Gene sequences of the *pil* operon reveal relationships between symbiotic strains of *Vibrio fischeri*

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Abstract

Symbiosis between the bobtail squid *Euprymna scolopes* (Mollusca: Cephalopoda) and *Vibrio fischeri* bacteria has been a well-studied model for understanding the molecular mechanisms of colonization and adherence to host cells. For example, pilin expression has been observed to cause subtle variation in colonization for a number of Gram-negative bacteria with eukaryotic hosts. To investigate variation amongst *pil* genes of closely related strains of vibrios, we amplified *pil* genes *A*, *B*, *C* and *D* to determine orientation and sequence similarity to other symbiotic vibrios. The *pilA* gene was found to be upstream from all other *pil* genes, and not contiguous with the rest of the operon. The *pilB*, *pilC* and *pilD* loci were flanked at the 3′ end by *yacE*, followed by a conserved hypothetical gene. DNA sequences of each *pil* gene were aligned and analysed phylogenetically using parsimony for both individual and combined gene trees. Results demonstrate that certain *pil* loci (*pilB* and *pilD*) are conserved among strains of *V. fischeri*, but *pilC* differs in sequence between symbiotic and free-living strains. Phylogenetic analysis of all *pil* genes gives better resolution of Indo-west Pacific *V. fischeri* symbionts compared with analysis of the 16S rRNA gene. Hawaiian and Australian symbiotic strains form one monophyletic tree, supporting the hypothesis that *V. fischeri* strain specificity is selected by the geographical location of their hosts and is not related to specific squid species.

INTRODUCTION

The occurrence of bacterial symbionts with host partners is quite extensive and has been a topic for many evolutionary and developmental microbiologists (Nyholm & McFall-Ngai, 2004; Visick & Ruby, 2006). *Vibrio fischeri* belongs to the highly diverse family *Vibrionaceae* of the *Gammaproteobacteria* (Nishiguchi & Nair, 2003; Thompson *et al.*, 2004a). These luminous bacteria specifically colonize the light organ of sepiolid squids and monocentrid fishes (McFall-Ngai, 1994; McFall-Ngai & Ruby, 1998; Jones & Nishiguchi, 2004). The association begins after hatching, where bacteria are acquired environmentally via the light organ pores, which are continuously exposed to surrounding seawater. Although all environmental bacteria are in contact with the pores, only specific strains of *V. fischeri* can colonize and persist therein, out-competing non-native strain even during direct competition (Nishiguchi *et al.*, 1998; Nishiguchi, 2002).

Features known to play significant roles in success of the squid–symbiont relationship include chemotaxis, specific adherence, colonization, persistence and out-competing other bacterial strains (Graf *et al.*, 1994; Millikan & Ruby, 2004; Nyholm & McFall-Ngai, 2004).

Likewise, competition studies have determined that both ecological and environmental factors may be responsible for the evolutionary history of specificity between the partners (Nishiguchi *et al.*, 1998; Nishiguchi, 2000, 2002). Although a number of biochemical and molecular mechanisms are found to increase the ability of *V. fischeri* to colonize and persist in the light organ, phenotypic characteristics such as pili have been observed to increase the surface area between bacterium and host (Nair, 2006). Pili are long, filamentous projections on the surface of both Gram-positive and Gram-negative bacteria, and are known to be important in both colonization and twitching motility (Darzins & Russell, 1997). They also mediate adherence or attachment to both abiotic and biotic surfaces (Stabb & Ruby, 2003). Pili are formed by a number of proteins encoded by genes found in the *pil* operon, and are grouped as types I, II, III and IV based on the pathway by which they are secreted (Soto & Hultgren, 1999). For example, *V. fischeri* expresses type IV pili, which are secreted by a pathway homologous to the type II secretion apparatus (Nunn, 1999; Sandkvist, 2001). The *pilA* gene codes for the major structural pilus component and *pilB* is involved in nucleotide binding, which controls the extension of type IV pili by the process of polymerization of the pilin subunit (Villar *et al.*, 2001). *pilC* codes for a transmembrane protein that belongs to the GspF family of proteins and is involved in pilus assembly and adherence (Winther-Larsen *et al.*, 2001), while *pilD* serves as a leader peptidase for processing PilA precursors and *N*-methylation of the first amino acid of the mature protein. Previous observations of different wild-type *V. fischeri* strains isolated from both seawater and squid light organs exhibited differences in pili (both *in vitro* and *in vivo*; Nair, 2006), which may influence the specificity between sepiolid squids and their *Vibrio* symbionts and may be useful loci to determine subtle strain differences when determining the phylogenetic relatedness between symbiotic and free-living strains of *V. fischeri*. Therefore, we sequenced individual *pil* genes from strains of *V. fischeri* isolated from different geographical locations from both *Euprymna* and *Sepiola* squid hosts and two monocentrid fishes, as well as some free-living isolates. All sequences were used to construct individual and combined phylogenetic trees to determine the relatedness amongst *pil* genes and to determine whether these loci can reveal whether host specificity via pili is a common character between phylogenetically distinct *V. fischeri* strains. In addition, we established the gene order in each of the strains tested to determine whether differences existed in the *pil* operons of free-living and symbiotic *V. fischeri*.

METHODS

Bacterial strains

Bacteria used in this study were isolated previously from a number of sepiolid squids and monocentrid fishes, as well as free-living strains from seawater (Table 1) (Nishiguchi & Nair, 2003). Bacteria were initially plated from a frozen glycerol stock onto Luria– Bertani high-salt (32 %) (LBS) agar and grown at their optimum temperature (28 uC). A single colony-forming unit (c.f.u.) was then used to inoculate 5 ml LBS, grown for 12 h overnight at the optimum growth temperature and used subsequently for DNA isolation. Chromosomal DNA was extracted using the DNeasy kit (Qiagen) as described by the manufacturer. DNA was determined to be of PCR quality by electrophoresis on 1.0% agarose gels.

PCR amplifications

Individual loci of pilin genes were amplified by PCR in 25 µl reaction volumes. Primers were designed on the basis of the *V. fischeri* ES114 genome sequence (Ruby *et al.*, 2005) (http://www.ncbi.nlm.nih.gov/sites/entrez? Db=genomeprj&Cmd=Retrieve&list_uids=12986). Primers used in these experiments are listed in Table 2. All PCRs contained 2.5 mM $MgCl_2$, 1× reaction buffer (10 mM Tris/HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 2.5 mM each dNTP (Promega), 0.2 µM of each

primer and 0.2 U *Taq* polymerase (Promega) or AmpliTaq (Applied Biosystems). PCR conditions are described in Table 3. All reactions included a hot start for 2 min at 94 uC and a final termination for 7 min at 72 °C using a DNA Engine Dyad (MJ Research). Amplified products were visualized via gel electrophoresis on a 1% agarose gel stained with ethidium bromide.

Southern blotting

Southern blotting was used to test whether strains of *V. fischeri* that had unsuccessful PCR amplification of *pil* genes still possessed homologous regions of the *pil* operon. Genomic DNA was digested with *Hin*dIII, run on a 1% agarose gel and transferred to nylon blotting paper (Millipore) by a standard method (Sambrook *et al.*, 1989). Each blot was probed with ESP915pilA (designed from the *V. fischeri* ES114 genome) and labelled with ^{32}P. Reactions contained 10 pmol oligonucleotide (5.0 μl), 2.0 μl kinase buffer (50 mM Tris/HCl, 10 mM MgCl$_2$, 0.1 mM EDTA, 5 mM DTT, 0.1 mM spermidine), 2.0 μl sterile distilled water, 0.5 U polynucleotide kinase (1.0 μl) and 10 μl [γ-^{32}P]ATP (100 μCi; Amersham Quebec). Labelling reactions were incubated at 37 °C for 30 min. Membranes were hybridized overnight individually with each probe at the specific activity of 5×10^5 μCi ml^{-1} at 48 °C. The hybridization solution was discarded and the membranes were washed twice for 15 min each with washing buffer at 48 °C. Membranes were then exposed to X-ray film for 3 h to determine the presence of the *pil* operon.

Cloning and sequencing

Reactions that produced the desired PCR product were purified with the Geneclean II kit (Bio 101) and sequenced using Applied Biosystems Big Dye (version 3.1). PCR products that proved difficult to sequence directly were cloned into pGEM-T Easy vector (Promega) using the manufacturer's protocol. Plasmid DNA from positive clones was extracted using the Qiagen miniprep kit and sequenced. Excess fluorescently labelled dNTPs were removed via spin columns or plates (Edge Biosystems) and DNA was subsequently sequenced using an Applied Biosystems 3100 automated sequencer. Accession numbers for all sequences generated are listed in Table 1.

Gene walking to determine gene order

New primers corresponding to the 5′ and 3′ ends of each *pil* locus were designed using the derived sequences to link and determine the order of individual *pil* loci. Using the Universal Genome Walker kit (BD Biosciences), chromosomal DNA was digested separately with four restriction enzymes, *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. The reactions were purified with 100% saturated phenol (pH 8.0), with the upper aqueous layer re-extracted with chloroform. DNA was precipitated with 2 vols cold 95% ethanol, 20 μg glycogen and 0.1 vols 3 M sodium acetate. The mixture was centrifuged at 12 000 ***g*** for 10 min and dissolved in TE (10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5).

Ligation reactions included 4 μl digested DNA, 25 μM GenomeWalker adaptor, 1.6 μl ligation buffer and 0.5 μl (3 U) T4 DNA ligase. Reactions were performed overnight at 16 °C in a DNA Engine Dyad (MJ Research). Samples were diluted 1 : 100 with sterile distilled water and used for PCRs. PCR was performed using Herculase *Taq* DNA polymerase (Stratagene), AP1 primer and individual *pilC* primers (Table 2). The PCR was completed in 25 μl reaction volumes containing 2.5 μl 10× reaction buffer, 0.2 μl dNTPs (25 mM each), 0.5 μg each primer and 1.25 U Herculase *Taq* polymerase. The following conditions were followed: seven cycles at 94 °C for 25 s and the primer T_m for 3 min; 32 cycles of 94 °C for 25 s and the primer T_m minus 3 °C for 3 min and a final extension at 61 °C for 7 min. PCR products were purified using Geneclean II and sequenced directly (ABI Big Dye v. 3.1) or cloned into pGEM-T Easy vector as described above. Fluorescently labelled products were

purified using Edge DTR columns (Edge Biosystems) and analysed on an ABI 3100 automated sequencer.

Sequence alignment and phylogenetic analyses

Double-stranded DNA sequences were assembled and edited using the program Sequencher version 4.0 (Gene Codes). Sequences were translated to determine the correct reading frame and promoter region to establish whether the genes were in one continuous operon. Comparison between strains was essential, since the arrangement of *pil* genes was not universal among all *V. fischeri* strains tested, as well as the presence of all loci. Each sequence was compared with the NCBI Blastx search engine to determine similarity to other available *pil* sequences.

Molecular data were analysed with the computer program POY (Wheeler *et al.*, 2002) using the direct optimization method, with parsimony as the optimality criterion (Wheeler, 1996). This method assesses the number of DNA sequence transformations (evolutionary events) required by a phylogenetic topology directly, without the use of multiple sequence alignment. This is accomplished by generalization of existing character optimization procedures, including insertion and deletion events (indels) in addition to base substitutions. This method treats indels as processes, as opposed to the patterns implied by multiple sequence alignment (Wheeler, 1995).

Nodal support was calculated in POY using Farris's parsimony jackknifing procedure (Farris *et al.*, 1996) for 100 replicates (Using the commands –jackboot –replicates 100). Tree searches were conducted in parallel at Harvard University on a 19 dual-processor cluster using pvm (parallel virtual machine). Commands for load balancing of spawned jobs were used to optimize parallelization procedures (–parallel –dpm –jobspernode 2). Trees were built via a random addition sequence procedure (10 replicates) followed by a combination of branch-swapping steps [SPR (subtree pruning and regrafting) and TBR (tree bisection and reconnection)] and tree fusing (Goloboff, 1999) in order to improve tree length minimization further. Discrepancies between heuristic and actual tree length calculations were addressed by adjusting slop values (–slop5 – checkslop10). Phylogenetic trees were obtained using parsimony with a gap/ts/tv cost of equal weighting. The final tree was drawn with TreeView (Win32) and consensus trees were analysed in PAUP version 4.02b (Swofford, 2002). Nodal support for all jackknife calculations was performed in POY by the procedure described in Nishiguchi & Nair (2003). Individual gene trees for each of the *pil* loci were produced, as well as total molecular trees combining all *pil* loci and all *pil* loci with the 16S rRNA gene.

RESULTS AND DISCUSSION

Analysis of *pil* gene order and content

The amplified *pilA* gene from *V. fischeri* ESP915 was used as a probe (designed in this study) and hybridized to 2.5 kb fragments from *V. fischeri* strains EM17, ES114, ET101, SA1G and SR5 using Southern blot verification. This demonstrated the presence of a homologous *pilA* gene in those strains. In addition, a 4.5–5.0 kb band was observed in *V. fischeri* strains EB12 and WH1 and *Vibrio logei* strain SR18-1 (Fig. 1). This is expected, since previous evidence had demonstrated that the *V. fischeri* genome contains ten individual clusters of *pil* genes, of which two are *pilA* homologues (Ruby *et al.*, 2005). Although similarities existed among the symbiotic strains of *V. fischeri* probed, no hybridization occurred with DNA from either *Vibrio harveyi* ATCC 14126T or *Vibrio cholerae* RGELP40. This may be due to the fact that both *V. cholerae* and *V. harveyi* are pathogenic bacteria, and may have evolved a different pilin structure necessary for the infection of their respective host tissues. In a phylogenetic context, *V. cholerae* and *V.*

harveyi are not closely related to one another or to *V. fischeri*, which again provides evidence that colonization and infection mechanisms have evolved multiple times within the *Vibrionaceae* (Nishiguchi & Nair, 2003; Thompson *et al.*, 2004a).

Availability of the *V. fischeri* ES114 genome sequence also enabled the design of primers for the *pilB*, *pilC* and *pilD* loci. Targeted genes were amplified as well as an additional 350 bp from *V. fischeri* strains ET101, ESP915 and WH1. *V. fischeri* EB12 produced an additional 650 bp fragment, while *V. fischeri* strains ET101, SA1G, SA1J and SI1E produced an additional 550 bp band with the JBSpilB primers (Table 2). All *V. fischeri* strains except for ATCC 7744, a free-living isolate, were successfully amplified with the JBSpilC primers. *Vibrio parahaemolyticus* ATCC 17802T, *Photobacterium phosphoreum* PP21 and *V. fischeri* ATCC 7744 yielded a smaller 850 bp fragment, with sequence similarity to other *pilB* sequences. All of these vibrios as well as *V. fischeri* ATCC 7744 cannot colonize squid light organs (M. K. Nishiguchi, unpublished) and may therefore be missing an important functional group of the *pil* gene cluster that enables symbiotic bacteria to colonize animal tissues successfully.

When comparing all *V. fischeri* DNA sequences from this study, it was noted that all sequences were A+T rich (61 mol%). For the *pilA* locus, a 740 bp fragment was amplified and sequenced from all bacteria. Blastx demonstrated that the initial 502 bp was related to *pilA* and the remaining 309 bp was homologous to the polar flagellar sequence of other vibrios, with the only exception being *V. harveyi*. CLUSTAL W alignment demonstrated that *pilA* was 86–95% conserved in nucleotide sequence among all strains examined in this study. A multiple alignment of the in-frame amino acid sequences exhibited the same conservation. Results for the *pilB* comparisons gave 93% similarity. This was not surprising, since previous evidence has demonstrated that *pilA* can be replaced by corresponding genes from non-transformable species (Graupner *et al.*, 2000). The *pilA* locus is a pre-pilin gene that is flanked by other *pil*-like genes in numerous Gram-negative bacteria but, in the *V. fischeri* strains examined, no other *pil*-like genes were found in the vicinity (Stabb & Ruby, 2003). From our genome-walking experiments, only proteins whose functions are unknown (as determined for the *V. fischeri* ES114 genome; Ruby *et al.*, 2005) were within the 1000 bp region examined. Previous studies in other Gram-negative bacteria have indicated that the *pilA* and *pilD* genes are 100% conserved, with variation observed most widely at the *pilC* locus (Fullner & Mekalanos, 1999). We found that the *pilC* genes sequenced in this study were 94–97% similar, with the exception of *V. fischeri* WH1 and *V. fischeri* MDR7, both of which are freeliving isolates (100% sequence similarity to each other at the *pilC* locus, but less than 90% similarity to symbiotic isolates). *pilC* has been one of the *pil* loci identified as critical for adhesin in the binding of *Neisseria gonorrhoeae* to human epithelial cells (Rudel *et al.*, 1995b). Likewise, a *pilC* gene homologue has also been observed in *Pseudomonas aeruginosa* and has been linked to virulence in this species (Rudel *et al.*, 1995a; Alm *et al.*, 1996), suggesting that the *pilC* gene is important for the infectivity of these pathogenic bacteria. Interestingly, neither of our free-living strains (WH1 and MDR7) can colonize or persist inside squid light organs, and the 10% sequence difference observed at this locus may account for a loss of symbiotic competence. Future experiments to complement free-living *V. fischeri* strains such as WH1 and MDR7 with *pil* genes may help to determine whether improved colonization can be achieved with this operon alone, or whether other factors may be responsible for colonization and persistence in squid light organs.

The order of *pil* genes in the *V. fischeri* strains examined also differed from pathogenic *V. cholerae* and *V. parahaemolyticus*. *pilA* was determined to be monocistronic in *V. fischeri* and was separated from the other *pil* loci, which were present downstream and not in one continuous reading frame (Fig. 2). This is different from *pilA* found in both *V. cholerae* and *V. parahaemolyticus*. *pilB* (1674 bp) and *pilC* (1239 bp) were both translated in the second

reading frame, while *pilD* (933 bp) was translated in the first reading frame. There were 19 bases between *pilB* and *pilC* (the pilin assembly/biogenesis protein), 59 bases between *pilC* and *pilD* (the pilin peptidase), a 24 base overlap of the dephospho-CoA kinase (CoA, or *yacE* in *V. cholerae*) and 6 bases between *yacE* and a conserved hypothetical protein (HP) when compared to the *V. fischeri* ES114 genome. Using PsortB (http://psort.nibb.ac.jp/form.html), the following predictions were made for the *V. fischeri* strains examined in this study: (i) PilB has the conformation of a cytoplasmic protein, (ii) PilC is similar to an internal membrane protein that contains four internal helices and (iii) PilD has the conformation of an inner-membrane protein (Pepe & Lory, 1998). Additionally, a fragment which proved to be homologous to the membrane-bound phosphotransferase system (*pts*), which phosphorylates the *manXYZ* operon (Erni & Zanolari, 1985), was found to follow the hypothetical protein (not shown).

Phylogeny of symbiotic vibrios using the *pil* operon

All individual and total molecular gene trees were outgrouped with *V. fischeri* WH1 and MDR7, both of which are free-living isolates. We did not use *V. fischeri* ATCC 7744 as a free-living outgroup, since earlier studies suggested that this strain was divergent from the other *V. fischeri* strains used in this study (Nishiguchi & Nair, 2003). Phylogenetic trees based on individual *pil* loci produced phylogenies that were similar to each other, with most Indo-west Pacific vibrios ('E' strains) grouping together (Fig. 3). This was expected from our sequence analysis, since individual sequences for each locus were highly conserved with little variation. The two symbiotic fish strains (MJ101 and CG101) did not group together in all three individual gene analyses, or in the combined molecular analysis of all *pil* loci (Fig. 4). The three Mediterranean strains ('S' strains) analysed in our study were also included in clades containing fish and squid 'E' strains (Fig. 3). Thus, no geographical or host separation was made evident by any single gene phylogeny or the combined phylogenetic analysis of the entire *pil* gene operon (Fig. 4). The combined *pil* gene analysis (*pilB*, *pilC* and *pilD*) did group all ET and ES strains into a single clade, but this clade also included *V. fischeri* SR5 (Mediterranean) and ATCC 7744 (Fig. 4). A combined analysis of *pilB*, *pilC*, *pilD* and the 16S rRNA locus also indicated that all ET and ES strains were grouped within one clade (again including SR5 and ATCC 7744; Fig. 5).

Both combined tree analyses verify previous work using haplotype comparisons and nested clade analysis that demonstrated that ET and ES strains are closely related to one another and occupy populations of *Euprymna* that span a large geographical range (Hawaii, Australia and Thailand; Jones *et al.*, 2006). *Vibrio* strains did not cluster together based on geographical location or host specificity, as hypothesized previously based on competition experiments (Nishiguchi *et al.*, 1998; Nishiguchi, 2002). However, these earlier studies were based on a single clone isolated from an individual squid host and were not representative of the large genetic breadth exhibited by *Vibrio* (Thompson *et al.*, 2004b; Thompson & Swings, 2006). Likewise, with more *Vibrio* genomes becoming available, there is growing evidence that horizontal gene transfer events and the number of increased mutation events (particularly those *Vibrio* species that are subject to host control, such as *V. fischeri*) are responsible for the rapid radiation of this dynamic group of bacteria. Recent work has demonstrated that vibrios have the capability to expand their environmental niches (both free-living and symbiotic; Jones *et al.*, 2006), which may explain why so many symbiotic *V. fischeri* strains have the capability to infect other species of host squid (Nishiguchi, 2002). In addition, recent work has demonstrated that *V. harveyi* is also capable of infecting and colonizing the light organs of loliginid squids (Guerrero-Ferreira & Nishiguchi, 2007), which exemplifies how *Vibrio* bacteria are adept at expanding into new ecological niches. The fact that *V. fischeri* and *V. logei* had previously been identified as the only two species of light-organ-inhabiting bacteria illustrates how little we know about the vast number of

ecological niches that are available to vibrios and whether they are more susceptible to environmental selection or horizontal gene transfer events that may lead to incongruence in phylogenetic topologies (Thompson *et al.*, 2004b). Although this study was focused mainly on determining whether pilin gene sequences could distinguish between closely related *V. fischeri* strains, further studies need to be completed that will determine whether changes in any of the *pil* genes influence colonization specificity observed among closely related *Vibrio* symbionts. This would include constructing mutations within the *pil* genes to determine how deletions will affect adhesion and colonization specificity. Also, *in vivo* adherence assays of mutant *V. fischeri* strains to light organ epithelia cells would determine whether a change in colonization efficiency between wild-type and mutant vibrios is a main factor for driving recognition among closely related host–symbiont pairs. Whether variability between these genes is caused by rapid evolution, host selection or other gene transfer events remains to be determined and will provide yet another window into the evolution of this interesting group of gammaproteobacteria.

Acknowledgments

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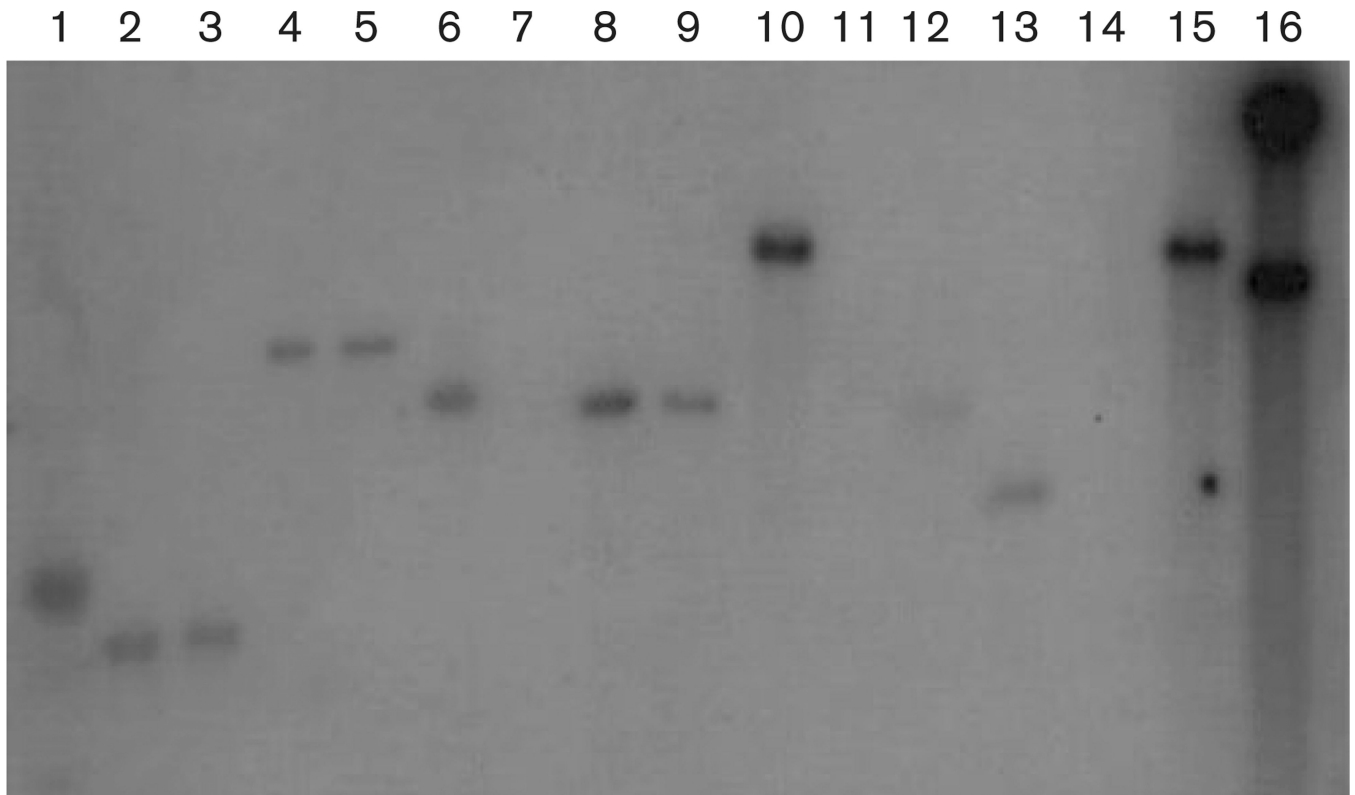
Fig. 1.
Southern blot of *Hin*dIII digests probed with the *pilA* gene from *V. fischeri* ESP915. Lanes: 1, 1 kb ladder; 2, *V. fischeri* EB12; 3, *V. fischeri* EM17; 4, *V. fischeri* ES114; 5, *V. fischeri* ET101; 6, *V. fischeri* SA1G; 7, *V. logei* SR18-1; 8, *V. fischeri* SR5; 9, *V. fischeri* ESP915; 10, *V. harveyi* ATCC 14126[T]; 11, *Photobacterium leiognathi* RM1; 12, *V. fischeri* WH1; 13, *V. parahaemolyticus* ATCC 17802[T]; 14, *V. logei* ATCC 35077; 15*, E. coli* O157 : H7-84/24; 16, *V. cholerae* RGELP40.
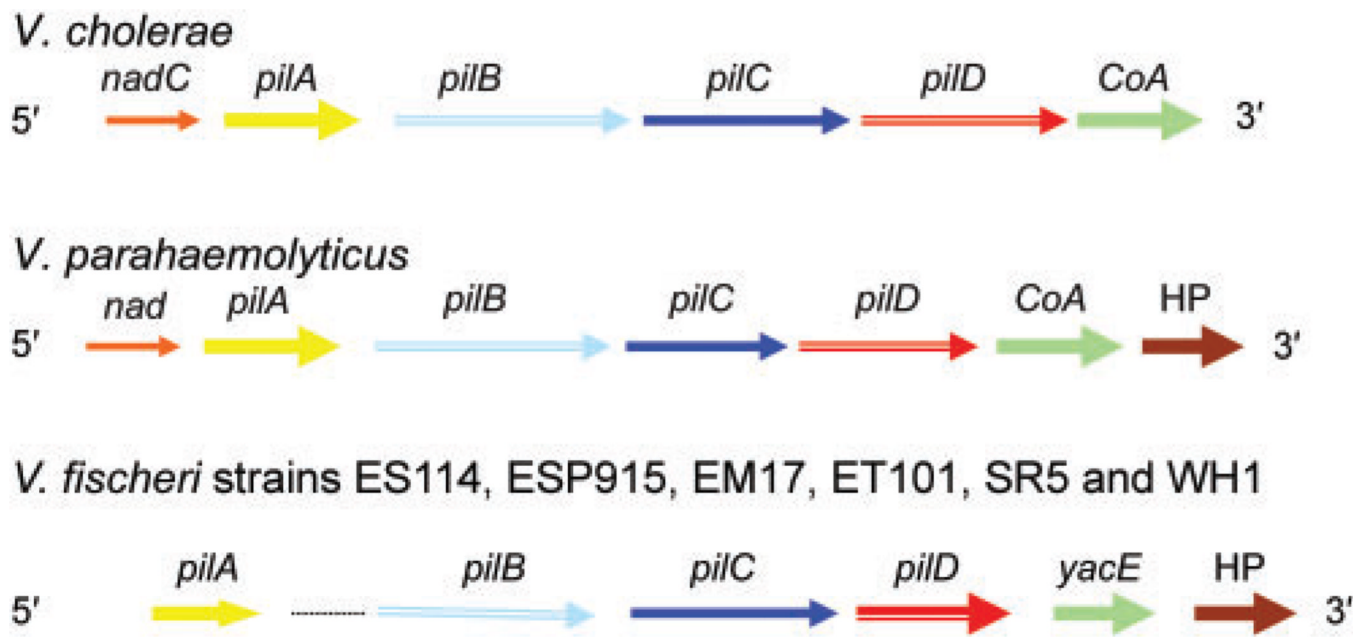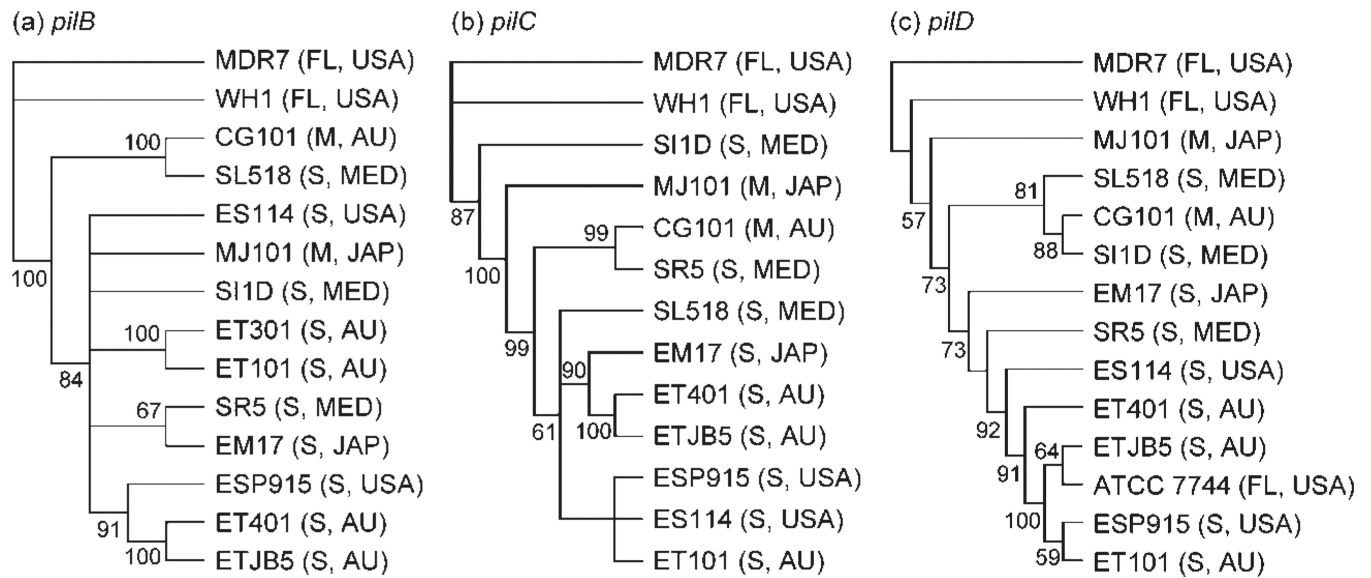
Fig. 2.
Gene order of each of the *pil* operons from five symbiotic (ES114, ES915, EM17, ET101 and SR5) and one free-living (WH1) strain of *V. fischeri* compared with *V. cholerae* RGELP40 and *V. parahaemolyticus* ATCC 14126[T]. Sequences of ~5 kb were utilized to construct the gene maps for all strains.

Fig. 3.
Gene trees for *pilB* (a), *pilC* (b) and *pilD* (c) for equal-weighted parameter sets (indels/is/tv) that minimized overall incongruence using POY with parsimony as the criterion. Jacknife values >50% are shown at nodes. Outgroups are *V. fischeri* strains MDR7 and WH1 (free-living). FL, Free-living; S, sepiolid symbiont; M, monocentrid symbiont; USA, United States isolate; AU, Australian isolate; MED, Mediterranean Sea isolate; JAP, Japanese isolate. See Table 1 for sequence accession numbers.
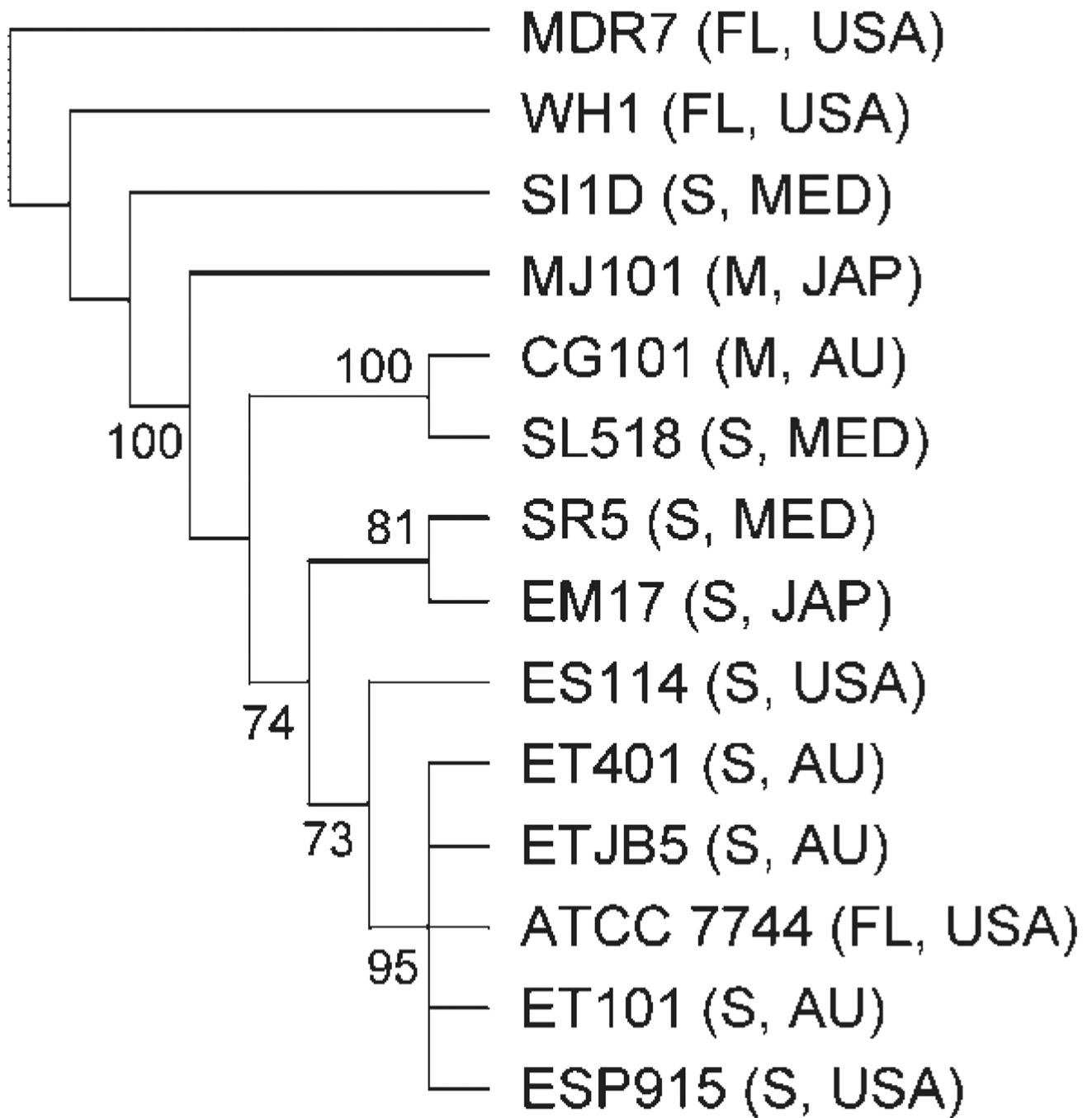
Fig. 4.
Total combined *pil* loci (*B*, *C* and *D*) tree for equal-weighted parameter set (indels/ts/tv) that minimized overall incongruence of the three genes using POY with parsimony as the criterion. See legend to Fig. 3 for further details.
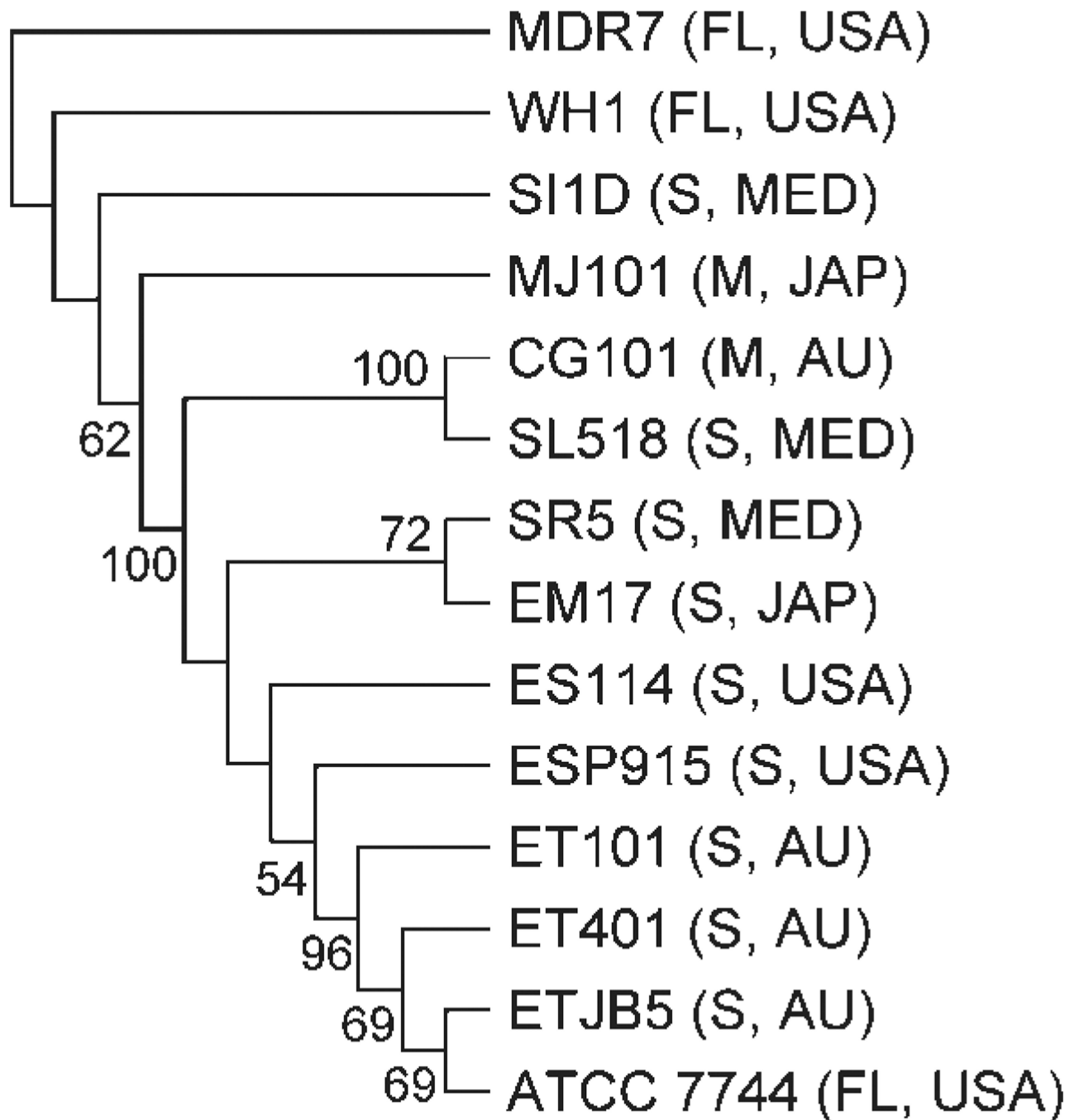
Fig. 5.
Total molecular tree combining all *pil* loci (*B*, *C* and *D*) and the 16S rRNA locus for equal-weighted parameter set (indels/ts/tv) that minimized the overall incongruence of the four genes using POY with parsimony as the criterion. See legend to Fig. 3 for further details.

Table 1

Bacterial isolates used in this study

Accession numbers respectively represent 16S rRNA gene and *pilB, pilC* and *pilD* sequences unless indicated.
–, Not sequenced.

Strain	Host	Source	GenBank accession numbers
V. fischeri ATCC 7744	Free living	Not known	AY292938, –, –, DQ207634
V. fischeri ESP915	*Euprymna scolopes*	USA (Paiko, HI)	AY292920, DQ093340 (*B, C, D*)
V. fischeri ES114	*Euprymna scolopes*	USA (Kaneohe Bay, HI)	AY292919, DQ093345 (*B, C, D*)
V. fischeri EM17	*Euprymna morsei*	Japan (Tokyo Bay)	AY292922, DQ093341 (*B, C, D*)
V. fischeri WH1	Free living	USA (Woods Hole, MA)	AY292930, DQ093344 (*B, C, D*)
V. fischeri CG101	*Cleidopus gloriamaris*	Australia (Townsville)	AY292939, DQ207619, DQ207627, DQ207636
V. fischeri ET101	*Euprymna tasmanica*	Australia (Crib Point)	AY292923, DQ093342 (*B, C, D*)
V. fischeri ET301	*Euprymna tasmanica*	Australia (Botany Bay)	–, DQ207620, –, –
V. fischeri ET401	*Euprymna tasmanica*	Australia (Magnetic Island)	AY292943, DQ207621, DQ207628, DQ207637
V. fischeri ETJB5	*Euprymna tasmanica*	Australia (Jervis Bay)	–, DQ207622, DQ207629, DQ207638
V. fischeri SR5	*Sepiola robusta*	France (Banyuls sur mer)	AY292926, DQ093343 (*B, C, D*)
V. fischeri SI1D	*Sepiola intermedia*	France (Banyuls sur mer)	AY292948, DQ207625, DQ207632, DQ207635
V. fischeri SL518	*Sepiola ligulata*	France (Banyuls sur mer)	AY292950, DQ207626, DQ207633, DQ207641
V. fischeri MJ101	*Monocentrus japonicus*	Japan	AY292946, DQ207624, DQ207631, DQ207640
V. fischeri MDR7	Free living	USA (Marina del Rey, CA)	AY292945, DQ207623, DQ207630, DQ207639
Photobacterium leiognathi RM1	*Rondeletiola minor*	France (Banyuls sur mer)	AY292947, –, –, –

Table 2
Primers used in this study for amplification of *pil* loci

Primers designed in this study were based on the *V. fischeri* ES114 genome sequence, as described in the text.

Primer	Sequence (5′–3′)	T_m (°C)	Reference
ESpilA47	GCAGAGCTCATATTAAAGCTCCTTCGTG	64.6	Stabb & Ruby (2003)
ESpilA48	CGTCGTATTGAGGCGATGCGTGAG	70.7	Stabb & Ruby (2003)
PilC2	CCAACTTGAGGGCTTGCACAATGG	66.3	This study
PilC3	GTAACGAGTGTACGGGCGGCTATT	66.3	This study
JBSpilB F	GCACACCAACCTCGCCTC	59.3	This study
JBSpilB R	GAGCACGGTTTAGTTCTTG	51.9	This study
JBSpilC F	GCTTTCAATGGCGTGGC	55.6	This study
JBSpilC R	GCCAATAACACTCATAAG	45.7	This study
JBSpilD F	GATTATTACCCTTGGCTT	41.7	This study
JBSpilD R	GTGGCAAACCTAGAATG	48.0	This study
pilC 3′1	CCGCCGGTAATCTTTACTATCAAGAAGCCA	63.7	This study
5′1pilC	TGAACAACWGGAACACCTGAGGCGAGC	64.1	This study
pilB1-175	GAACTCCACTAGTTGCACTGAGTTAAAACT	60.7	This study
pilD1-3860	GCTATTGCTTGGGTTTGCCTATTATGG	65.1	This study
AP1	GTAATACGACTCACTATAGGC	59.0	BD Biosciences

Table 3

PCR conditions used in this study

Gene target	Cycles (*n*)	Temperature (°C)		
		Denaturing	**Annealing**	**Extension**
pilA	35	94	58	72
pilB	30	94	49	72
pilC (primers PilC2 and PilC3)	30	94	53	72
pilC (other primers)	30	94	43	72
pilD	30	94	43	72