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Base changes in the *fliC* gene of *Edwardsiella tarda*: possible effects on flagellation and motility

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ABSTRACT: *Edwardsiella tarda* is a broad host-range pathogen infecting both animals and humans. *E. tarda* isolates from red sea bream *Pagrus major* are non-motile, whereas isolates from Japanese eel *Anguilla japonica* and Japanese flounder *Paralichthys olivaceus* are motile with peritrichous flagella. We compared the *fliC* gene coding for flagellin (FliC) in motile and non-motile *E. tarda* strains isolated from diseased fish. Twenty-two amino acid residues differed in the predicted FliC amino acid sequences between non-motile and motile strains. There were no significant differences either in the upstream sequences regulating transcription of the *fliC* gene or in the *fliC* transcript levels between motile and non-motile strains. The predicted secondary structure of FliC in non-motile *E. tarda* differed from that of motile strains, and the modeled data suggested that the secondary structure may be the important factor responsible for non-flagellation in the non-motile strains.

KEY WORDS: *Edwardsiella tarda* · *fliC* gene sequence · Base changes · Flagellation · Motility · Red sea bream · Japanese flounder

INTRODUCTION

Edwardsiella tarda, a member of the family *Enterobacteriaceae*, was first reported as a pathogen associated with the so-called 'red disease' of the Japanese eel *Anguilla japonica* (Hoshina 1962). Since then, the bacterium has been isolated from humans (Jordan & Hadley 1969, Clarridge et al. 1980, Wilson & Waterer 1989, Janda & Abbott 1993, Mowbray et al. 2003) and other animals including fish (Meyer & Bullock 1973, Wakabayashi & Egusa 1973), amphibians (Sharma et al. 1974, Kourany et al. 1977), reptiles (Otis & Behler 1973, Goldstein et al. 1981), birds (Winsor et al. 1981, Cook & Tappe 1985), and mammals (Kourany et al. 1977, Van Assche 1991).

Edwardsiella tarda causes systemic infection in a wide variety of cultured seawater and freshwater fish (Plumb 1999, Muroga 2001). The disease itself is called edwardsiellosis, and is a serious problem for the aquaculture industry throughout the world owing to a lack of effective chemotherapeutics and commercially available vaccines. In Japan, the presence of 2 phenotypes, typical and atypical, has long been recognized among *E. tarda* isolates from diseased fish. Typical strains are motile with peritrichous flagella and produce no acids from arabinose, sucrose, trehalose, or mannitol (Yasunaga et al. 1982, Sakazaki 2001), and have been isolated from the freshwater Japanese eel and the marine Japanese flounder *Paralichthys olivaceus* (Wakabayashi & Egusa 1973, Nakatsugawa 1983). In contrast, atypical strains, which are non-motile and produce acids from arabinose and mannitol but not from trehalose or sucrose, have been isolated from the marine crimson sea bream *Evynnis japonicus* and red sea bream *Pagrus major* (Kusuda et al. 1977, Yasunaga et al. 1982). There is no description of the non-motile atypical strains of *E. tarda* in Bergey's Manual (Sakazaki 2001). Matsuyama et al. (2005) reported a difference in experimental pathogenicity in some marine fish between typical (motile) and atypical (non-motile) strains. The typical strain is highly pathogenic to Japanese flounder but not to red sea bream,

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while the atypical strain exhibits high virulence only in red sea bream.

Bacterial flagella are important structures for pathogenic bacteria because they provide motility and increase adhesion to mucosal surfaces (Ramos et al. 2004). The flagellar filament is composed of approximately 20 000 subunits of the protein known as flagellin (FliC). Expression of flagella can be controlled by various genes in response to environmental changes. McIntosh & Austin (1991) showed that another fish pathogen, *Aeromonas salmonicida*, expresses flagella only at supra-optimal environmental temperatures (from 30 to 37°C). In *Edwardsiella tarda*, the *fliC* gene coding for the FliC protein was previously cloned and sequenced from strain PPD130/91, a motile strain (Tan et al. 2002). The other conditions and genes related to the expression of flagella are unknown in *E. tarda*.

In this study we determined the mechanism of motility expression and flagellation in typical and atypical strains of *Edwardsiella tarda*. We show that non-motile strains do not form flagella *in vitro* in response to changing environmental temperatures. The data suggests that an alteration of the *fliC* gene sequence in non-motile strains is likely to be responsible for the deficiency in flagella formation in atypical strains.

MATERIALS AND METHODS

Bacterial strains and media. Thirteen *Edwardsiella tarda* strains isolated from diseased Japanese flounder or diseased red sea bream were used (Table 1). The *E. tarda* strains were grown in Triptic-Soy broth (TSB, Eiken) or on Tryptic-Soy agar (TSA, Nissui) at 30°C unless otherwise indicated.

Motility tests. The motility of the test strain was examined using a wet mount method with a light microscope (the direct method), and sulfide indole motility (SIM) media (Eiken) (the indirect method) at a variety of temperatures (15, 20, 25, 30, 35, or 40°C). For the direct wet mount method, the test strains were grown in TSA at 30°C.

Utilization of carbohydrates. Bacteria were inoculated into bromocresol purple semisolid medium (Eiken) containing each of the 4 carbohydrates L-arabinose, sucrose, trehalose, and D-mannitol at 1% and incubated at 30°C for 2 wk.

Flagella staining. *Edwardsiella tarda* strains were incubated in TSB at 25°C for 12 h and the cells were fixed by addition of 0.5% neutralized formalin. The bacterial flagella were stained using the Nishizawa-Sugawara method (Shitara 1988).

Analysis of the *fliC* gene and its upstream sequence. The open reading frame (ORF) of the *fliC* gene was amplified using PCR with primers 5′-Fla (ATG) and 3′-Fla (stop) (Table 2) that were designed from the reported sequence of a motile strain, PPD130/91 (GenBank Accession No. AF487406) (Tan et al. 2002). PCR amplification was performed using *Taq* polymerase (Ex Taq; Takara Biochemicals), and the thermal cycling conditions were 30 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1.5 min, followed by a final extension step at 72°C for 5 min. The amplified PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced. Using the *fliC* gene sequence of PPD130/91, we designed 3 primers, 3′-Fla-TSP1, 3′-Fla-TSP2, and 3′-Fla-TSP3 (Table 2). PCR amplification of fragments containing the 199 bp sequence upstream of the *fliC* ORF was performed

Table 1. *Edwardsiella tarda*. Motility, flagellation and carbohydrate utilization of 13 strains used in this study. Motility was assessed using a direct wet-mount method, and an indirect test with sulphide indole motility medium. Flagella presence was confirmed using light microscopy

Strain	Isolation			Motility		Flagella	Utilization of			
	Source	Place	Year	Direct	Indirect		L-arabinose	sucrose	trehalose	D-mannitol
FK1051	Japanese flounder	Hiroshima	2001	+	+	+	–	–	–	–
NUF251	Japanese flounder	Nagasaki	1986	+	+	+	–	–	–	–
NUF82	Japanese flounder	Nagasaki	1984	+	+	+	–	–	–	–
PE113	Japanese flounder	Ehime	2001	+	+	+	–	–	–	–
E01-33	Japanese flounder	Ehime	2001	+	+	+	–	–	–	–
E01-37	Japanese flounder	Ehime	2001	+	+	+	–	–	–	–
NE8003	Japanese flounder	Ehime	1980	+	+	+	–	–	–	–
E01-14	Red sea bream	Ehime	2001	–	–	–	+	–	–	–
E01-17	Red sea bream	Ehime	2001	–	–	–	+	–	–	–
E01-40	Red sea bream	Ehime	2001	–	–	–	+	–	–	–
REF179	Red sea bream	Mie	2001	–	–	–	+	–	–	–
REF146	Red sea bream	Mie	1996	–	–	–	+	–	–	–
REF0001	Red sea bream	Mie	1996	–	–	–	+	–	–	–

Table 2. *Edwardsiella tarda*. Oligonucleotides used for PCR amplification of the *fiC* gene in this study

Designation	Nucletide position	Sequence
5′-Fla (ATG)	200–220[a]	5′-ATGGCACAAGTAATTAATACC-3′
5′-Fla (968)	968–985[a]	5′-GGTACTACAGCCGTAACC-3′
3′-Fla (stop)	1430–1450[a]	5′-TTAACGCAGCAGAGACAGGAC-3′
3′-Fla-TSP1	282–300[a]	5′-GAAGACAGACGCTCGATGG-3′
3′-Fla-TSP2	239–261[a]	5′-GATTTGTTCAGGTTGGTTCTGTGC-3′
3′-Fla-TSP3	218–238[a]	5′-CATCAGCGACAGGCTGTTGG-3′
3′-Fla-TSP3-2	209–239[a]	5′-CCATCAGCGACAGGCTGTTGGTATTAATTAC-3′
5′-Fla-1	1–21[a]	5′-CGATGGGTCAATAGAAGCAGG-3′
5′-Fla-2	1–34[a]	5′-CGATGGGCCAATAGAAGCAGGAAAATGGGGGCTT-3′
5′-ET-gyrA	428–447[b]	5′-GATGTTCGGGATGGCCTGAA-3′
3′-ET-gyrA	709–729[b]	5′-GCCAACAGCTCATGAGCAATC-3′

[a]Nucleotide positions in Fig. 2
[b]Nucleotide positions in GenBank Accession No. DQ019315

using the 3 primers with a DNA walking kit (DNA Walking *SpeedUp* Premix Kit, Seegene) according to the manufacturer's protocol. The amplified PCR products were cloned into the pGEM-T easy vector and sequenced. A search for the promoter sequence (–10 and –35 regions) in the 199 bp sequence was performed using the software GENETYX-MAC version 12.2.5 (GENETYX).

***lacZ* transcriptional fusion assay.** A plasmid-borne *lacZ* transcriptional fusion was constructed by cloning the DNA fragment containing the presumed promoter region (the 199 bp upstream sequence) and the N-terminal coding region of the *fliC* gene into the *lacZ* fusion site of the pUJ8 vector (de Lorenzo et al. 1990). Briefly, the 240 bp sequence comprising the 199 bp sequence upstream of the *fliC* ORF and the 41 bp 5′ proximal sequence of the *fliC* ORF was amplified by PCR using primers 5′-Fla-1 and 3′-Fla-TSP3 (for FK1051 and E01-17) or primers 5′-Fla-2 and 3′-Fla-TSP3-2 (for REF146) (Table 2). The amplicons were cloned into the pUJ8 vector, and the resultant plasmids were transformed into *Escherichia coli* DH5α. The β-galactosidase assay of the transformants was performed as described previously (Okuda & Nishibuchi 1998). The β-galactosidase activities were compared using the Student's *t*-test for significant differences ($p < 0.05$).

***fliC* transcript analysis by RT-PCR.** For reverse transcription (RT)-PCR, bacterial cells were cultured in TSB at 25°C overnight. Total RNAs from 13 *Edwardsiella tarda* strains isolated from diseased Japanese flounder or diseased red sea bream (Table 1) were isolated with ISOGEN (Nippongene) using the method recommended by the manufacturer. The RNAs were purified again after treatment with RNase-free DNase I (Takara), and the amount and purity of the RNAs were determined by measuring A_{260} and A_{280}. A total of 100 ng of the RNA was used to amplify the *fliC* and *gyrA* transcripts by RT-PCR. The *gyrA* transcript level was used as the internal control for RT-PCR as described previously (Tanaka et al. 2005). RT-PCR was performed using the SuperScript One-Step RT-PCR with Platinum *Taq* system (Invitrogen) and primers for *fliC* (5′-Fla [968] and 3′-Fla [stop] primers) and *gyrA* (5′-ET-gyrA and 3′-ET-gyrA primers designed from the *E. tarda gyrA* sequence in GenBank Accession No. DQ019315) (Table 2). The RT-PCR was performed with the following cycle profiles: 30 cycles for *fliC* annealing at 52°C, and 30 cycles for *gyrA* annealing at 50°C. The RT-PCR products (483 bp for *fliC* and 302 bp for *gyrA*) were visualized using ethidium bromide after 2% agarose gel electrophoresis. Semiquantitative RT-PCR assay was carried out as described previously (Okuda et al. 2005) with slight modifications. The relative expression level of the *fliC* transcript was measured by normalizing the PCR product of the *fliC* gene to that of the *gyrA* gene. After the densitometric intensity of the RT-PCR products was quantified with NIH Image software, the relative expression level of the *fliC* transcript was calculated as the ratio of the final RT-PCR product of the *fliC* gene to that of the *gyrA* gene (*fliC*:*gyrA* ratio). The amount of contaminating chromosomal DNA in each sample was determined in control reactions without reverse transcriptase.

Prediction of FliC secondary structure. We predicted the secondary structure of the FliC using the Chou-Fasman algorithm from the deduced amino acid sequence (Chou & Fasman 1978) using the software GENETYX-MAC version 12.2.5.

Nucleotide sequence accession numbers. The *fliC* nucleotide sequences of *Edwardsiella tarda* FK1051, E01-17, and REF146 were lodged in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database under accession numbers AB271128, AB271129, and AB271130, respectively.

RESULTS

Motility, flagellation, and carbohydrate utilization

All 7 *Edwardsiella tarda* strains isolated from diseased Japanese flounder showed motility using both the direct and indirect tests, whereas all 6 *E. tarda* strains isolated from diseased red sea bream did not (Table 1). Photographs of representative strains using the indirect motility test are shown in Fig. 1A. In addition, when the motile and non-motile strains were grown in the SIM medium (indirect test) at a variety of temperatures (15, 20, 25, 30, 35, or 40°C), the results were the same: motile and non-motile strains were unchanged (data not shown).

Flagella were observed only in the motile strains and not with non-motile strains (Table 1). Photographs of representative strains are shown in Fig. 1B.

None of the 7 *Edwardsiella tarda* strains isolated from diseased Japanese flounder utilized L-arabinose, sucrose, trehalose, or D-mannitol; in contrast, all 6 *E. tarda* strains isolated from diseased red sea bream produced acid and gas from arabinose, but not from mannitol, trehalose, or sucrose (Table 1).

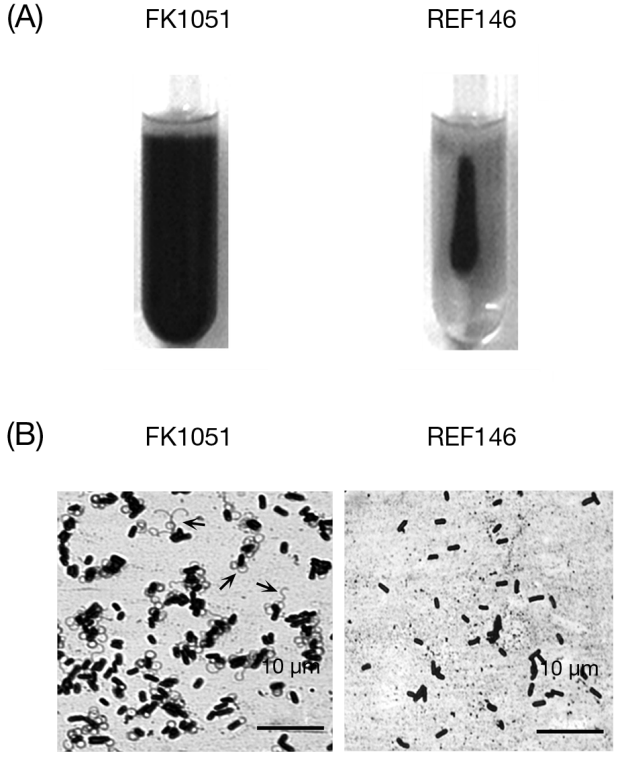


Fig. 1. *Edwardsiella tarda*. (A) Indirect motility test and (B) direct detection of flagella for motile (FK1051) and non-motile (REF146) strains. Black arrows in (B) indicate representative flagella

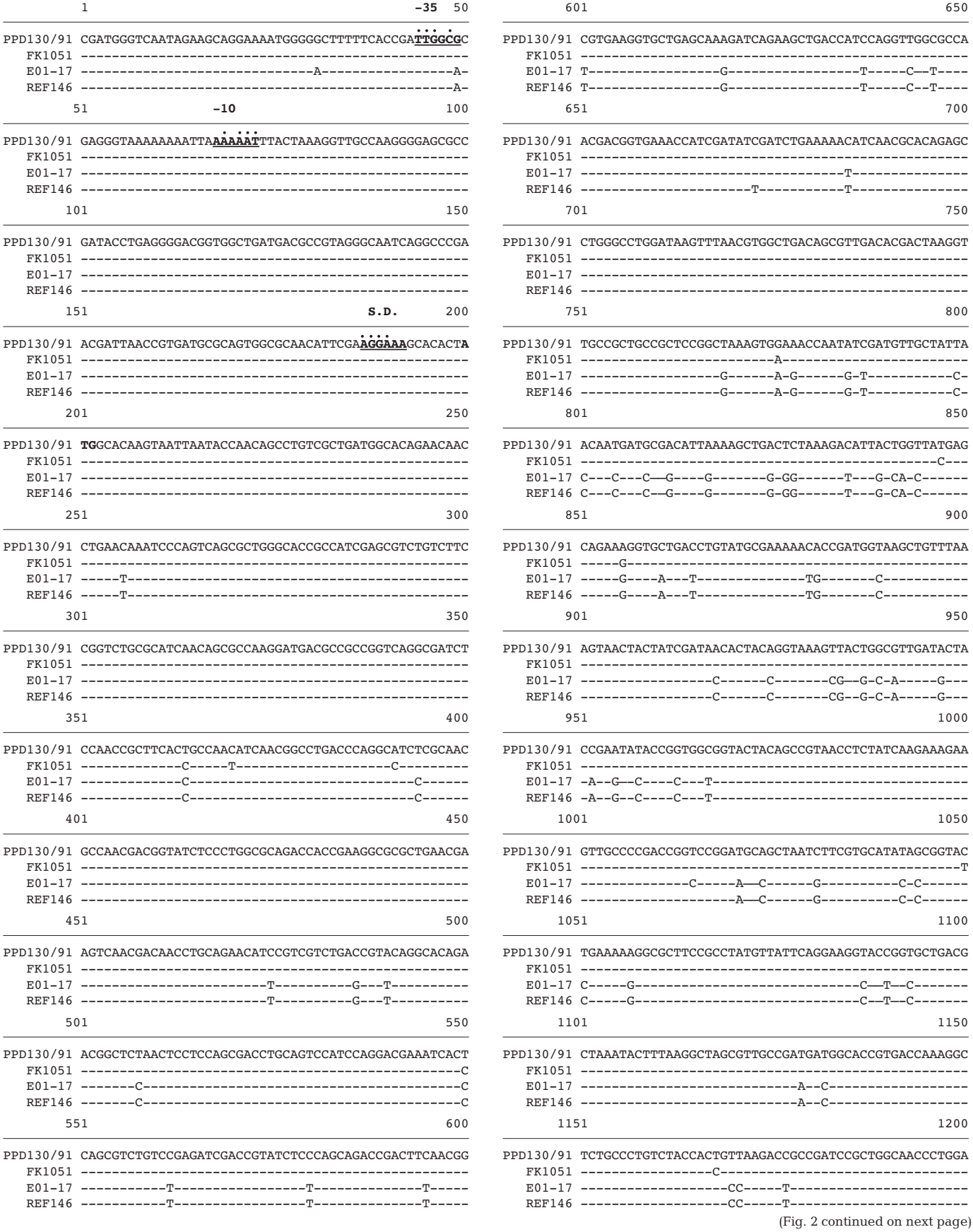
Comparison of the *fliC* ORF

The *fliC* ORF sequence was successfully amplified from all 6 non-motile strains as well as from the 7 motile strains. The amplified PCR products from FK1051 representing a motile strain and E01-17 and REF146 representing non-motile strains were cloned and sequenced. The *fliC* ORF sequence of a motile strain, PPD130/91, was obtained from the reported sequence (Tan et al. 2002). Many base differences were detected in the *fliC* ORFs of the 2 non-motile strains compared with those of the motile strains PPD130/91 and FK1051 (Fig. 2), and all the *fliC* ORFs were the same length (Fig. 2). The deduced amino acid sequences of the *fliC* gene cloned from E01-17 and REF146 were compared with those from PPD130/91 and FK1051 (Fig. 3). The amino acid sequence of the 2 motile strains PPD130/91 and FK1051 differed by only 2 residues. The amino acid sequences of the 2 non-motile strains E01-17 and REF146 were identical, and 22 residues differed between motile and non-motile strains. The influence of these residue differences on the FliC structure was investigated using computer structural modeling software. As shown in Fig. 4, the secondary structure of FliC predicted from the amino acid sequences of the non-motile E01-17 and REF146 (Fig. 4B) is clearly different from that of motile FK1051 (Fig. 4A). The distinct difference in the FliC secondary structure of the non-motile strains compared with that of motile FK1051 is the presence of an additional β-turn from amino acids 219 to 222. Owing to the additional β-turn, the secondary structure of the FliC protein of non-motile strains is an extended form (Fig. 4B), whereas that of motile FK1051 has a hairpin loop structure (Fig. 4A).

Comparison of sequences upstream of the *fliC* ORF, and *fliC* transcript levels

We then determined whether the transcription of the *fliC* gene in non-motile strains was down-regulated because of mutations in the *fliC* promoter or the

Fig. 2. *Edwardsiella tarda*. Comparison of strains using alignment of the *fliC* gene ORF and its 199 bp upstream region. PPD130/91 and FK1051 are motile strains; E01-17 and REF146 are non-motile strains. Sequences showing homology with consensus sequences such as a Shine-Dalgarno sequence (S.D.), and –10 and –35 regions of the promoter are indicated by dots above sequences. The ATG and TTA in **bold** at nucleotide positions 200 to 202 and 1448 to 1450 are the start and stop codons of the *fliC* gene, respectively. Identical nucleotides are indicated by dashes



(Fig. 2 continued on next page)

```
      1201                                                1250

PPD130/91 TAAAGCCCTGTCTCAGGTTGATGACCTGCGCAGCGGCCTGGGTGCGGTAC
   FK1051 --------------------------------------------------
   E01-17 --------------------------------------------------
   REF146 --------------------------------------------------
      1251                                                1300

PPD130/91 AGAACCGTTTCGATTCCGTTATCAACAACCTGAACAGCACCGTGAACAAC
   FK1051 --------------------------------------------------
   E01-17 ------------------A-------------------------------
   REF146 ------------------A-------------------------------
      1301                                                1350

PPD130/91 CTGTCCGCTTCCCGTTCACGTATTCAGGACGCTGACTACGCGACCGAAGT
   FK1051 --------C-----------------------------------------
   E01-17 --------------------------------------------------
   REF146 --------------------------------------------------
      1351                                                1400

PPD130/91 GTCCAACATGAGCCGTGCGCAGATCCTGCAGCAGGCCGGTACCTCCGTAC
   FK1051 ------------------------------------------------T----
   E01-17 --------------------------------------------------
   REF146 --------------------------------------------------
      1401                                                1450

PPD130/91 TGGCTCAGGCTAACCAGTCTACCCAGAACGTCCTGTCTCTGCTGCGTTAA
   FK1051 ----C-----------------------------------------------
   E01-17 ----------C-----------------------------------------
   REF146 ----------C-----------------------------------------
```

Fig. 2 (continued)

sequence involved in the control of *fliC* transcription. The 199 bp sequence located upstream of the *fliC* ORF contains the presumed promoter sequence (Fig. 2). This was amplified from FK1051, E01-17 and REF146 using PCR. The amplified PCR fragments were cloned and sequenced. Two bp differences were detected in the 199 bp upstream sequences of E01-17 and REF146 compared with that of FK1051 (Fig. 2). One base difference at nucleotide position 49 (guanine vs. adenine) was common to E01-17 and REF146. To examine the influence of these base differences on the transcription of the *fliC* gene, the ability of the 199 bp upstream sequence to initiate transcription of the downstream gene was compared. A *lacZ* transcriptional fusion was constructed using a *lacZ* fusion vector plasmid as described in 'Materials and methods'. The 240 bp DNA sequence comprising the 199 bp sequence upstream of the *fliC* ORF and the 41 bp 5′ proximal sequence of the *fliC* ORF was fused to *lacZ*. The β-galactosidase activities of the promoter-fusions in an *E. coli* background were compared. There was no significant difference in the ability of the 199 bp upstream sequence to stimulate downstream transcription using either the motile strain or the non-motile strain's promoter regions (Table 3).

```
            1                                                  40                                                  80

PPD130/90   MAQVI NTNSL SLMAQ NNLNK SQSAL GTAIE RLSSG LRINS AKDDA AGQAI SNRFT ANING LTQAS RNAND GISLA QTTEG
   FK1051   ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
   E01-17   ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
   REF146   ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----

         81                                                 120                                                 160

PPD130/90   ALNEV NDNLQ NIRRL TVQAQ NGSNS SSDLQ SIQDE SIQDE SEIDR ISQQT DFNGV KVLSK DQKLT IQVGA NDGET IDIDL
   FK1051   ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
   E01-17   ----- ----- ----- S---- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
   REF146   ----- ----- ----- S---- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----

            161                                                200                                                 240

PPD130/90   KNINA QSLGL DKFNV ADSVD TTKVA AAAPA KVETN IDVAI NNDAT LKADS KDITG YEQKG ADLYA KNTDG KLFKV TTIDN
   FK1051   ----- ----- ----- ----- ----- ----- --K-- ----- ----- ----- ----- ----- ----- ----- ----- -----
   E01-17   ----- ----- ----- ----- ----- ----- --K-- V---- T--PA ---GG ---AS ----- D---- --A-- ----- ----T
   REF146   ----- ----- ----- ----- ----- ----- --K-- V---- T--PA ---GG ---AS ----- D---- --A-- ----- ----T

            241                                                280                                                 320

PPD130/90   TTGKV TGVDT TEYTG GGTTA VTSIK KEVAP TGPDA ANLRA YSGTE KGASA YVIQE GTGAD AKYFK ASVAD DGTVT KGSAL
   FK1051   ----- ----- ----- ----- ----- ----- ----- ----- ---I- ----- ----- ----- ----- ----- ----- -----
   E01-17   ----A -AI-A ----A ----- ----- ----- ---E- --V-- -T--- ----- ----- ----- ----- ----E ----- -----
   REF146   ----A -AI-A ----A ----- ----- ----- ---E- --V-- -T--- ----- ----- ----- ----- ----E ----- -----

            321                                                360                                                 400

PPD130/90   STTVK TADPL ATLDK ALSQV DDLRS GLGAV QNRFD SVINN LNSTV NNLSA SRSRI QDADY ATEVS NMSRA QILQQ AGTSV
   FK1051   ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
   E01-17   ---A- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
   REF146   ---A- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- -----

            401              416

PPD130/90   LAQAN QSTQN VLSLL R
   FK1051   ----- ----- ----- -
   E01-17   ----- ----- ----- -
   REF146   ----- ----- ----- -
```

Fig. 3. *Edwardsiella tarda*. Comparison of strains using alignment of the deduced FliC amino acid sequences. PPD130/91 and FK1051 are motile strains; E01-17 and REF146 are non-motile strains. Identical residues are indicated by dashes

Fig. 4. *Edwardsiella tarda*. Predicted secondary structure of FliC from the deduced amino acid sequence. Secondary structure prediction was performed using the Chou-Fasman algorithm (Chou & Fasman 1978). (A) Motile strain FK1051. (B) Non-motile strains E01-17 and REF146

Table 3. β-galactosidase activities of *E. coli* DH5α derivatives carrying *fliC-lacZ* transcriptional fusions. Values are mean ± SD of 3 independent experiments

Origin (strain) of the *fliC* gene	β-galactosidase activity (units)
FK1051	2174 ± 622
E01-17	2247 ± 216
REF146	2051 ± 375

Furthermore, we examined whether the *fliC* gene is actually transcribed to the same level in motile and non-motile strains by RT-PCR, as described in 'Materials and methods'. To rule out the possibility that reverse-transcribed cDNA contained genomic DNA, we also examined the amount of contaminating chromosomal DNA in each sample and found that there was no amplification in control reactions without reverse transcriptase (data not shown). As shown in Fig. 5, the *fliC* transcripts were detected in all *Edwardsiella tarda* strains examined. To perform relative quantification of the *fliC* transcript levels, the relative expression level of the *fliC* transcript was measured by normalizing the PCR product of the *fliC* gene to that of the *gyrA* gene. The relative expression level of the *fliC* transcript was calculated as the ratio of the RT-PCR product of the *fliC* gene to that of the *gyrA* gene (the *fliC*:*gyrA* ratio) (Fig. 5). There was no significant difference in the relative *fliC* transcript levels (the *fliC*:*gyrA* ratio) between motile and non-motile strains: the *fliC*:*gyrA* ratios ranged from 0.8 to 1.5 in the 7 motile strains and from 0.9 to 1.4 in the 6 non-motile strains (Fig. 5). These results confirmed that the *fliC* gene is actually transcribed in motile and non-motile strains to the same level.

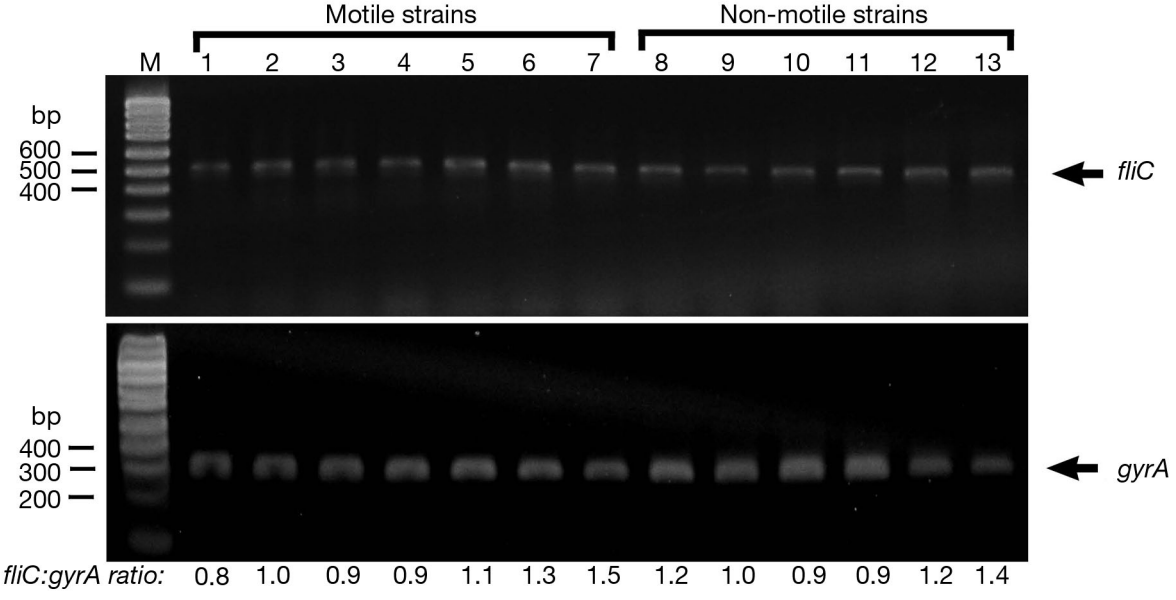


Fig. 5. *Edwardsiella tarda*. RT-PCR analysis of the *fliC* transcript levels. Lanes: M, 100 bp DNA ladder marker; 1, FK1051; 2, NUF251; 3, NUF82; 4, PE113; 5, E01-33; 6, E01-37; 7, NE8003; 8, E01-14; 9, E01-17; 10, E01-40; 11, REF179; 12, REF146; 13, REF0001. The *gyrA* gene was used as an internal control. The relative expression level of the *fliC* transcript was calculated as the ratio of the RT-PCR product of the *fliC* gene to that of the *gyrA* gene (*fliC*:*gyrA* ratio)

DISCUSSION

Bergey's manual (Sakazaki 2001) describes 2 types of *Edwardsiella tarda*, whereby most strains are motile with peritrichous flagella and produce no acids from L-arabinose, sucrose, trehalose, or D-mannitol; in comparison, *E. tarda* strains belonging to Biogroup 1 (Grimont et al. 1980) are also motile but produce acids from L-arabinose, sucrose, and D-mannitol. The *E. tarda* strains isolated for this study from Japanese flounder all showed the same characteristics as the former strains in Bergey's manual. The atypical isolates from red sea bream were differentiated from the typical strains by the lack of motility and the ability to utilize L-arabinose only; however, the previously reported non-motile *E. tarda* strains produced acid from D-mannitol as well as from L-arabinose (Kusuda et al. 1977, Yasunaga et al. 1982). There is no specific description of non-motile *E. tarda* strains in Bergey's manual. Our results suggest it may be appropriate to classify *E. tarda* into 3 biogroups, with the non-motile strains belonging to a new biogroup.

Motility was detected only in *Edwardsiella tarda* strains isolated from Japanese flounder, not in those isolated from red sea bream, and motility was associated with flagella formation (Table 1). Unlike *Aeromonas salmonicida*, motility and flagella formation were not influenced by the incubation temperature. The possibility that an occurrence of *fliC* ORF disruption by nonsense mutation or frame-shift mutation in non-motile strains was ruled out because full-length FliCs were encoded in the *fliC* ORFs of non-motile strains. However, amino acid residue substitutions at 22 positions were detected in the predicted FliC amino acid sequence of the non-motile strains (Fig. 3). Some of these substitutions significantly influenced the secondary structure of FliC in computer generated models (Fig. 4, discussed below). We next examined the possibility of mutations in the 199 bp upstream sequences of the *fliC* ORF containing the presumed *fliC* promoter. The sequence and *lacZ* fusion analyses suggested that FliC would be produced in non-motile strains at similar levels as in motile strains unless some other unknown factors are involved in *fliC* expression control. We then examined if the *fliC* gene is actually transcribed by RT-PCR in motile and non-motile strains to the same level. As shown in Fig. 5, the *fliC* transcripts were detected in all *E. tarda* strains examined, and there was no significant difference in the relative *fliC* transcript levels (*fliC*:*gyrA* ratio) between motile and non-motile strains.

We therefore consider that an alteration in the secondary structure of FliC in non-motile strains may be an important factor responsible for non-flagellation in non-motile strains. The hairpin loop structure predicted for FliC in the motile strain FK1051 was disrupted in non-motile strains E01-17 and REF146 owing to the additional β-turn, and an extended form was predicted instead for the secondary structure (Fig. 4). The N- and C-terminal conserved domains of flagellin (the D0 and D1 domains) form packed α-helix structures, whereby the packed α-helix regions are embedded in the flagellum inner core and are essential for filament architecture and motility functions (Ramos et al. 2004). In FK1051, the N- and C-terminal domains of flagellin (the D0 and D1 domains) form packed α-helix structures owing to the hairpin loop structure of FliC. Because of the disruption of the hairpin loop structure in non-motile strains, the N- and C-terminal domains would not form packed α-helix structures, and may result in a failure in the construction of the filament architecture. Another possible explanation is failure to transport a defective form of FliC in non-motile strains. FliC is synthesized in the cytoplasm and exported through the central channel by the flagellum-specific export apparatus to the site of assembly at the distal end of the growing filament (MacNab 2004). Non-flagellated strain FliC might not be transported through the central channel because of the drastic alteration in the secondary structure in non-motile strains. These possibilities need to be studied in the future.

In this study, we speculate about the possible role of a modified FliC in the non-motile behavior of certain *Edwardsiella tarda* strains. However, there are several dozen proteins involved in the export and assembly of flagella and the motor-export complex, and mutations in any of their corresponding genes may result in nonfunctional motility and lack of flagellin export. Transformation of FliC knockout bacterial models with the non-motile *E. tarda* FliC gene would help clarify its effect on motility and flagella formation.

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