 and 3 ORFs in pSH4 and pSH12, respectively, were common and highly similar in both plasmids (Table 1).

Both plasmid sequences resulted in the presence of the same ORFs, except for the presence of an extra 43 bp region in pSH4 that introduced a stop codon that split in two the equivalent of ORF5 in pSH12 giving rise to ORF5 and ORF6 in pSH4. The localization and orientation of all ORFs of pSH12 and pSH4 are shown in the circular map in Fig. 4. Since unique EcoRI and EcoRV cleavage sites were found in both plasmids, they were used for cloning and sequencing.

Prediction of functional proteins

Based on blastp and Phyre 2, ORF5 and ORF6 of pSH4, and ORF5 of pSH12 presented a high identity to a plasmid replication initiator protein of the repB family of *Shewanella algae* (Table 2). The proteins encoded by these ORFs were predicted to show structural analogy with a crystal structure of the plasmid initiator protein of the repB family, with a nucleic acid as ligand and functions such as DNA replication and plasmid copy control (Table 2). Similarly, the proteins encoded by ORF7 of pSH4 and ORF6 of pSH12 showed analogy with the Type II toxin-antitoxin system PemK/MazF family toxin from *Avibacterium paragallinarum,* presenting DNA as ligand and functions of cell growth inhibitor by cleaving mRNA with high sequence-specificity as part of the bacterial stress response, and as toxic component for plasmid maintenance (Table 2). No reliable predictions were obtained for the remaining ORFs that showed similarities to hypothetical or unknown proteins.

286

287 **Genomic and plasmid comparison between strains**

288 Comparison of the genomes of SH4 and SH12 and their respective plasmids yielded high similarity
289 at the plasmid level with 94.24% coverage (Fig. S2), differing only by an additional 43 pb region
290 in pSH4 as explained above. At the same time, genome-wide comparison of SH4 and SH12 strains
291 also showed high similarity composed of 46 and 58 scaffolds, respectively with 99.42% coverage
292 between each other (Fig. S2). These percentage values support the idea that SH4 and SH12 are
293 different strains belonging to the same species.
294 In addition, a blastn alignment was carried out to check for a possible insertion of these plasmids
295 or any of their ORFs in the complete genome of the other strains under study. A total absence of
296 plasmids was found in the rest of NPS genomes, as wells as in the genome of the probiotic *S.*
297 *putrefaciens* Pdp11.

298

299 **Phylogenetic analysis of the repB protein**

300 The repB protein sequences found in pSH12 (ORF5) and pSH4 (ORF5 and ORF6) were compared
301 extensively and a phylogenetic analysis was performed (Fig. 5) to look for similar disruptions in
302 the repB sequences and their possible effects on functionality. The disruption in the repB protein
303 in pSH4 gave rise to ORF5 and ORF6, two short repB sequences encoding 127 and 186 amino
304 acids, respectively, indicating that the ORF6 was the longest conserved region of the repB protein
305 in pSH4, close to the only 228 amino acid protein encoded by ORF5 in pSH12. Phylogenetic
306 analysis showed that the pSH12 protein was conserved and structurally similar to other repB
307 proteins described in *Shewanella* species (Fig. 5). In addition, the repB proteins of both plasmids
308 also were closely related to the repB protein of *Pseudoalteromonas* sp, *Thiomicrospira* sp. and
309 other *Shewanella* species (Fig. 5).

310

311 ## Discussion

312 The reason why different strains of the same bacterial species can be either pathogenic or probiotic
313 remains elusive, but knowledge of the molecular basis may help to understand it (Seoane et al.
314 2019). In this work, the study of plasmids from different strains of *Shewanella putrefaciens*, a
315 bacterium of commercial and biotechnological importance for aquaculture, has been addressed.

316 The presence of plasmids in the *Shewanella* group is often associated with resistance traits, as is
317 the case for plasmid pSFKW33 of *Shewanella* sp. 33B (Werbowy, Cieśliński, and Kur 2009),
318 plasmid pSMX33 of *Shewanella xiamenensis* BC01 (Zhou and Ng 2016) or plasmid pSheB, of
319 *Shewanella* sp. O23S (Uhrynowski, Radlinska, and Drewniak 2019). The horizontal mobile
320 genetic element (MGE) products encoded by plasmids could be at the root of the differences
321 between probiotic and pathogenic strains, as has been described for the *Enterococcus* group
322 (Santos et al. 2020). Therefore, the absence of plasmids in *S. putrefaciens* strain Pdp11 could
323 underlie its probiotic nature. However, this fact does not guarantee probiosis, since three of the
324 five pathogenic strains tested (strains SH6, SH9 and SH16) did not contain any plasmids. Given
325 that plasmids were only detected in two of the five pathogenic strains, it cannot be ruled out that
326 virulence factors in *S. putrefaciens* are chromosomally encoded nor that no other plasmids were
327 detected because of the extraction methodology used in this work. The pathogenic strains SH4 and
328 SH12 had one plasmid each, pSH4 and pSH12, respectively, which did not show much similarity
329 to the plasmids in the databases. Only two ORFs with a high percentage of identity to known
330 plasmid proteins were identified, while the rest of the ORFs belonged to hypothetical or unknown
331 proteins. Since only 1 to 29 % of plasmid sequences are usually found in bacterial databases
332 (Maguire et al. 2020), especially in the genus *Shewanella*, their characterization is of interest. In
333 this work, we found that the main ORFs conserved in both plasmids pSH4 and pSH12 belong to
334 the replication protein initiator repB superfamily, and to the type II toxin-antitoxin system
335 superfamily (PemK/PemI family protein).
336 Rep proteins are especially important as they are primarily responsible for the initial DNA binding
337 and nicking activities, which represent the first steps in plasmid replication and conjugative
338 plasmid transfer (Stolz 2014). It is generally composed of repA (helicase), repB (initiator protein)
339 and repC (initiator protein) proteins (Wawrzyniak et al. 2019) but only repB protein has been
340 identified in pSH4 and pSH12. Some bacterial strains such as *Agrobacterium tumefaciens* require
341 the products of the repABC operon for plasmid replication, as they encode its rep proteins (Chai
342 and Winans 2005; Pinto, Pappas, and Winans 2012). Since these proteins recruit several other
343 DNA polymerases and helicases from the bacterial host to proceed with plasmid replication (San
344 Millan and Maclean 2019), it seems plausible that SH4 and SH12 may use this mechanism to
345 replicate. In addition, repB protein in pSH4 supposed major attention due to the disruption of it,
346 which can affect its oligomerization and functionality as occurs in rep proteins Rep68/78 as

described (Zarate-Perez et al. 2012). The results of the phylogenetic analysis suggest that the larger and more conserved ORF5 may retain its functionality, whereas future studies will be necessary to determine the functionality of ORF6.

In any case, the identification of the repB protein is interesting because it has been previously explored in *S. xiamenensis* BC01 and *S. oneidensis* MR-1 (Zhou and Ng 2016) as functional ori for stable plasmid replication in *Shewanella*. Furthermore, the combination of repB with other promoters such as placI favoured the expression of *gfp* and *mtr* genes by recombinant DNA technology in *S. oneidiensis* MR-1 (Ng et al. 2018). In this way, specific plasmids could be constructed and incorporated into other *Shewanella* species to favour the expression of exogenous genes of interest (Antonio-Hernández et al. 2019). According to this compatibility, the above application could be attractive for future studies to improve the toolbox of the probiotic strain Pdp11.

Another major ORF identified is the PemK protein, which is part of the type II PemK-PemI toxin-antitoxin (TA) system. This system is found both in bacterial chromosomes and in MGEs such as plasmids and prophages (Bukowski et al. 2019). A type II TA system typically consists of two genes located in an operon encoding a stable toxin that disrupts essential cellular processes and a labile antitoxin that forms a tight protein complex with the cognate toxin to neutralize its activity (Yao et al. 2018). Activities that have been associated with type II TA systems are maintenance of genetic material, bacterial virulence, biofilm formation, phage inhibition, and different types of stress management, including antibiotic tolerance and persister formation (Bleriot et al. 2020). In this work, the gene encoding the PemK protein has been detected in the plasmids of pathogenic strains of *Shewanella*, suggesting that its unstable antitoxin could consist of a protein or a non-coding RNA (Yamaguchi, Park, and Inouye 2011). On the other hand, strains SH4 and SH12 might not carry the complementary antitoxin PemI, allowing the toxin to exert its virulent effect. Furthermore, since virulence and drug-resistant genes had been detected in different species of *Shewanella*, such as *S. algae*, *S. putrefaciens*, *S. xiamenensis*, *S. oneidensis* and *S. frigidimarina*, mainly on the chromosome associated or not with MGEs (Yousfi et al. 2017), a genomic and plasmid comparison of SH4 and SH12 with the rest of the study strains was performed to check the presence of these plasmid sequences and in particular, of the TA system. The results allowed to exclude the probiotic Pdp11 since it lacks the TA type II system as a virulence factor and its self-regulatory characteristics.

Phenotypic and pathogenic differences had been identified between the pathogenic strains SH4 and SH12. The lethal doses (LD50) of strains SH4 and SH12 were 2.4 x 10^6 and 2.8 x 10^6, respectively, in addition to obvious differences in metabolic characteristics between these two strains (Esteve, Merchán, and Alcaide 2017). However, taking into account previous results on phenotypic (Esteve, Merchán, and Alcaide 2017) and genomic (Seoane et al. 2019) characteristics, our results seem to indicate a greater similarity between both strains, and may even suggest considering them as possible clones with specific differences. In any case, the absence in Pdp11 of plasmids carrying virulence genes supports the idea of its probiotic nature and opens the way for future research on biotechnological applications. To comply with EU regulations on GMOs, these could be aimed at obtaining a higher probiotic value through natural processes (conjugation, transformation and transduction). The use of genetic engineering to improve derived products such as metabolites and extracellular products, among others, could also be considered.

Conclusions

The presence of plasmids is expected to be associated with bacterial survival and, commonly, with virulence factors. The present work has characterized two new plasmids present in two strains of *S. putrefaciens*, SH4 and SH12, which despite having similar genomic and plasmid profiles with high levels of identity, turned out to be different strains of the same species. The plasmids identified encode several proteins of known or unknown function. Among the former, two were found, one belonging to an essential protein, repB, necessary for plasmid replication, and the other, pemK, corresponding to a virulence factor. The probiotic strain *S. putrefaciens* Pdp11 did not present plasmids, which may be behind its probiotic nature, making it unique in comparison to other *Shewanella putrefaciens* strains.

Acknowledgements

A.G-P acknowledges to the BIO-264 Research Group from the Consejería de Economía, Innovación y Ciencia de la Junta de Andalucía, with the support of the European Regional Development Fund (ERDF) and the European Social Fund (ESF). The authors are grateful for the computing resources and technical support provided by the Plataforma Andaluza de Bioinformática of the University of Malaga.

Funding

This study was supported by MINECO and co-financed by FEDER funds (Grant AG-2017-509 83370-C3-3-R).

Competing interest

Ana Grande Pérez is an Academic Editor for PeerJ.

References

Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215(3): 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Antonio-Hernández, Francisco Miguel, Selene Silva-Perez, Alma Reyn Osorio-Martínez, Rodolfo Daniel Alday-Reyes, Ma. Guadalupe Aguilera-Arreola, Graciela Margarita González-Lugo, Pérez-Valdespino Abigail, Rosa Margarita Hernández-Velez, Rosa Lilia Guzmán Hernández, and Araceli Contreras-Rodríguez. 2019. "Identification, and Characterization of Shewanella Haliotis and Shewanella Chilikensis Isolated from Ready-to-Eat Seafood and Their Capacity to Release Outer Membrane" *Journal of Bioengineering and Biomedicine Research* 3(3): 1-12.

Assefa, Ayalew, and Fufa Abunna. 2018. "Maintenance of Fish Health in Aquaculture: Review of Epidemiological Approaches for Prevention and Control of Infectious Disease of Fish." *Veterinary Medicine International*. https://doi.org/10.1155/2018/5432497.

Ayad, Lorraine A.K., and Solon P. Pissis. 2017. "MARS: Improving Multiple Circular Sequence Alignment Using Refined Sequences." *BMC Genomics 18(1): 1-10*. https://doi.org/10.1186/s12864-016-3477-5.

Bankevich, Anton, Sergey Nurk, Dmitry Antipov, Alexey A. Gurevich, Mikhail Dvorkin, Alexander S. Kulikov, Valery M. Lesin, et al. 2012. "SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing." *Journal of Computational Biology 19(5): 455-477*. https://doi.org/10.1089/cmb.2012.0021.

Besemer, John, Alexandre Lomsadze, and Mark Borodovsky. 2001. "GeneMarkS: A Self-Training Method for Prediction of Gene Starts in Microbial Genomes. Implications for Finding

Sequence Motifs in Regulatory Regions." *Nucleic Acids Research* 29(12): 2607–18. https://doi.org/10.1093/nar/29.12.2607.

Bleriot, Ines, Lucia Blasco, Mercedes Delgado-Valverde, Ana Gual de Torella, Anton Ambroa, Laura Fernandez-Garcia, Maria Lopez, et al. 2020. "Mechanisms of Tolerance and Resistance to Chlorhexidine in Clinical Strains of *Klebsiella Pneumoniae* Producers of Carbapenemase: Role of New Type II Toxin-Antitoxin System, PemIK." *Toxins* 12(9): 1–17. https://doi.org/10.3390/toxins12090566.

Bowden, T. J., I. R. Bricknell, and B. M. Preziosi. 2018. "Comparative Pathogenicity of *Vibrio* spp., *Photobacterium damselae* ssp. *damselae* and Five Isolates of *Aeromonas salmonicida* ssp. *achromogenes* in Juvenile *Atlantic Halibut* (Hippoglossus Hippoglossus)." *Journal of Fish Diseases* 41 (1): 79–86. https://doi.org/10.1111/jfd.12679.

Bukowski, Michal, Rafal Piwowarczyk, Anna Madry, Rafal Zagorski-Przybylo, Marcin Hydzik, and Benedykt Wladyka. 2019. "Prevalence of Antibiotic and Heavy Metal Resistance Determinants and Virulence-Related Genetic Elements in Plasmids of *Staphylococcus aureus*." *Frontiers in Microbiology* 10(4): 1–14. https://doi.org/10.3389/fmicb.2019.00805.

Bushnell, Brian. 2015. "BBMap." Https://Sourceforge.Net/Projects/Bbmap/. 2015.

Chabrillón, M., R. M. Rico, M. C. Balebona, and M. A. Moriñigo. 2005. "Adhesion to Sole, *Solea senegalensis* Kaup, Mucus of Microorganisms Isolated from Farmed Fish, and Their Interaction with *Photobacterium damselae* subsp. *piscicida*." *Journal of Fish Diseases* 28(4): 229–37. https://doi.org/10.1111/j.1365-2761.2005.00623.x.

Chai, Yunrong, and Stephen C. Winans. 2005. "RepB Protein of an *Agrobacterium tumefaciens* Ti Plasmid Binds to Two Adjacent Sites between RepA and RepB for Plasmid Partitioning and Autorepression." *Molecular Microbiology* 58(4): 1114-1129. https://doi.org/10.1111/j.1365-2958.2005.04886.x.

Chevreux, Bastien. 2005. "MIRA: An Automated Genome and EST Assembler." *Duisburg: Heidelberg*.

Conlan, Sean, Pamela J. Thomas, Clayton Deming, Morgan Park, Anna F. Lau, John P. Dekker, Evan S. Snitkin, et al. 2014. "Single-Molecule Sequencing to Track Plasmid Diversity of Hospital-Associated Carbapenemase-Producing Enterobacteriaceae." *Science Translational Medicine* 6 (254). https://doi.org/10.1126/scitranslmed.3009845.

Cordero, Héctor, Francisco A. Guardiola, Silvana Teresa Tapia-Paniagua, Alberto Cuesta, José

Meseguer, M. Carmen Balebona, M. Ángel Moriñigo, and M. Ángeles Esteban. 2015. "Modulation of Immunity and Gut Microbiota after Dietary Administration of Alginate Encapsulated *Shewanella putrefaciens* Pdp11 to Gilthead Seabream (*Sparus aurata* L.)." *Fish and Shellfish Immunology* 45(2): 608-618. https://doi.org/10.1016/j.fsi.2015.05.010.

Cui, Yifang, Erwin Märtlbauer, Richard Dietrich, Hailing Luo, Shuangyang Ding, and Kui Zhu. 2019. "Multifaceted Toxin Profile, an Approach toward a Better Understanding of Probiotic *Bacillus cereus*." *Critical Reviews in Toxicology* 49(4): 342–56. https://doi.org/10.1080/10408444.2019.1609410.

Díaz-Rosales, P., S. Arijo, M. Chabrillón, F. J. Alarcón, S. T. Tapia-Paniagua, E. Martínez-Manzanares, M. C. Balebona, and M. A. Moriñigo. 2009a. "Effects of Two Closely Related Probiotics on Respiratory Burst Activity of Senegalese Sole (*Solea senegalensis*, Kaup) Phagocytes, and Protection against *Photobacterium damselae* subsp. *piscicid*a." *Aquaculture* 293 (1–2): 16–21. https://doi.org/10.1016/j.aquaculture.2009.03.050.

Esteve, C., R. Merchán, and E. Alcaide. 2017. "An Outbreak of *Shewanella putrefaciens* Group in Wild Eels *Anguilla anguilla* L. Favoured by Hypoxic Aquatic Environments." *Journal of Fish Diseases* 40 (7): 929–39. https://doi.org/10.1111/jfd.12574.

FAO. 2020. *De La Pesca Y La Acuicultura*. *Marine Pollution Bulletin*. Vol. 3. http://dx.doi.org/10.1016/j.marpolbul.2013.01.032%0Ahttp://dx.doi.org/10.1016/j.tws.2012.02.007%0Ahttp://www.fao.org/publications/es.

Galata, Valentina, Tobias Fehlmann, Christina Backes, and Andreas Keller. 2019. "PLSDB: A Resource of Complete Bacterial Plasmids." *Nucleic Acids Research 47*(D1), D195-D202. https://doi.org/10.1093/nar/gky1050.

Galtier, N., M. Gouy, and C. Gautier. 1996. "Seaview and Phylo_ Win: Two Graphic Tools for Sequence Alignment and Molecular Phylogeny." *Bioinformatics* 12(6): 543-548. https://doi.org/10.1093/bioinformatics/12.6.543.

García de La Banda, Inés, Carmen Lobo, Juan M. León-Rubio, Silvana Tapia-Paniagua, M. Carmen Balebona, Miguel A. Moriñigo, Xabier Moreno-Ventas, et al. 2010. "Influence of Two Closely Related Probiotics on Juvenile Senegalese Sole (*Solea senegalensis*, Kaup 1858) Performance and Protection against *Photobacterium damselae* subsp. *piscicida*." *Aquaculture* 306 (1–4): 281–88. https://doi.org/10.1016/j.aquaculture.2010.05.008.

Ghattargi, Vikas C., Meghana A. Gaikwad, Bharati S. Meti, Yogesh S. Nimonkar, Kunal Dixit,

Om Prakash, Yogesh S. Shouche, Shrikant P. Pawar, and Dhiraj P. Dhotre. 2018. "Comparative Genome Analysis Reveals Key Genetic Factors Associated with Probiotic Property in *Enterococcus faecium* Strains." *BMC Genomics* 19(1): 1–16. https://doi.org/10.1186/s12864-018-5043-9.

Giess, Adam, Louise J Pankhurst, Alison Vaughan, Christopher J Grim, Heather L Cox, and Anthony J Yeh. 2016. "Dissemination of the Carbapenem Resistance Gene Bla KPC." *American Society for Microbioloy* 60 (6): 3767–78. https://doi.org/10.1128/AAC.00464-16.Address.

Gish, Warren, and David J. States. 1993. "Identification of Protein Coding Regions by Database Similarity Search." *Nature Genetics* 3(3): 266-272. https://doi.org/10.1038/ng0393-266.

Kechin, Andrey, Uljana Boyarskikh, Alexander Kel, and Maxim Filipenko. 2017. "CutPrimers: A New Tool for Accurate Cutting of Primers from Reads of Targeted Next Generation Sequencing." *Journal of Computational Biology* 24(11): 1138-1143. https://doi.org/10.1089/cmb.2017.0096.

Kelley, Lawrence A., Stefans Mezulis, Christopher M. Yates, Mark N. Wass, and Michael J.E. Sternberg. 2015. "The Phyre2 Web Portal for Protein Modeling, Prediction and Analysis." *Nature Protocols* 10(6): 845-858. https://doi.org/10.1038/nprot.2015.053.

Langmead, Ben, and Steven L. Salzberg. 2012. "Fast Gapped-Read Alignment with Bowtie 2." *Nature Methods* 9(4): 357-359. https://doi.org/10.1038/nmeth.1923.

Langmead, Ben, Cole Trapnell, Daehwan Kim, Rone Charles, Chris Wilks, and Valentin Antonescu. 2017. "Bowtie 2 Manual." Tuxedo Suite.

Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, and Richard Durbin. 2009. "The Sequence Alignment/Map Format and SAMtools." *Bioinformatics* 25(16): 078-2079. https://doi.org/10.1093/bioinformatics/btp352.

Lobo, Carmen, Silvana Tapia-Paniagua, Xabier Moreno-Ventas, F. Javier Alarcón, Covadonga Rodríguez, Ma Carmen Balebona, Miguel A. Moriñigo, and Inés García de La Banda. 2014. "Benefits of Probiotic Administration on Growth and Performance along Metamorphosis and Weaning of Senegalese Sole (*Solea senegalensis*)." *Aquaculture* 433: 183–95. https://doi.org/10.1016/j.aquaculture.2014.06.011.

Macrina, Francis L., Dennis J. Kopecko, Kevin R. Jones, Deborah J. Ayers, and Sara M.

McCowen. 1978. “A Multiple Plasmid-Containing *Escherichia coli* Strain: Convenient Source of Size Reference Plasmid Molecules.” *Plasmid* 1(3): 417–20. https://doi.org/10.1016/0147-619X(78)90056-2.

Maguire, Finlay, Baofeng Jia, Kristen L. Gray, Wing Yin Venus Lau, Robert G. Beiko, and Fiona S.L. Brinkman. 2020. “Metagenome-Assembled Genome Binning Methods with Short Reads Disproportionately Fail for Plasmids and Genomic Islands.” *Microbial Genomics* 6(10): 1–12. https://doi.org/10.1099/mgen.0.000436.

Marcos-López, M., P. Gale, B. C. Oidtmann, and E. J. Peeler. 2010. “Assessing the Impact of Climate Change on Disease Emergence in Freshwater Fish in the United Kingdom.” *Transboundary and Emerging Diseases* 57(5): 293–304. https://doi.org/10.1111/j.1865-1682.2010.01150.x.

Medina, Alberto, Miguel Ángel Moriñigo, and Salvador Arijo. 2020. “Selection of Putative Probiotics Based on Antigen-Antibody Cross-Reaction with *Photobacterium damselae* subsp. *piscicida* and *Vibrio harveyi* for Use in Senegalese sole (*Solea senegalensis*).” *Aquaculture Reports* 17(5): 100366. https://doi.org/10.1016/j.aqrep.2020.100366.

Minkin, Ilya, Anand Patel, Mikhail Kolmogorov, Nikolay Vyahhi, and Son Pham. 2013. “Sibelia: A Scalable and Comprehensive Synteny Block Generation Tool for Closely Related Microbial Genomes.” In *Lecture Notes in Computer Science* (pp. 215-229). Springer, Berlin, (Heidelberg). https://doi.org/10.1007/978-3-642-40453-5_17.

Ng, I. Son, Yanlan Guo, Yunli Zhou, Jhe Wei Wu, Shih I. Tan, and Ying Chen Yi. 2018. “Turn on the Mtr Pathway Genes under Placi Promoter in *Shewanella oneidensis* MR-1.” *Bioresources and Bioprocessing* 5(1): 1-11. https://doi.org/10.1186/s40643-018-0221-9.

Orlek, Alex, Nicole Stoesser, Muna F. Anjum, Michel Doumith, Matthew J. Ellington, Tim Peto, Derrick Crook, et al. 2017. “Plasmid Classification in an Era of Whole-Genome Sequencing: Application in Studies of Antibiotic Resistance Epidemiology.” *Frontiers in Microbiology* 8 (2): 1–10. https://doi.org/10.3389/fmicb.2017.00182.

Paździor, Ewa. 2016. “Shewanella Putrefaciens - A New Opportunistic Pathogen of Freshwater Fish.” *Journal of Veterinary Research (Poland)* 60(4): 429–34. https://doi.org/10.1515/jvetres-2016-0064.

Paździor, Ewa, Agnieszka Pękala-Safińska, and Dariusz Wasyl. 2019. “Phenotypic Diversity and Potential Virulence Factors of the *Shewanella putrefaciens* Group Isolated from Freshwater

564 Fish." *Journal of Veterinary Research* 63 (3): 321–32. https://doi.org/10.2478/jvetres-2019-
565 0046.
566 Pesaro, Manuel, Franco Widmer, Gilles Nicollier, and Josef Zeyer. 2003. "Effects of Freeze-Thaw
567 Stress during Soil Storage on Microbial Communities and Methidathion Degradation." *Soil*
568 *Biology and Biochemistry* 35 (8): 1049–61. https://doi.org/10.1016/S0038-0717(03)00147-0.
569 Pinto, Uelinton M., Katherine M. Pappas, and Stephen C. Winans. 2012. "The ABCs of Plasmid
570 Replication and Segregation." *Nature Reviews Microbiology* 10(11): 755-765.
571 https://doi.org/10.1038/nrmicro2882.
572 Quainoo, Scott, Jordy P. M. Coolen, C Sacha A. F. T. van Hijum, c Willem J. G. Melchers Martijn
573 A. Huynen, E Willem van Schaik, and Heiman F. L. Wertheim. 2017. "Crossm Whole-
574 Genome Sequencing of Bacterial Pathogens : The Future of Nosocomial." *Clinical*
575 *Microbiology Reviews* 30 (4): 1015–64. https://doi.org/10.1128/CMR.00016-17
576 Sáenz De Rodrigáñez, M. A., P. DÍaz-Rosales, M. ChabrillÓn, H. Smidt, S. Arijo, J. M. LeÓn-
577 Rubio, F. J. AlarcÓn, et al. 2009. "Effect of Dietary Administration of Probiotics on Growth
578 and Intestine Functionality of Juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858)."
579 *Aquaculture Nutrition* 15 (2): 177–85. https://doi.org/10.1111/j.1365-2095.2008.00581.x.
580 San Millan, Alvaro, and R. Craig Maclean. 2019. "Fitness Costs of Plasmids: A Limit to Plasmid
581 Transmission." *Microbial Transmission* 5(5): 5-5.
582 https://doi.org/10.1128/9781555819743.ch4.
583 Santos, Dayane Da Silva, Priscilla Régia De Andrade Calaça, Ana Lúcia Figueiredo Porto, Paulo
584 Roberto Eleutério De Souza, Nara Suzy Aguiar De Freitas, and Maria Taciana Cavalcanti
585 Vieira Soares. 2020. "What Differentiates Probiotic from Pathogenic Bacteria? The Genetic
586 Mobility of *Enterococcus faecium* Offers New Molecular Insights." *OMICS A Journal of*
587 *Integrative Biology* 24 (12): 706–13. https://doi.org/10.1089/omi.2020.0078.
588 Seegers, Jos F. M. L., Thi Phuong Nam Bui, and Willem M. de Vos. 2021. *Remarkable Metabolic*
589 *Versatility of the Commensal Bacteria Eubacterium hallii* and *Intestinimonas*
590 *butyriciproducens:* Potential Next-Generation Therapeutic Microbes. In *Probiotic Bacteria*
591 *and Postbiotic Metabolites: Role in Animal and Human Health* (pp. 139-151). Springer,
592 Singapore. https://doi.org/10.1007/978-981-16-0223-8_5.
593 Seoane, Pedro, Silvana T. Tapia-Paniagua, Rocío Bautista, Elena Alcaide, Consuelo Esteve,
594 Eduardo Martínez-Manzanares, M. Carmen Balebona, M. Gonzalo Claros, and Miguel A.

595 Moriñigo. 2019. “TarSynFlow, a Workflow for Bacterial Genome Comparisons That
596 Revealed Genes Putatively Involved in the Probiotic Character of *Shewanella putrefaciens*
597 Strain Pdp11.” *PeerJ* 7, e6526. https://doi.org/10.7717/peerj.6526.
598 Sharifuzzaman, S.M., and Brian Austin. 2017. “Probiotics for Disease Control in Aquaculture.”
599 *Diagnosis and Control of Diseases of Fish and Shellfish*, 189–222.
600 https://doi.org/10.1002/9781119152125.ch8.
601 Sievers, Fabian, and Desmond G. Higgins. 2014. “Clustal Omega.” *Current Protocols in*
602 *Bioinformatics* 48(1): 3-13. https://doi.org/10.1002/0471250953.bi0313s48.
603 Solovyev, Victor, Asaf Salamov, Igor Seledtsov, Denis Vorobyev, and Alexander Bachinsky.
604 2011. “Automatic Annotation of Bacterial Community Sequences and Application to
605 Infections Diagnostic.” *Proceedings of the International Conference on Bioinformatics*
606 *Models, Methods and Algorithms* (1): 346–53. DOI: 10.5220/0003333703460353
607 Sonnenborn, Ulrich, and Jürgen Schulze. 2009. “The Non-Pathogenic *Escherichia coli* Strain
608 Nissle 1917-Features of a Versatile Probiotic.” *Microbial Ecology in Health and Disease* 21
609 (3–4): 122–58. https://doi.org/10.3109/08910600903444267.
610 Stolz, Andreas. 2014. “Degradative Plasmids from Sphingomonads.” *FEMS Microbiology Letters*
611 350 (1): 9–19. https://doi.org/10.1111/1574-6968.12283.
612 Tanizawa, Yasuhiro, Takatomo Fujisawa, and Yasukazu Nakamura. 2018. “DFAST: A Flexible
613 Prokaryotic Genome Annotation Pipeline for Faster Genome Publication.” *Bioinformatics*
614 34(6): 1037-1039. https://doi.org/10.1093/bioinformatics/btx713.
615 Tapia-Paniagua, Silvana Teresa, Mariana Chabrillón, Patricia Díaz-Rosales, Inés García de la
616 Banda, Carmen Lobo, Ma Carmen Balebona, and Miguel Angel Moriñigo. 2010. “Intestinal
617 Microbiota Diversity of the Flat Fish *Solea senegalensi*s (Kaup, 1858) Following Probiotic
618 Administration.” *Microbial Ecology* 60 (2): 310–19. https://doi.org/10.1007/s00248-010-
619 9680-z.
620 Uhrynowski, Witold, Monika Radlinska, and Lukasz Drewniak. 2019. “Genomic Analysis of
621 *Shewanella* sp. O23s—the Natural Host of the Psheb Plasmid Carrying Genes for Arsenic
622 Resistance and Dissimilatory Reduction.” *International Journal of Molecular Sciences* 20
623 (5): 1018. https://doi.org/10.3390/ijms20051018.
624 Untergasser, Andreas, Ioana Cutcutache, Triinu Koressaar, Jian Ye, Brant C. Faircloth, Maido
625 Remm, and Steven G. Rozen. 2012. “Primer3-New Capabilities and Interfaces.” *Nucleic*

626 *Acids Research* 40(15): e115-e115.https://doi.org/10.1093/nar/gks596.
627 Wawrzyniak, Paweł, Agnieszka Sobolewska-Ruta, Piotr Zaleski, Natalia Łukasiewicz, Paulina
628 Kabaj, Piotr Kierył, Agata Gościk, et al. 2019. "Molecular Dissection of the Replication
629 System of Plasmid PIGRK Encoding Two In-Frame Rep Proteins with Antagonistic
630 Functions." *BMC Microbiology* 19(1): 1-15. https://doi.org/10.1186/s12866-019-1595-3.
631 Werbowy, Katarzyna, Hubert Cieśliński, and Józef Kur. 2009. "Characterization of a Cryptic
632 Plasmid PSFKW33 from *Shewanella* sp. 33B." *Plasmid* 62(1): 44–49.
633 https://doi.org/10.1016/j.plasmid.2009.03.003.
634 Wijegoonawardane, Priyanjalie K.M., Nusra Sittidilokratna, Natthida Petchampai, Jeff A. Cowley,
635 Nicholas Gudkovs, and Peter J. Walker. 2009. "Homologous Genetic Recombination in the
636 Yellow Head Complex of Nidoviruses Infecting *Penaeus monodon* Shrimp." *Virology* 390
637 (1): 79–88. https://doi.org/10.1016/j.virol.2009.04.015.
638 Yamaguchi, Yoshihiro, Jung Ho Park, and Masayori Inouye. 2011. "Toxin-Antitoxin Systems in
639 Bacteria and Archaea." *Annual Review of Genetics* 45: 61–79.
640 https://doi.org/10.1146/annurev-genet-110410-132412.
641 Yao, Jianyun, Yunxue Guo, Pengxia Wang, Zhenshun Zeng, Baiyuan Li, Kaihao Tang, Xiaoxiao
642 Liu, and Xiaoxue Wang. 2018. "Type II Toxin/Antitoxin System ParESO/CopASO Stabilizes
643 Prophage CP4So in *Shewanella oneidensis*." *Environmental Microbiology* 20 (3): 1224–39.
644 https://doi.org/10.1111/1462-2920.14068.
645 Yousfi, Khadidja, Abdelaziz Touati, Brigitte Lefebvre, Éric Fournier, Jean Charles Côté, Hafid
646 Soualhine, Matthew Walker, Djamila Bougdour, Cécile Tremblay, and Sadjia Bekal. 2017.
647 "A Novel Plasmid, PSx1, Harboring a New Tn1696 Derivative from Extensively Drug-
648 Resistant *Shewanella xiamenensis* Encoding OXA-416." *Microbial Drug Resistance* 23 (4):
649 429–36. https://doi.org/10.1089/mdr.2016.0025.
650 Zarate-Perez, Francisco, Martino Bardelli, John W. Burgner, Maria Villamil-Jarauta, Kanni Das,
651 Demet Kekilli, Jorge Mansilla-Soto, R. Michael Linden, and Carlos R. Escalante. 2012. "The
652 Interdomain Linker of AAV-2 Rep68 Is an Integral Part of Its Oligomerization Domain: Role
653 of a Conserved SF3 Helicase Residue in Oligomerization." Edited by Craig Meyers. *PLoS*
654 *Pathogens* 8 (6): e1002764. https://doi.org/10.1371/journal.ppat.1002764.
655 Zhou, Yunli, and I. Son Ng. 2016. "Explored a Cryptic Plasmid PSXM33 from *Shewanella*
656 *xiamenensis* BC01 and Construction as the Shuttle Vector." *Biotechnology and Bioprocess*

657 *Engineering* 21 (1): 68–78. https://doi.org/10.1007/s12257-015-0618-7.
658

Figure 1

Figure 1. Flowchart depicting the workflow used to obtain fully assembled plasmid DNA sequences from *S. putrefaciens* strains SH4 and SH12.

Within a dashed box, the two sources of plasmid sequences are shown. Quality trimming steps (A) performed with the BBDuk software leading to capture read set are indicated by the letters B, and those leading to assembly read set by the letters C. The Bowtie and Samtools programs, denoted with the letter D, were used to capture specific Illumina sequences. The output of the Mira and Spades programs was a sequence generated from Illumina and Sanger reads (E),and circularized by the MARS method (F). Plasmids were aligned by the Clustal method of the Seaview program (G) to finally obtain the plasmid consensus sequences. The consensus sequence was mapping by sanger, illumina and PCR reads for assembly validation.

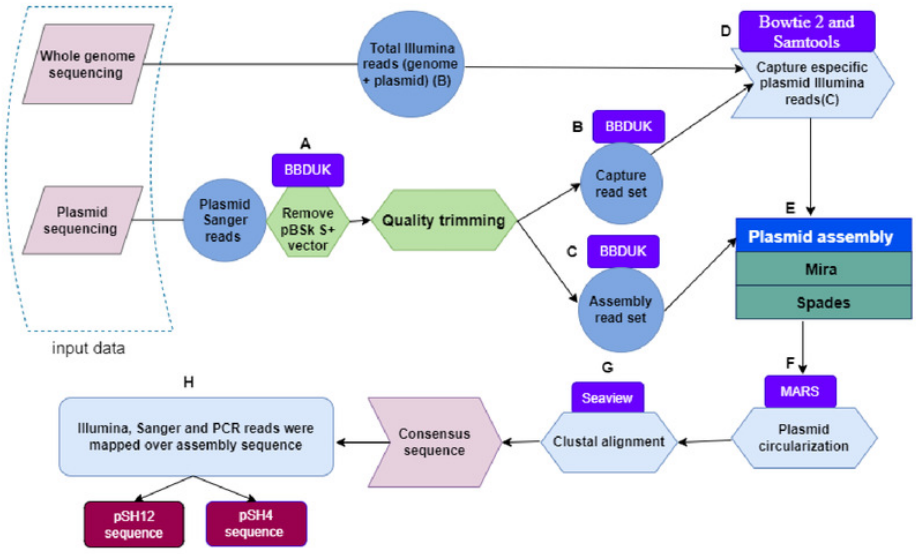
Whole genome sequencing
Total Illumina reads (genome + plasmid) (B)
D
Bowtie 2 and Samtools
Capture especific plasmid Illumina reads(C)
B
BBDUK
Capture read set
A
BBDUK
Plasmid sequencing
Plasmid Sanger reads
Remove pBSk S+ vector
Quality trimming
C
BBDUK
Assembly read set
E
Plasmid assembly
Mira
Spades
input data
F
MARS
Plasmid circularization
G
Seaview
Clustal alignment
Consensus sequence
H
Illumina, Sanger and PCR reads were mapped over assembly sequence
pSH12 sequence
pSH4 sequence

Figure 2

Figure 2. Plasmid DNA isolated from different *S. putrefaciens* strains (SH4, SH12, SH6, SH16, SH9, Pdp11, SdM1 and SdM2) separated by agarose gel electrophoresis.

All strains were grown under the optimal growth conditions of 23°C in TSBs under shaking for 24h. The sizes of the individual plasmids contained in strains SH4 and SH12 as well as the five plasmids detected of the positive control E. coli O157, grown in LB broth, are indicated.

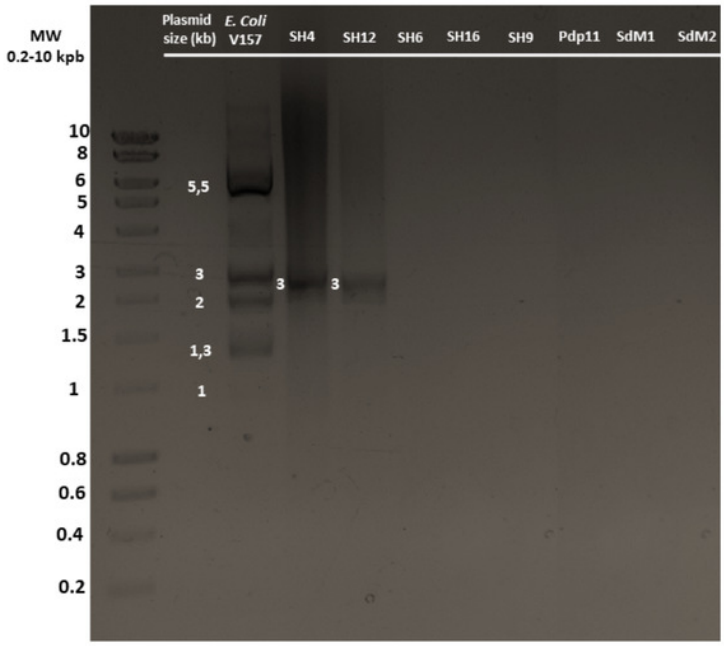

Figure 3

Figure 3. Complete consensus sequences of pSH4 (A) and pSH12 (B) showing the unvalidated regions (blue line) resolved by mapping with Illumina and Sanger sequences.

Both unvalidated regions in the consensus sequences were subjected to validation by PCR amplification (A) The unvalidated region of pSH4 supported by 22 Illumina sequence (red bars) and one Sanger sequence (green bar) led to a reliable sequence between positions 1107 and 1176 in the pSH4 consensus sequence. (B) The unvalidated region of pSH12, located between nucleotides 2021 and 2090, was poorly supported by Illumina sequences and no Sanger sequences, so the consensus sequence of pSH12 turned out to be 30 nucleotides shorter than that of SH4.

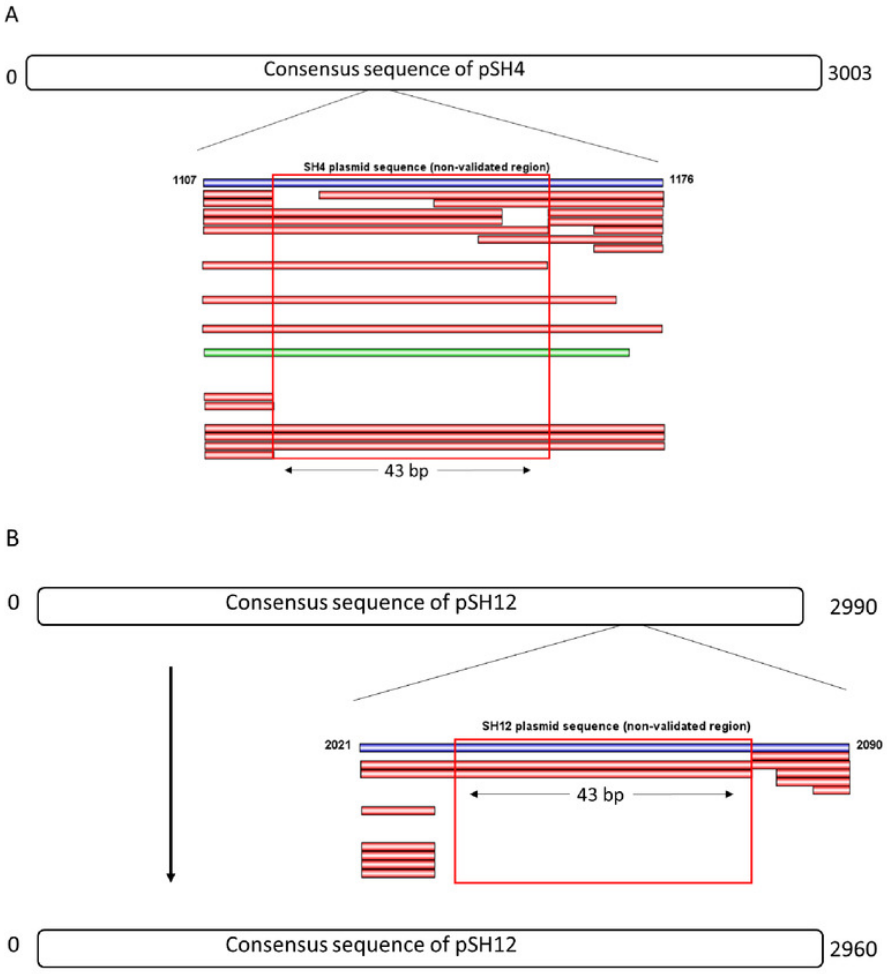
A
0
Consensus sequence of pSH4
3003
SH4 plasmid sequence (non-validated region)
1107
1176
43 bp
B
0
Consensus sequence of pSH12
2990
SH12 plasmid sequence (non-validated region)
2021
2090
43 bp
0
Consensus sequence of pSH12
2960

Figure 4

Figure 4. Circular map of pSH4 (A) and pSH12 (B).

The main circle that shapes the plasmid is coloured according to the percentage of G+C shown in the bottom bar. Solid arrows indicate the approximate position of the ORF and the orientation of the transcript. ORFs encoded by the (+) strand of the nucleotide sequence are outside the circle and ORFs encoded by the (-) strand inside. The range of ORF colours on the map reveals differences according to the analysis of the putative translation products and their respective functions. The blue colour corresponds to informative ORFs, whose amino acid sequences showed similar homologies with partial proteins of known function and hypothetical proteins in both plasmids, according to protein searches. In addition, they are labeled as complete genes by the algorithms used. The rest of ORFs are drawn with pink arrows.

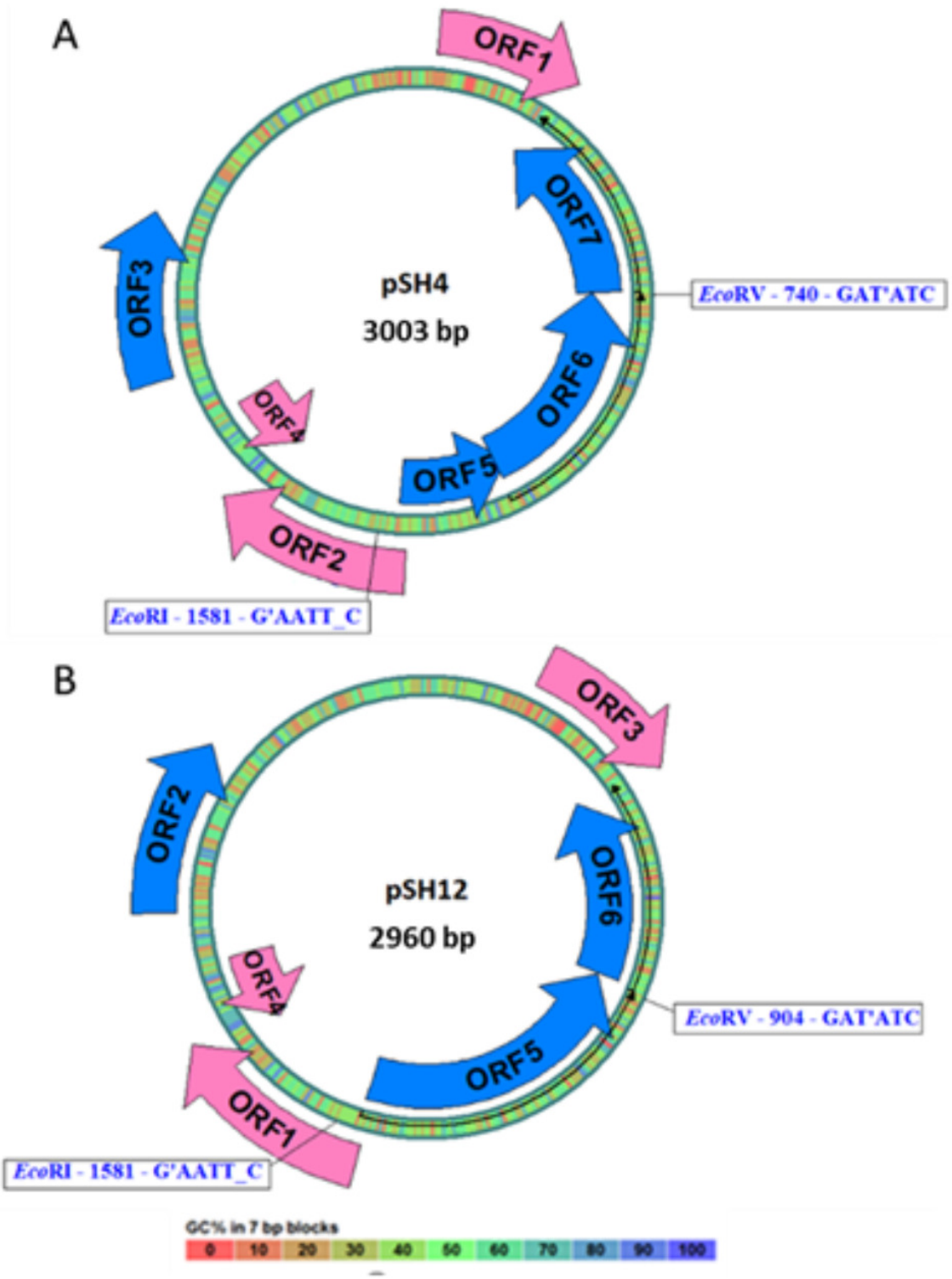
A
ORF1
ORF7
ORF3
pSH4
3003 bp
EcoRV - 740 - GAT'ATC
ORF4
ORF6
ORF5
ORF2
EcoRI - 1581 - G'AATT_C
B
ORF3
ORF2
ORF6
pSH12
2960 bp
ORF4
EcoRV - 904 - GAT'ATC
ORF5
ORF1
EcoRI - 1581 - G'AATT_C
GC% in 7 bp blocks
0 10 20 30 40 50 60 70 80 90 100

Figure 5

Figure 4. Phylogenetic tree using repB protein sequences of pSH12 and pSH4.

The different protein sequences were downloaded from the NCBI database, and alignment was performed by Clustal method followed by PhyML algorithm to infer distances by using the Seaview software.

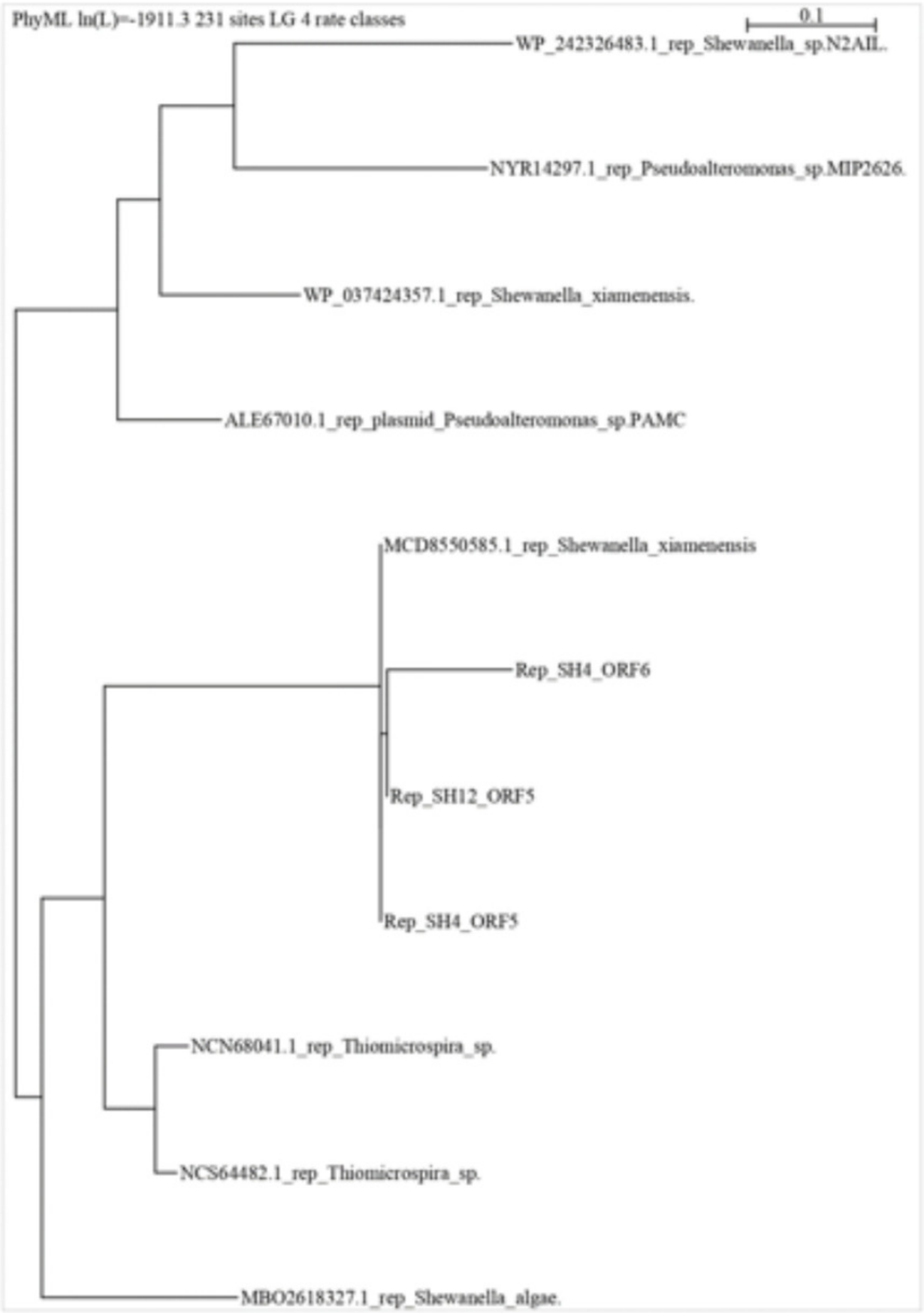
PhyML ln(L)=-1911.3 231 sites LG 4 rate classes
0.1
WP_242326483.1_rep_Shewanella_sp.N2AIL.
NYR14297.1_rep_Pseudoalteromonas_sp.MIP2626.
WP_037424357.1_rep_Shewanella_xiamenensis.
ALE67010.1_rep_plasmid_Pseudoalteromonas_sp.PAMC
MCD8550585.1_rep_Shewanella_xiamenensis
Rep_SH4_ORF6
Rep_SH12_ORF5
Rep_SH4_ORF5
NCN68041.1_rep_Thiomicrospira_sp.
NCS64482.1_rep_Thiomicrospira_sp.
MBO2618327.1_rep_Shewanella_algae.

Table 1(on next page)

Table 1. Common ORFs found in pSH4 and pSH12 in all predictions tools tested.

[a] ORFs were named as ORFn followed by the plasmid name (pSH12 or pSH4), where n is the ORF id.

Strain	ORF[a]	Strand	Start	Stop	Lenght (nt|aa)	G+C content (%)
SH4	3	+	2109	2420	312 | 103	36.74
	5	-	1541	1158	384 | 127	57.22
	6	-	1288	728	561 | 186	44.15
	7	-	727	275	453 | 150	44.35
SH12	2	+	2143	2454	312 | 103	42.47
	5	-	1575	889	687 | 228	46.44
	6	-	888	436	453 | 150	48.76

1

Table 2(on next page)

Table 2. Functional annotation of the main ORFs of pSH4 and pSH12 by blastp and Phyre2.

Strain		Blastp					Phyre 2	
	ORF	Accesion ID	Protein	Identity (%)	Coverage (%)	Organism	Superfamily/family	Confidence
SH4	3	MBP7664129.1	Hypothetical protein	78,48	94,00	*Shewanella* sp	Family: BH3980-like	29,9
	5	WP_208142329.1	repB family plasmid replication initiator protein	76,92	89,00	*Shewanella algae*	Family:Replication initiation protein	100
	6	WP_208142329.1	repB family plasmid replication initiator protein	67,74	100,00	*Shewanella algae*	Superfamily:“Winged helix" DNA-binding domain Family: Replication initiation protein	100
	7	WP_115615842.1	Type II toxin-antitoxin system PemK/MazF family toxin	54,17	96,00	*Avibacterium paragallinarum*	Superfamily: Cell growth inhibitor/plasmid maintenance toxic component Family: Kid/PemK	99,4
SH12	2	NCS64480.1	Hypothetical protein	87,18	92,00	*Thiomicrospira* sp.	BH3980-like	29,9
	5	WP_208142329.1	repB family plasmid replication initiator protein	72,89	100	*Shewanella algae*	Superfamily:“Winged helix" DNA-binding domain Family: Replication initiation protein	100
	6	WP_115615842.1	Type II toxin-antitoxin system PemK/MazF family toxin	54,17	96,00	*Avibacterium paragallinarum*	Superfamily: Cell growth inhibitor/plasmid maintenance toxic component Family: Kid/PemK	99,4

1