Characterization of six flagellin genes in the H3, H53 and H54 standard strains of *Escherichia coli*

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Six flagellin genes in three H standard *Escherichia coli* strains for H3, H53 and H54 were characterized. Each strain has two flagellin genes, one of which is expressed as its standard H antigen. A pair of flagellin genes *flkA3* (encoding for H3 antigen) and *fliC16* (H16) was cloned from Bi7327-41, *flkA53* (H53) and *fliC-53* from E480-68, and *flmA54* (H54) and *fliC-54* from E223-69. Two *fliC* genes, *fliC-53* and *fliC-54*, are nonfunctional owing to the insertions of IS1 and IS1222, respectively. The *flkA* and *flmA* regions are located in the 3' end of the *rnpB* gene and near the *nlpA* gene, respectively. Each of them is followed by a gene homologous to *fljA*, which is known to repress the expression of *fliC(i)* in *Salmonella enterica* serovar Typhimurium. These results suggest that they are derived from the same origin of the *fljBA* operon. However, these regions contain neither the *hin* gene nor the invertible H segment. The four flagellin genes, *fliC16*, *flkA3*, *flkA53* and *flmA54*, share high homology in nucleotide and amino-acid sequences with one another and with the *S. enterica* serovar Typhimurium flagellin genes. The promoter sequence of *fliC16* is homologous to that of *fliC(i)*, whereas the promoter sequences of *flkA* and *flmA* are homologous to that of *fljB*. The terminator sequences of the *fliC16*, *fliC-53* and *fliC-54* genes are conserved among themselves and identical with that of the *E. coli* *fliC48* gene. Three FljA repressors, FljA3, FljA53 and FljA54, are homologous highly with one another and moderately with FljA of *Salmonella*. These results indicate that six flagellin genes analyzed are markedly similar to the *Salmonella* flagellin genes, suggesting their lateral transfer from *Salmonella*.

Key words: *Escherichia coli*, flagellin gene, H antigen, lateral transfer

INTRODUCTION

Although there is little evolutionary divergence between *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Ochman and Wilson, 1987), one remarkable difference between them is in the flagellar gene system: in *E. coli* K-12 one flagellin gene *fliC48* (encoding for H48 antigen) located at 43.1 min is constitutively expressed, while in *S. enterica* serovar Typhimurium two flagellin genes *fliC(i)* and *fljB*, located at 42.5 min and 60 min, respectively, are expressed alternatively by the phase variation mechanism (Zieg et al., 1977). This mechanism is controlled by orientation of the invertible H segment (Zieg and Simon, 1980) upstream of the *fljBA* operon: in the H(+) orientation the operon is expressed and the *fljA* product (FljA) represses expression of the *fliC(i)* gene (Silverman et al., 1979; Hanafusa et al., 1993)

resulting in only FljB flagellin being expressed, whereas in the H(–) orientation the operon is inactive, resulting in *fliC(i)* flagellin being expressed. The H inversion is mediated by a DNA invertase encoded by the *hin* gene within the H segment (Zieg and Simon, 1980).

Recent studies for the flagellin genes in natural isolates of *E. coli* (Ratiner, 1982, 1987, 1998) demonstrated that some standard *E. coli* strains for H antigens have another flagellin gene beside *fliC*. For example, two new flagellin genes, named *flkA* and *flmA*, were mapped near *argG* (69 min) and *mtlA* (81 min), respectively. In addition to flagellin, the *flkA* and *flmA* loci show a repressor activity similar to that of *Salmonella* FljA, and this repressor represses the expression of some *fliC* genes of standard *E. coli* strains but not that of *fliC48* (Ratiner, 1998). This work was initiated to gain an insight into diversity of flagellar antigens in natural isolates of *E. coli*. This paper describes molecular characterization of the *flkA* and *flmA* loci of *E. coli*. Structures of the silent *fliC* genes in the *flkA*- or *flmA*-expressing strains are also

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described. On the basis of the obtained results, I discuss the evolutionary aspect of the divergence of these flagellin genes.

MATERIALS AND METHODS

Bacterial strains, plasmids and media Bacterial strains used are listed in Table 1. Cloning vectors used were plasmids pBR322 (Bolivar et al., 1977), pACYC184 (Chang and Cohen, 1978) and pSTV28 (Takara). TLY broth, nutrient agar and nutrient semisolid agar (NSS) were as described (Enomoto et al., 1983). Chloramphenicol (Sigma), tetracycline (Sigma) and ampicillin (Sigma) were used at final concentrations of 12.5, 25 and 50 µg/ml, respectively. Anti-i serum (Enomoto et al., 1985) was used at a final concentration of 0.05%.

Table 1. Bacterial strains used

Strain	Relevant genotype ^a	Reference
<i>Escherichia coli</i>		
EJ2060	<i>fliD1101 recA56</i>	Tominaga et al. 1994
EJ2061	<i>fliA4181 recA56</i>	Tominaga et al. 1994
EJ2282	<i>ΔfliC48 recA56</i>	Tominaga et al. 1991
PM336	<i>fliC16^{on}</i> (H16)	Ratiner 1998
Bi7327-41	<i>flkA3^{on}, fliC^{off}</i> (H3)	Ratiner 1982
E480-68	<i>flkA53^{on}</i> (H53)	Ratiner 1987
E223-69	<i>flmA54^{on}</i> (H54)	Ratiner 1998
<i>Salmonella enterica</i>		
KK1004	<i>ΔfliJBA fliC(i)^{on}</i>	Kutsukake et al. 1988

a. The H antigen number is postfixed to each flagellin gene. Superscripts: on, expressed; off, cryptic state. The flagellar antigens expressed are indicated in the parentheses.

DNA manipulation and sequencing Genomic DNA was prepared by the method described (Tominaga et al., 1991). Isolation of plasmid DNA, construction of recombinant plasmids, restriction analysis of cloned fragments and transformation were performed using standard methods (Sambrook et al., 1989). Restriction endonucleases and T4 DNA ligase were purchased from Takara and used as recommended. The nonfunctional *fliC* regions were PCR-amplified with the primers *fliA2* (5'-AACCGCCG-AAACTGTACCGAGA-3') and *fliD-S3* (5'-TCCGGATC-CGATTGCAAACAGTAG-3') and cloned into pSTV28. Nucleotide sequence was determined by primer walking using a dye terminator cycle-sequencing ready-reaction kit (Applied Biosystems). Nucleotide and amino-acid sequences were analyzed using GENETYX information processing software (Software Development Co.).

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB128916 to AB128921.

RESULTS AND DISCUSSION

Cloning of the flagellin genes from the H3 standard strain The standard strain for H3 (Bi7327-41) has two flagellin genes, *fliC16* and *flkA3*, which are in a cryptic and expressed state, respectively. The expression of the *fliC16* gene is repressed by an FljA-like activity in this strain (Ratiner, 1982).

For cloning of the *fliC16* gene, strain PM336 expressing the *fliC16* gene transduced from Bi7327-41 was used as a DNA donor. DNA of PM336 digested with *Bam*HI was inserted into pBR322 and introduced into a non-motile strain EJ2282 (*ΔfliC*). One motile clone was isolated, and a recombinant plasmid within the clone was named pECH16-1. This plasmid was found to contain a 12.6-kb *Bam*HI fragment and confer motility on the strains EJ2282 (*ΔfliC*), EJ2060 (*fliD*) and EJ2061 (*fliA*) (Fig. 1A). As the *fliC* gene is flanked by the *fliA* and *fliD* genes in *E. coli* K-12, this result suggests that this flagellin gene locates in the authentic *fliC* locus. According to subcloning experiments, the *fliC16* gene was finally localized in a 4.5-kb *Cla*I fragment carried by pECH16-2.

For cloning of the *flkA3* gene, DNA of Bi7327-41 was digested with *Eco*RI, inserted into pACYC184 and introduced into strain EJ2282. One of the motile clones was isolated, and a recombinant plasmid within the clone was named pECH3-1. This plasmid was found to contain a 19.3-kb *Eco*RI fragment and confer motility on EJ2282 but not on either EJ2060 or EJ2061 (Fig. 1B). According to subcloning experiments, the *flkA3* gene was finally localized onto a 5.5-kb *Cla*I fragment carried by pECH3-3.

Cloning of the flagellin genes from the standard strains for H53 and H54 The standard strains for H53 (E480-68) and H54 (E223-69) have single functional flagellin genes, *flkA53* and *flmA54*, respectively, and have no functional *fliC* gene (Ratiner, 1998). For cloning of the *flkA53* and *flmA54* genes, DNA of E480-68 or E223-69 was digested with *Bam*HI, *Eco*RI or *Sal*I, inserted into pACYC184 and introduced into strain EJ2282. Several motile clones were isolated. One recombinant plasmid pECH53-1 derived from E480-68 contains a 30.6-kb *Sal*I fragment. Two recombinant plasmids pECH54-1 and pECH54-2 derived from E223-69 contain a 25.7-kb *Bam*HI fragment and a 13.6-kb *Eco*RI fragment, respectively (Fig. 1C, 1D). According to subcloning experiments, the *flkA53* and *flmA54* genes were finally localized onto 3.6-kb *Hind*III and 4.6-kb *Bam*HI-*Sal*I fragments carried by pECH53-3 and pECH54-3, respectively.

To clone the nonfunctional *fliC* genes from these strains, the *fliC* genes were PCR-amplified with the primers, *fliA2* and *fliD-S3*, and inserted into pSTV28. Recombinant plasmids pECC-53 and pECC-54 contain 2.7-kb E480-68 and 2.4-kb E223-69 fragments, respectively.

Fig. 1. Restriction maps of the cloned *E. coli* DNA fragments containing flagellin genes. Abilities to complement *fliD*, *fliC* and *fliA* mutants and FljA activity to repress the *fliC(i)* gene of *Salmonella* are also indicated. The size and transcriptional direction of the flagellin gene and the *fliA* gene are shown by solid and open arrows, respectively. Cloned fragments were derived from PM336 (A), Bi7327-41 (B), E480-68 (C) and E223-69 (D). Restriction cleavage sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I. NT, not tested.

FljA repressor activity To detect the FljA-like repressor activity in the cloned *flkA* and *flmA* loci, each recombinant plasmid (pECH3-1, pECH53-1 or pECH54-1) was introduced into a *Salmonella* Δ *fljBA* mutant (KK1004 *fliC(i)^{on}*), and the motility was examined on an NSS plate with or without anti-i serum. All recombinant plasmids conferred motility even in the presence of anti-i serum, though the motility of KK1004 was completely inhibited by anti-i serum. This indicates that the *fliC(i)* gene was repressed by the FljA-like activity on the plasmids. This

FljA-like activity was also detected on smaller plasmids (pECH3-3, pECH53-3 and pECH54-3). Genes for this repressor activity were named *fljA3*, *fljA53* and *fljA54*, respectively.

Sequence analysis of the *flkA* and *flmA* loci The *flkA* and *flmA* genes and their flanking regions were sequenced. Gene organization deduced from the determined nucleotide sequences are summarized in Fig. 2. Comparison to the genome organization of *E. coli* K-12

Fig. 2. Gene organization of the flagellin genes, *flkA* and *flmA* and their flanking regions. Thin lines indicate DNA regions unique to the strains analyzed, whereas thick lines indicate the DNA regions conserved in *E. coli* K-12. Arrows above the lines show *E. coli* K-12 genes. Newly identified *orfs* are indicated by open arrows and named after number of amino acids they encode. IS elements are indicated by rectangles. They are homologous more than 92% to the known elements in DDBJ/EMBL/GenBank databases.

indicates that the *flkA* loci in strains Bi7327-41 and E480-68 are located at the identical position in the 3' region of the *rnpB* gene, which exists at 70.4 min in the *E. coli* K-12 genome. This suggests that these *flkA* regions might have been derived from a common ancestor. The *flmA* region in E223-69 is flanked by truncated *yicC* and *yicK* genes, which locate 19.4 kb apart from each other near 82.5 min in *E. coli* K-12. This suggests that the 19.4-kb DNA region of K-12 might have been replaced by the *flmA* locus in E223-69. The *yicC* and *yicK* genes are transcribed in the same direction in *E. coli* K-12 but in the opposite directions in E223-69, suggesting that this DNA region had been subjected to genomic rearrangement after or at the time of its integration.

Flagellin genes were identified on the basis of the homology with known flagellin genes (Fig. 2). The predicted gene sizes of *flkA3*, *flkA53* and *flmA54* are 1,587 bp (529 amino-acid residues [a.a.], 55,567 Da), 1269 bp (423 a.a., 44,887 Da) and 1548 bp (516 a.a., 54,451 Da), respectively. All of these flagellin genes are followed by genes homologous to *Salmonella fljA*, which are named *fljA3*, *fljA53* and *fljA54*, respectively. They consist of 522 bp (174 a.a., 19,734 Da), 522 bp (174 a.a., 19,750 Da) and 525 bp (175 a.a., 19,746 Da), respectively. However, these flagellin genes are not accompanied with either the *hin* gene or the invertible H segment, suggesting these are expressed constantly.

The replaced DNA regions have no sequence homology to any sequences in DDBJ/EMBL/GenBank databases except for IS elements and flagellum-related sequences.

Though the two *flkA* regions in Bi7327-41 and E480-68 are homologous to each other, the regions downstream of the *fljA* gene are divergent. In Bi7327-41, that region contains an *ISEhe3* element (Guo et al., 2002), into which an *IS30* element (Dalrymple et al., 1984) is inserted further. A predicted ORF211 of E480-68 is partially homologous to a hypothetical protein YxkC (213 a.a.) of *Bacillus subtilis* (Kunst et al., 1997).

More than 4-kb sequences upstream of the two *flkA* genes are 98% homologous to each other, and the amino-acid sequences of the predicted ORFs, ORF204 of Bi7327-41 and ORF210 of E480-68, ORF240 of both strains and ORF486 of both strains exhibit more than 99% homology. Both ORF204 and ORF210 are 43.8% homologous to the PinR protein (196 a.a.) of lambdoid prophage Rac (Blattner et al., 1997), which belongs to the DNA invertase/resolvase family as well as the *Salmonella* Hin protein (Johnson, 2002). The organization of *orf204*, *flkA3* and *fljA3* in Bi7327-41 or *orf210*, *flkA53* and *fljA53* in E480-68 is reminiscent of that of the *hin* gene and the *fljBA* operon in *S. enterica* serovar Typhimurium, suggesting that these three loci might have been derived from a common ancestor. ORF240 and ORF486 are partially homologous to a hypothetical protein VV2259 (212 a.a.) of *Vibrio vulnificus* (Chen et al., 2003) and a probable integrase/recombinase family protein XCC1597 (488 a.a.) of *Xanthomonas campestris* (da Silva et al., 2002), respectively.

The *flmA* locus in E223-69 contains insertions of one *ISEhe3* with an internal deletion and two *IS1s* (Umeda and Ohtsubo, 1991), one of which is disrupted by a sec-

Table 2. Homology of the 5' and 3' constant sequences among flagellin genes and molecules

Gene		<i>flkA3</i>	<i>flkA53</i>	<i>flmA54</i>	<i>fliC16</i>	<i>fliC48</i>	<i>fliC(i)</i>	<i>fljB</i>
<i>flkA3</i>	N		0.78 (518)	0.78 (520)	0.79 (520)	0.74 (520)	0.77 (512)	0.77 (512)
	A		0.84 (173)	0.86 (173)	0.92 (173)	0.84 (173)	0.83 (173)	0.83 (173)
<i>flkA53</i>	N	0.80 (275)		0.79 (515)	0.75 (518)	0.74 (511)	0.78 (511)	0.77 (511)
	A	0.86 (92)		0.86 (173)	0.86 (173)	0.79 (173)	0.86 (173)	0.86 (173)
<i>flmA54</i>	N	0.79 (276)	0.78 (260)		0.80 (520)	0.73 (520)	0.79 (520)	0.78 (520)
	A	0.87 (92)	0.83 (93)		0.89 (173)	0.84 (171)	0.91 (173)	0.91 (173)
<i>fliC16</i>	N	0.83 (280)	0.80 (274)	0.77 (277)		0.75 (520)	0.78 (514)	0.76 (518)
	A	0.91 (93)	0.84 (92)	0.89 (92)		0.84 (173)	0.84 (173)	0.84 (173)
<i>fliC48</i>	N	0.68 (274)	0.64 (274)	0.65 (264)	0.70 (272)		0.79 (513)	0.78 (512)
	A	0.73 (91)	0.67 (91)	0.70 (91)	0.75 (91)		0.79 (173)	0.79 (173)
<i>fliC(i)</i>	N	0.79 (276)	0.76 (251)	0.78 (279)	0.78 (277)	0.71 (271)		0.98 (520)
	A	0.87 (92)	0.81 (93)	0.87 (93)	0.85 (92)	0.69 (91)		0.99 (173)
<i>fljB</i>	N	0.79 (276)	0.74 (251)	0.75 (279)	0.78 (277)	0.70 (278)	0.89 (280)	
	A	0.87 (92)	0.82 (92)	0.87 (92)	0.85 (92)	0.71 (91)	0.97 (92)	

A pair of nucleotide sequences, 520 bp for the 5' sequence and 280 bp for the 3' sequence, was compared by maximum homology alignment. Predicted amino-acid sequences were also compared. The numbers in parentheses show the numbers of nucleotides or amino acids used for comparison. Homology in the 5' and 3' sequences is shown above and under the slanted line, respectively. Flagellin genes are named after their H antigen types. N, nucleotide sequence; A, amino-acid sequence.

ondary insertion of IS1222 (Steibl and Lewecke, 1995). The insertion site of the intact IS1 is flanked by 9-bp direct repeats. The nine bp direct repeat might be target duplication, suggesting that this insertion was a recent event of evolution. Among six ORFs predicted,

only three (ORF74, ORF125 and ORF148) show amino-acid sequence homology to those in protein databases. ORF74 is 37% homologous to a hypothetical protein Rsc0962 of *Ralstonia solanacearum* (Salanoubat et al., 2002), which has a DNA-binding domain of the transcrip-

Fig. 3. Comparison of the upstream (A, B) and downstream (C, D) sequences of the flagellin genes. A, B: The sequences are numbered from the first nucleotide (+1) of the start codon. σ^{28} -dependent promoter and SD sequences are underlined. The boxed sequence in *fljB* is the right one of the inverted repeats (IRR) of the H segment (Zieg and Simon, 1980). C, D: The sequences including the stop codon (TAA) for the flagellin gene and the start codon (ATG or GTG) for the next gene (*fljA* or *fljA*) are shown. Putative terminator sequences are shown by converging arrows.

tional regulator AlpA of the cryptic prophage CP4-57. ORF125 is 54% homologous to the fimbrial protein MrfJ of *Photobacterium luminescens* (Duchaud, et al., 2003), which contains a helix-turn-helix motif specific for DNA-binding proteins. ORF148 is partially homologous to a putative lipoprotein Reut4794 of *Ralstonia metallidurans*.

Sequence analysis of the *fliC* genes Three *fliC* genes cloned were also sequenced. The functional *fliC16* gene from Bi7327-41 consists of 1572 bp (524 a.a., 55,039 Da). Two nonfunctional *fliC* genes, *fliC-53* from E480-68 and *fliC-54* from E223-69, are interrupted by insertions of IS1 and IS1222, respectively. A target duplication of 8 bp is present at the insertion site of IS1 into *fliC-53*. The presumed intact *fliC-53* gene consists of 1476 bp (492 a.a., 51,660 Da) and is highly homologous (98%) to the *fliC* gene for H40 antigen. In the *fliC-54* gene, no target duplication can be detected at either side of IS1222. If this IS1222 sequence is removed, the resulting *fliC* gene should encode a protein too small to form flagellar filaments (Kuwajima 1988). Therefore, it is assumed that two IS1222 elements might have been integrated independently into different sites of the ancient *fliC* gene and a subsequent homologous recombination between them resulted in deletion of the central region.

Comparison of protein and gene sequences of flagellin and FljA The molecular masses of flagellin from strains PM336, Bi7327-41, E480-68 and E223-69 were estimated by SDS-PAGE to be 54 kDa, 54 kDa, 43 kDa and 53 kDa, respectively, which are in a reasonable agreement with those deduced from nucleotide sequences of *fliC16*, *flkA3*, *flkA53* and *flmA54* genes, respectively (data not shown).

Flagellin genes are generally composed of three structural parts, 5'-constant, central variable and 3'-constant regions. Comparison of the 5'- or 3'-constant sequence of the newly analyzed flagellin genes with those of *E. coli* K-12 and *S. enterica* serovar Typhimurium is shown in Table 2. The *flkA*, *flmA* and *fliC16* genes show higher similarity with those of *Salmonella* for both 5'- and 3'-constant sequences, suggesting that these three genes are close relatives of the *Salmonella* flagellin genes. Similarities were also observed in the amino-acid sequences predicted from the nucleotide sequences. Marked similarities are observed in the promoter regions among the *flkA*, *flmA* and *fljB* genes. However, the inverted repeat sequence responsible for invertible recombination of the H segment is absent in the *flkA* and *flmA* genes (Fig. 3A). The 105-bp regions upstream of the *fliC16*, *fliC-53* and *fliC-54* are almost identical with that of *fliC(i)* of *Salmonella* (Fig. 3B), which contains its promoter and operator sequences (Hanafusa et al., 1989, Ide et al., 1999, Inoue et al., 1989). This suggests that both *fliC-53* and

fliC-54 genes are also repressed by FljA. The *fliC* genes might have been inactivated by IS insertions during their repressed state in strains E480-68 and E223-69. The intergenic regions between the *fliC* (*fliC16*, *fliC-53* or *fliC-54*) and *fliA* genes are identical except for 3-bp mismatch. Each of these regions contains a ρ -independent terminator-like sequence identical to that of *fliC48* (Fig. 3D). These regions show no sequence similarity to the region downstream of the *Salmonella fliC* gene.

Comparison of the FljA repressors is shown in Table 3. FljA3 and FljA53 show 98% homology with each other and 83% and 85% homology with FljA54, respectively. Homology of the FljA repressors of *E. coli* with that of *Salmonella* is less than 70%, indicating that the *fljA* genes in *E. coli* had diverged from that of *Salmonella* and have been evolving independently. A ρ -independent terminator, that is known to attenuate the *fljA* expression in *Salmonella* (Hanafusa et al., 1993), is also present in the intergenic regions of *flkA* or *flmA* and *fljA* (Fig. 3C).

Table 3. Homology of FljA repressor

Gene \ Protein	FljA3	FljA53	FljA54	FljA
<i>fljA3</i>		0.99 (174)	0.83 (174)	0.68 (174)
<i>fljA53</i>	0.97 (522)		0.84 (174)	0.68 (174)
<i>fljA54</i>	0.85 (521)	0.85 (521)		0.69 (173)
<i>fljA</i>	0.71 (520)	0.71 (520)	0.70 (520)	

Nucleotide sequences and predicted amino-acid sequences were compared by maximum homology alignment. The numbers in parentheses show the numbers of nucleotides or amino acids used for comparison. Homology between the amino-acid sequences and between the nucleotide sequences is shown above and under the slant line, respectively.

Conclusions Flagellin genes from the H3, H53 and H54 standard strains of *E. coli* were sequenced and compared with known flagellin genes. All the flagellin genes show marked similarity to the *Salmonella* flagellin genes, suggesting their lateral transfer from *Salmonella*. The *flkA* and *flmA* genes are flanked by IS elements and genes involved in the recombination of mobile DNAs, suggesting that these genes might have been transferred as parts of transposable units and subjected to many structural changes such as rearrangements mediated by IS elements.

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