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Genetic and molecular analyses of the interaction between the flagellum-specific sigma and anti-sigma factors in *Salmonella typhimurium*

Kazuhiro Kutsukake[1,4], Sunao Iyoda[1], Kouhei Ohnishi [2,3] and Tetsuo Iino[2]

[1]Faculty of Applied Biological Science, Hiroshima University, Kagamiyama 1-4-4, Higashi-Hiroshima 724 and [2]School of Human Sciences, Waseda University, Tokorozawa, Saitama 359, Japan
[3]Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114, USA
[4]Corresponding author

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More than 50 genes are required for flagellar formation and function in *Salmonella typhimurium*. According to the cascade model of flagellar regulon, the flagellar operons are divided into three classes, 1, 2, and 3, with respect to transcriptional hierarchy. FliA is an alternative sigma factor specific for transcription of the class 3 operons, while FlgM is an anti-sigma factor which binds to FliA and prevents its association with RNA polymerase core enzyme. In the present study, we isolated a number of *fliA* mutants in which the altered FliA proteins become insensitive to inhibition by FlgM. Sequence analysis of their mutation sites revealed that most of them caused the amino acid substitutions in region 4 of the conserved amino acid sequences of sigma factors which lies near the C-terminal end of FliA. Using a set of *fliA* deletion mutants in a high-expression plasmid, we demonstrated that polypeptides containing the C-terminal portion of FliA could titrate the intracellular FlgM protein resulting in derepression of the class 3 operons. This result indicates that the C-terminal region of FliA contains the FlgM-binding domain. This was confirmed by a chemical cross-linking experiment with FlgM and truncated FliA proteins.

Key words: alternative sigma factor/anti-sigma factor/flagellar regulon/protein–protein interaction/*Salmonella typhimurium*

Introduction

The bacterial flagellum is a complex organelle consisting of three distinctive structural parts, the basal body, the hook and the filament (for a recent review, see Macnab, 1992). The filament extends into the extracellular space and is connected by the hook to the basal body located in the cell membrane. Assembly of the flagellum begins with the basal body, proceeds with the hook and is completed with the filament (Suzuki *et al.*, 1978). In *Salmonella typhimurium*, >50 genes have been shown to be required for flagellar formation and function. These genes constitute at least 13 different operons on the chromosome (Kutsukake *et al.*, 1988). In the expression cascade of flagellar regulon, the flagellar operons are grouped into three classes, 1, 2 and 3, with respect to transcriptional hierarchy (Kutsukake *et al.*, 1990). Class 1 contains only one operon, *flhD*, consisting of two genes, *flhD* and *flhC*, both of which are believed to be positive regulators of the transcription of class 2. Class 2 contains seven operons, *flgA, flgB, flhB, fliA, fliE, fliF* and *fliL*. All the class 2 genes except *fliA* are involved in the formation of the hook–basal body complex. The *fliA* gene encodes an alternative sigma factor specific for class 3 operons (Ohnishi *et al.*, 1990). Class 3 contains three operons, *fliC, fliD* and *flgK*, involved in filament formation, and at least two operons, *motA* and *tar*, involved in flagellar rotation and chemotaxis. Mutants defective in any one of the class 2 hook–basal body genes cannot express class 3 operons. Sequential transcription of the flagellar operons is thus coupled with the assembly process of flagellar structure.

On the basis of the observation that a *fliD*::Tn*10* mutation enhanced the expression of class 3, the *fliD* operon has been postulated to contain a negative regulatory gene, *rflA* (Kutsukake *et al.*, 1990). However, because the *fliD*::Tn*10* mutation could not relieve the repression of the class 3 operons in the hook–basal body mutant background (K.Kutsukake, unpublished result), *rflA* is unlikely to be involved in coupling of class-3 expression with flagellar assembly. Based on genetic analysis of the mutants that could express one class 3 operon, *fliC*, in the hook–basal body mutant background, Gillen and Hughes (1991a,b) identified another negative regulatory gene, *flgM*, which couples class 3 expression with flagellar assembly. Our biochemical study indicated that FlgM is an anti-sigma factor which binds to FliA and prevents its association with RNA polymerase core enzyme (Ohnishi *et al.*, 1992). Recently, it was shown that FlgM monitors the state of flagellar assembly and is secreted from the cell through the hook–basal body structures formed by the function of the class 2 hook–basal body genes (Hughes *et al.*, 1993; Kutsukake, 1994). Because mutants defective in the class 2 hook–basal body genes cannot complete the assembly of the hook–basal body structures, the FlgM proteins accumulate within the cell, resulting in repression of the class 3 operons. FlgM secretion was shown to be enhanced by the *fliD*::Tn*10* mutation (Kutsukake, 1994), indicating that the *rflA* gene in the *fliD* operon is involved in modulating FlgM secretion.

This work was performed in order to understand the molecular details of the interaction betweeen FliA and FlgM. To this end, we isolated a number of *fliA* mutants in which the altered FliA proteins had become insensitive to inhibition by FlgM. Sequence analysis of their mutation sites revealed that most of them caused amino acid substitutions near the C-terminal end of FliA. We demonstrated that polypeptides containing the C-terminal portion of FliA expressed in excess allowed the class 3 operons

to be expressed even in the hook-basal body mutant background. These results indicate that the C-terminal portion of FliA contained the FlgM-binding domain and titrated the intracellular FlgM protein, resulting in derepression of the FliA-dependent transcription of class 3 operons. This was further confirmed by a chemical cross-linking experiment with intact FlgM and truncated FliA proteins. Based on these results, we discuss the regulatory mechanism involving interplay between sigma and anti-sigma factors.

Results

Isolation and linkage analysis of derepression mutants

In order to understand the mechanism of protein–protein interaction between FliA and FlgM, we attempted to isolate *fliA* mutants which encoded altered FliA proteins that could direct transcription of class 3 flagellar operons even in the presence of FlgM. Such mutants were expected to be obtained among the derepression mutants in which the class 3 operons were allowed to be expressed in the hook–basal body mutant background. Strain KK1355T carried a *tar–lac* fusion gene and a deletion mutation in the *flhB* operon. Because the *tar* and *flhB* operons belong to class 3 and class 2 respectively, this strain displayed a Lac^- phenotype owing to the repression of *tar–lac* by FlgM. Cells of this strain were spread on minimal lactose plates and incubated for 1 day a ±37°C. Colonies that formed on the plates were purified and their Lac phenotypes were tested on MacConkey lactose plates. Sixty spontaneously induced Lac^+ mutants isolated independently were thus established.

Almost all of the flagellar genes are clustered in three regions of the chromosome called regions I, II and III (Macnab, 1992). Linkage of the derepression mutations with these flagellar regions was analysed by examining their cotransducibility with the *flgB* (region I), *tar* (region II) and *fliC* (region III) genes. Based on the results shown in Table I, the mutations were classified into four groups which were tentatively named *rflB, rflC, rflD* and *rflE*.

The *rflB* mutations were closely linked to the *flgB* gene. Because the *flgM* gene is located near the *flgB* gene (Gillen and Hughes, 1991a; Kutsukake *et al.*, 1994) and because there is no other regulatory gene in region I, it seems most plausible that the *rflB* mutations are located in the *flgM* gene. The *rflC* mutations were not linked to the known flagellar gene clusters. As described in Materials and methods, a strain which carried Tn*10* inserted near one of the *rflC* mutations (*rflC192*) was constructed. The *rflC192* mutation was 32% linked to the Tn*10* insertion. All the other *rflC* mutations were shown to be 20–40% linked to the Tn*10* insertion, suggesting that all the *rflC* mutations occur within a single gene. However, their map location has not been determined. The *rflD* mutations were closely linked to the *fliC* gene. Because the *fliC* gene is adjacent to the *fliA* gene (Kutsukake *et al.*, 1988), this result suggested that the *rflD* mutations might be located in the *fliA* gene. In order to confirm this, P22 phage lysates were propagated on these mutants and used to transduce a *fliA* deletion mutant, KK1361. In each case 20 $FliA^+$ transductants were selected. Next, both the *tar–lac* fusion gene and the *flhB*::Tn*10* mutation were introduced into each transductant by P22-mediated transduction, and the Lac phenotypes of the resulting transductants were examined on MacConkey lactose plates. In every case, all the transductants displayed a Lac^+ phenotype, indicating that the *rflD* mutations are located in the *fliA* gene. All the *rflE* mutations were closely linked to the *tar–lac* fusion gene. Because introduction of the *fliA*::Tn*10* mutation into these mutants did not abolish the expression of *tar–lac*, they may be promoter mutations which render the *tar–lac* fusion gene able to be expressed independently of FliA.

Table I. Classification of the derepression mutants

Mutation name	Linkage (%) with			FliA-independent expression[c]	No. of isolates	Presumed locus
	flgB[a]	*fliC*[a]	*tar–lac*[b]			
rflB	>75	<1	<1	–	21	*flgM*
rflC	<1	<1	<1	–	7	unknown
rflD	<1	>84	<1	–	22	*fliA*
rflE	<1	<1	>91	+	10	promoter

[a]Linkage of the *rfl* mutations with the *flgB* and *fliC* genes was examined as follows. P22 phage lysates propagated on KK2085 (*flgB*::Tn*10*) or KK2604 (*fliC*::Tn*10*) were used to transduce each *rfl* mutant and tetracycline-resistant transductants were selected. Their Lac phenotypes were examined on MacConkey lactose plates. In each cross, 100 transductants were tested.

[b]Linkage of the *rfl* mutations with the *tar–lac* fusion gene was examined as follows. P22 phage lysate propagated on each *rfl* mutant was used to transduce KK1355[Δ(*flhB–flhA*)] and ampicillin-resistant transductants were selected. Their Lac phenotypes were examined as above.

[c]The *fliA*::Tn*10* mutation was introduced into each *rfl* mutant by P22-mediated transduction. The Lac phenotypes were examined with the resulting transductants. Symbols + and – indicate the Lac^+ and Lac^- phenotypes respectively.

Sequence analysis of the rflD mutation sites

In order to determine the changes in DNA sequence that resulted in the *rflD* mutant phenotype, we cloned and sequenced the *fliA* genes from *rflD* mutants. Single base changes which resulted in amino acid substitutions were detected in all the *rflD* mutants except two (*rflD254* and *rflD405*). The mutations were named as in the following example: H14N contains a substitution of the histidine residue at position 14 with an asparagine residue (Figure 1). Though isolated independently, many of these mutations were found to be identical. Interestingly, most of them caused amino acid substitutions in the C-terminal region of FliA. We did not detect any change in DNA sequence in the *fliA*-coding region from the *rflD254* and *rflD405* mutants. Because their mutation sites were closely linked to *fliA*, they might be up-promoter mutants in the *fliA* gene. These two mutants were not analysed further in the present study.

Expression of the tar operon in rflD mutants

To quantify the effects of the *rflD* mutations on flagellar gene expression, we examined the activity of β-galactosidase from the *tar–lac* fusion gene in *rflD* mutants in both repressed and derepressed conditions (Table II). Derepression was achieved by introducing the *flgM*::*cat* mutation. The level of *tar–lac* expression in this condition was expected to reflect the actual sigma activity of the mutant FliA proteins. The H14N FliA protein exhibited slightly elevated sigma activity, while the L199R FliA

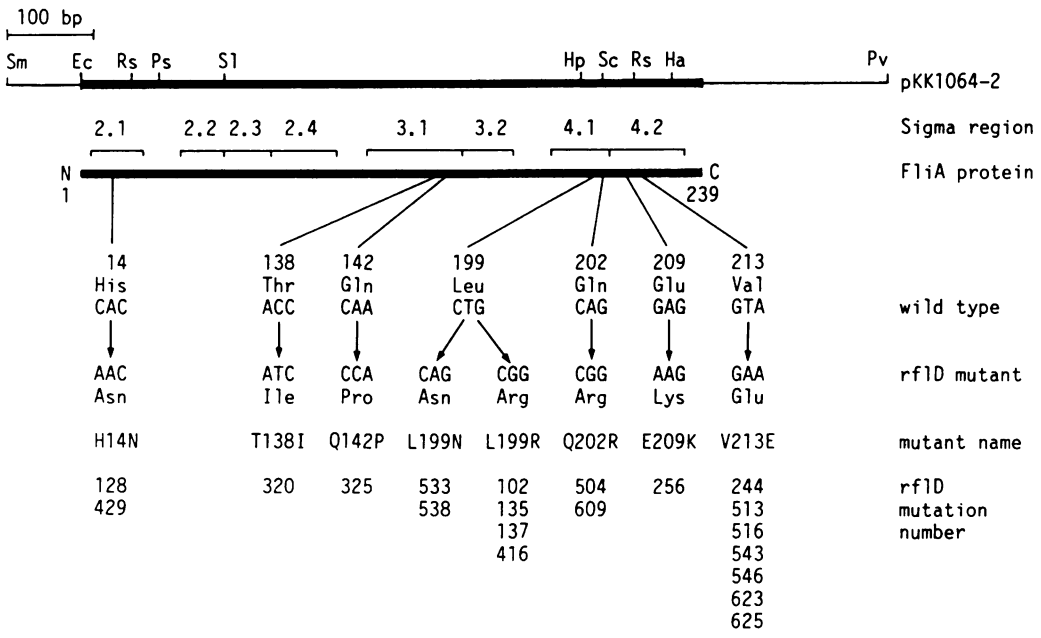

Fig. 1. Restriction map of the *fliA* gene and nucleotide and amino acid substitutions in the *rflD* mutants. Restriction sites used in the present study are shown using following abbreviations: Ec, *Eco*RI; Ha, *Hae*III; Hp, *Hpa*I; Ps, *Pst*I; Pv, *Pvu*II; Rs, *Rsa*I; Sl, *Sal*I; Sm, *Sma*I. The heavy line indicates the *fliA*-coding region. The unique *Eco*RI site exists just downstream of the initiation codon of the *fliA* gene. The FliA protein consists of 239 amino acids. N and C indicate the N- and C-termini of the FliA protein, respectively. The conserved regions of sigma factors were defined according to Ohnishi *et al.* (1990) and Lonetto *et al.* (1992). The nucleotide substitutions caused by the *rflD* mutations were determined by DNA sequencing.

protein showed impaired sigma activity. The other mutant FliA proteins showed sigma activity equivalent to or slightly weaker than that of the wild-type protein. Repression was achieved by introducing the *flhB*::Tn*10* mutation. As expected, in this condition, the level of *tar–lac* expression was at least 2-fold higher in the *rflD* mutants than in the *fliA*$^+$ strain. In particular, the V213E FliA protein showed 40-fold higher activity than the wild-type protein. The sensitivity of FliA to repression by FlgM can be defined as the ratio of the activity in the derepressed condition to that in the repressed condition. As shown in Table II, the H14N, T138I, Q142P and Q202R FliA proteins retained relatively high sensitivity to repression by FlgM, while the other four mutant FliA proteins showed low sensitivity. Interestingly, the L199R FliA protein showed higher activity in the repressed condition than in the derepressed condition, suggesting that the activity of this mutant FliA protein may be enhanced by FlgM. This supports the existence of direct interaction between FliA and FlgM. Except for the Q202R mutation, all the *rflD* mutants whose amino acid substitutions were located in the C-terminal region of FliA showed lowered sensitivity to repression by FlgM. This result suggests that the FlgM-binding site of FliA may be located in the C-terminal region.

Table II. Expression of the *tar–lac* fusion gene in the *rflD* mutants

Amino acid substitution in FliA	β-Galactosidase activity[a]		FlgM sensitivity[b]
	flhB::Tn*10*	*flgM*::*cat*	
None	3	209	70
H14N	7	288	41
T138I	8	181	23
Q142P	8	164	21
L199N	26	98	3.8
L199R	56	32	0.57
Q202R	12	151	13
E209K	30	128	4.3
V213E	125	194	1.6

[a]The β-galactosidase activity (Miller units) was assayed with the *rflD* mutants carrying the *tar–lac* fusion gene in the presence of the *flhB*::Tn*10* or *flgM*::*cat* mutation.
[b]FlgM sensitivity was defined as the ratio of the β-galactosidase enzyme units in the *flgM*-mutant background to those in the *flhB*-mutant background.

FlgM titration assay by truncated FliA proteins

In order to confirm and extend the result obtained above, we designed the *in vivo* assay method for FlgM titration by the truncated FliA proteins. For this purpose, various portions of the *fliA* gene were fused in-frame to the polylinker site of the expression vectors pTrc99A, B, and C. Because these vectors carried the *tac* promoter, the *lacZ* ribosome-binding site and the *lacI*^{q} gene, expression of the genes inserted in-frame into the polylinker site was driven by induction with isopropyl-β-D-thiogalactopyranoside (IPTG). As shown in Table III, the fusions progressed across the entire FliA polypeptide and were designed to include different portions of FliA. In order to assess the production of truncated FliA polypeptides, the plasmids were introduced into KK1361(Δ*fliA*) by transformation, and the protein products from the transformants treated with IPTG were separated by SDS–PAGE and analysed by Western blotting with a polyclonal antibody against FliA (Figure 2). In all the transformants except those harbouring pSIIA5 and pSIIA10, protein products which reacted with the antibody could be detected. However, such proteins were not detected from either pSIIA5 or

Table III. Sigma and FlgM-titration activities of the truncated FliA proteins

Plasmid	Amino acid residues of FliA[a]	β-Galactosidase activity[b]		
		KK1505IA	KK1505GM	KK1505HB
pTrc99A	None	6	239	2
pSIIA1	2–239	673	ND	ND
pSIIA2	31–239	7	245	202
pSIIA3	56–239	6	211	212
pSIIA4	154–239	6	248	166
pSIIA5	198–239	6	266	2
pSIIA6	2–227	6	276	22
pSIIA7	2–205	6	277	2
pSIIA8	2–197	6	218	2
pSIIA9	22–213	6	210	2
pSIIA10	154–227	5	244	10

[a]In addition to the amino acids from the FliA protein, the polypeptides encoded by the fusion genes on the plasmids contained 2–24 amino acids derived from the vector sequences.
[b]Each plasmid was introduced into KK1505IA (*fliA*), KK1505GM (*flgM*) and KK1505HB (*flhB*) by transformation. The β-galactosidase activity (Miller units) was assayed with each transformant in the presence of IPTG. The values in KK1505IA and KK1505HB reflect the residual sigma activity and the FlgM-titration activity of the truncated FliA proteins programmed by the plasmids, respectively. The values in KK1505GM indicate that none of the truncated FliA proteins have the activity to inhibit the transcription of the *tar–lac* fusion gene. ND, not done.

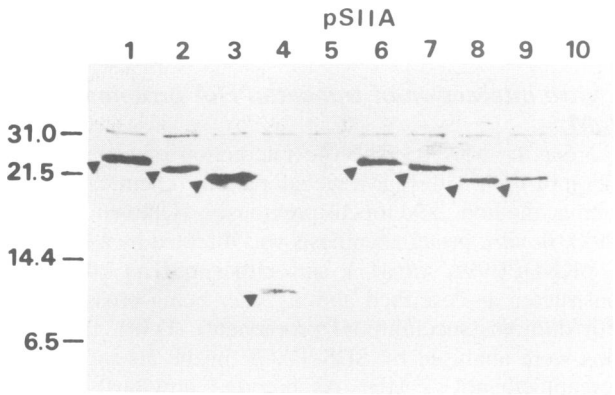

Fig. 2. Immunological detection of the truncated FliA proteins. Proteins from the cells of KK1361 (Δ*fliA*) harbouring one of the pSIIA plasmids were separated by SDS–PAGE and analysed by Western blotting using a polyclonal antibody against FliA as described in Materials and methods. Triangles indicate the proteins which reacted with the antibody. Numbers on the left indicate molecular masses in kDa.

pSIIA10, probably because their reactivity with the antibody may have been severely affected by truncation or because they may have been unstable within the cell.

Prior to FlgM titration assay, two control experiments were carried out. First, the sigma activity retained in the truncated polypeptides was examined. Hybrid plasmids were introduced into the strain KK1505IA by transformation, and the levels of β-galactosidase were assayed in the presence of IPTG (Table III). KK1505IA carried the *tar–lac* fusion gene and the *fliA*::Tn*10* mutation, and thus displayed a Lac$^-$ phenotype. Truncated polypeptides that retained sigma activity should be able to complement the *fliA* defect and so the transformants should display a Lac$^+$ phenotype. In fact, KK1505IA harbouring pSIIA1 which carried the entire *fliA* gene exhibited a high β-galactosidase activity. However, none of the transformants harbouring the other hybrid plasmids were found to show a Lac$^+$ phenotype, indicating that all the truncated polypeptides programmed by these plasmids lacked sigma activity.

Second, we tested the possibility that the truncated FliA polypeptides would inhibit the transcription of flagellar genes, because at least some of them might retain the ability to bind to the promoters or to RNA polymerase core enzyme. For this purpose, the plasmids were introduced into KK1505GM and the levels of β-galactosidase were assayed in the presence of IPTG (Table III). KK1505GM carried the *tar–lac* fusion gene and the *flgM* mutation, and thus showed high β-galactosidase activity. It was found that introduction of either of the plasmids did not reduce the β-galactosidase activity, indicating that none of the truncated FliA polypeptides could inhibit the expression of the *tar–lac* fusion gene.

Then, a FlgM titration assay was carried out. Hybrid plasmids were introduced by transformation into KK1505HB, and the levels of β-galactosidase were assayed in the presence of IPTG. KK1505HB carried the *tar–lac* fusion gene and the *flhB*::Tn*10* mutation, and thus displayed a Lac$^-$ phenotype owing to the repression of *tar–lac* by FlgM. If the truncated polypeptides retained the ability to bind to FlgM, the polypeptides expressed in excess in the cell should titrate the intracellular FlgM protein, resulting in the *tar–lac* fusion gene being derepressed. The results obtained are summarized in Table III. It was found that the plasmids directing the synthesis of the N-terminally truncated FliA proteins (pSIIA2, 3 and 4) could relieve the repression. This suggests that the N-terminal 153 amino acids are not involved in binding to FlgM. On the other hand, plasmids which directed the synthesis of C-terminally truncated FliA proteins could not relieve the repression. Because the polypeptide synthesized from pSIIA6 showed residual titration activity, the C-terminal critical point should be located between amino acids 214 and 227. Western blotting analysis of the protein product from pSIIA6 (Figure 2) suggested that removal of C-terminal amino acids 228–239 did not seem to affect the stability of the protein. The fact that the FlgM titration activity was drastically weakened in pSIIA6 therefore indicates that the C-terminal amino acids 228–239 play an important but ancillary role in the interaction with FlgM. Taken together, we concluded that the FlgM-binding domain lies within amino acids 154–227. This is consistent with the data on the distribution of the *rflD* mutation sites (Figure 1).

Hybrid plasmids which directed the synthesis of two different truncated forms of the *rflD*-mutant FliA proteins were constructed by inserting in-frame the appropriate restriction fragments from the *rflD*-mutant *fliA* genes into pTrc99A or pTrc99C. They consisted of amino acids 2–227 and 56–239 of the *rflD*-mutant FliA proteins which were equivalent to the polypeptides directed by pSIIA6 and pSIIA3, respectively. Production of the truncated proteins was confirmed by Western blotting analysis (Figure 3). These plasmids were introduced into KK1505HB by transformation and their effects on FlgM titration were examined as above (Table IV). Both forms of the polypeptides from the H14N, T138I, Q142P and E209K FliA proteins retained the titration activity, while those from the L199N, L199R and V213E FliA proteins

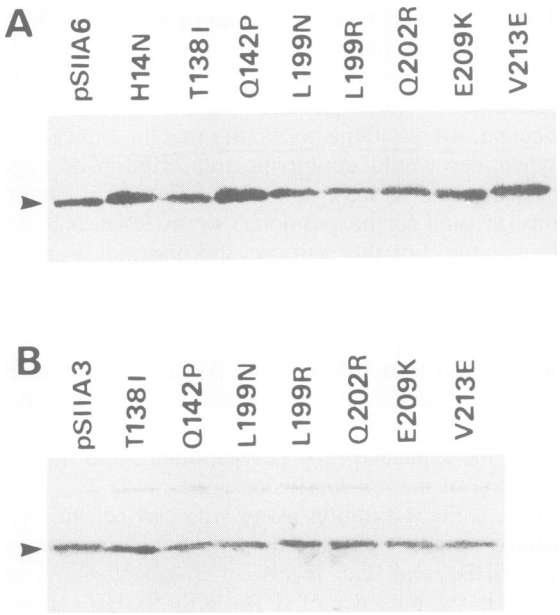

Fig. 3. Immunological detection of the truncated FliA proteins containing the *rflD* mutations. (**A**) Polypeptides consisting of amino acids 2–227. (**B**) Polypeptides consisting of amino acids 56–239. Detailed procedures were the same as those described in the legend to Figure 2. The arrowheads indicate the positions of the truncated FliA proteins.

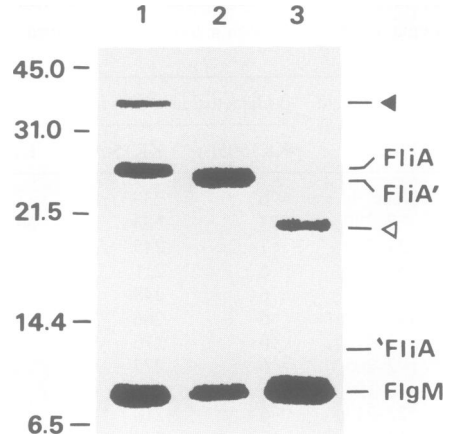

Fig. 4. Cross-linking of truncated FliA and intact FlgM proteins synthesized *in vitro*. Radiolabelled proteins were synthesized *in vitro*, treated with DSP, and separated immunologically using both anti-FliA and anti-FlgM antibodies. The samples were analysed by SDS–PAGE in the absence of 2-ME. Numbers on the left indicate molecular masses in kDa. Template plasmids were used as follows: lane 1, pKKOPB99A and pSIIA1; lane 2, pKKOPB99A and pSIIA7; lane 3, pKKOPB99A and pSIIA4. pKKOPB99A and pSIIA1 directed the synthesis of wild-type FlgM and FliA proteins, respectively. pSIIA4 and pSIIA7 directed the synthesis of N- and C- terminally truncated FliA proteins (`FliA and FliA′), respectively. Filled and open triangles indicate the positions of FliA–FlgM and `FliA–FlgM complexes, respectively. In lane 3, a faint band corresponding to `FliA is visible on the original autoradiogram but not in this figure.

Table IV. FlgM titration by the truncated *rflD*-mutant FliA proteins

Amino acid substitution in FliA	FlgM titration by polypeptide[a]	
	2–227	56–239
None	22	212
H14N	13	ND
T138I	11	163
Q142P	12	184
L199N	2	10
L199R	2	2
Q202R	<1	81
E209K	12	101
V213E	2	5

[a]Plasmids which generated two different polypeptides consisting of amino acids 2–227 and 56–239 of the wild-type and *rflD*-mutant FliA proteins were constructed as described in Materials and methods. The resulting plasmids were introduced into KK1505HB by transformation and the β-galactosidase activity was assayed with the resulting transformants in the presence of IPTG. FlgM-titration activity was expressed as the β-galactosidase activity (Miller units) of the transformants. ND, not done.

did not. These results are consistent with the data on FlgM sensitivity with the *rflD* mutants (Table II) except those with the E209K and Q202R proteins. Though the E209K protein exhibited a low FlgM sensitivity, it retained a high FlgM-titration activity. The result with the Q202R protein which retained high FlgM sensitivity was complicated. The 56–239 polypeptide showed high titration activity, while the 2–227 polypeptide showed severely impaired titration activity. At present, we cannot explain the mechanism that caused these discrepancies. Probably, these two mutations affect the intramolecular interaction of the FliA polypeptide which modulates the FlgM-binding activity.

In vitro interaction of truncated FliA proteins with FlgM

In order to show the specific interaction of a particular region of FliA with FlgM, we adopted the chemical cross-linking method developed previously (Ohnishi *et al.*, 1992). *In vitro* protein synthesis was directed by a mixture of pKKOPB99A (*flgM*$^+$) and either pSIIA1, 4 or 7, constructed as described above. After being cross-linked with dithio*bis*(succinimidyl propionate) (DSP), the proteins were analysed by SDS–PAGE in the absence of 2-mercaptoethanol (2-ME). As reported previously, when we used plasmid pSIIA1, which directed the synthesis of the entire FliA protein, a 40 kDa product cross-linked by DSP was detected in addition to the individual monomeric protein bands with apparent molecular masses of 28 kDa (FliA) and 8 kDa (FlgM) (Figure 4, lane 1). However, when we used plasmid pSIIA7, which directed the synthesis of C-terminally truncated FliA protein (FliA′), a product cross-linked by DSP could not be detected, though the individual monomeric protein bands with apparent molecular masses of 26 kDa (FliA′) and 8 kDa (FlgM) were both detected (Figure 4, lane 2). When we used plasmid pSIIA4, which directs the synthesis of the C-terminal portion of FliA (`FliA), a protein band with an apparent molecular mass of 20 kDa was detected in addition to the 8 kDa FlgM band (Figure 4, lane 3). In this combination, the protein band corresponding to the monomeric `FliA protein with an apparent molecular mass of 11 kDa was almost invisible, probably because the `FliA protein could not react efficiently with the anti-FliA antibody. Because the sum of the molecular masses of `FliA and FlgM is ~20 kDa and because FlgM does not form dimers or oligomers (Ohnishi *et al.*, 1992), it is most plausible that the 20 kDa protein was a complex of `FliA and FlgM. Therefore, we

concluded that the C-terminal 86 amino acids of FliA directed by pSIIA4 is sufficient to associate with FlgM.

Discussion

Multiple alignment of the primary sequences of many bacterial sigma factors defined four regions of similarity called regions 1, 2, 3 and 4 and these conserved amino acid sequences have been postulated to reflect common functional attributes. Further detailed comparison within the individual regions suggested that they are divided into subregions. Based on mutation and truncation studies, the functions of individual subregions in the process of transcription have been inferred (Helmann and Chamberlin, 1988; Dombroski *et al.*, 1992, 1993; Lonetto *et al.*, 1992). For example, subregion 2.4 may be involved in recognition of the −10 promoter sequence, whereas subregion 4.2, which contains the helix–turn–helix (HTH) motif, may be implicated in the specific interaction with the −35 promoter sequence. Subregion 2.1 is presumed to be responsible for binding to RNA polymerase core enzyme (Lesley and Burgess, 1989). FliA, the alternative sigma factor specific for transcription of the class 3 flagellar operons, also includes regions 2–4 of the conserved amino acid sequences of sigma factors (Figure 1). Unlike most sigma factors, the activity of FliA is negatively regulated by the anti-sigma factor, FlgM. Previously, we presented evidence suggesting that FlgM binds to FliA in a ratio of 1:1 and that the FliA–FlgM complex is unable to interact with RNA polymerase core enzyme (Ohnishi *et al.*, 1992). This suggested that the FlgM-binding domain might lie in subregion 2.1 which is located in the N-terminal region of FliA. However, all the data obtained in the present study indicated that the FlgM-binding domain lies not in the N-terminal region but in the C-terminal region of FliA.

In this study, we isolated a number of *fliA* mutants (named *rflD* mutants) in which one of the class 3 flagellar operons, *tar*, could be transcribed even in the presence of FlgM. According to the base changes in the *fliA* gene, these mutants were divided into eight different amino acid substitution mutations at seven different codons (Figure 1). Among them, only one mutation site was located in region 2. Most of the mutation sites were located in the C-terminal region which corresponds to region 4. All the mutant FliA proteins that had amino acid substitutions in regions 2 or 3 retained high sensitivity to repression by FlgM, whereas most of the region 4 mutants showed relatively low sensitivity. Of course, there must be many mutations in FliA which prevent the FlgM-binding activity but which would not be detected in the screening procedure adopted here because they also inactivate the sigma activity. However, we believe that these results suggest that the FlgM-binding domain is located in region 4. Consistent with this, the polypeptides containing the C-terminal region of FliA were capable of titrating the intracellular FlgM protein, when expressed in excess within the cells. Furthermore, the chemical cross-linking analysis showed that N-terminally truncated FliA protein could interact with FlgM but that C-terminally truncated FliA protein could not. In the *in vivo* FlgM titration assay with the truncated FliA proteins containing the *rflD* mutations, the L199R and V213E proteins were found to show drastically impaired activity of FlgM titration. This indicates that amino acids L199 and V213 of FliA play an important role in interaction with FlgM.

Recently, sequence information has become available for FliA-like sigma factors from five different organisms (Helmann, 1991; Starnbach and Lory, 1992; McCarter and Wright, 1993). Though considerable homology was observed throughout their primary sequences, the highest was obtained in region 4 (Lonetto *et al.*, 1992). Among the four *rflD* mutation sites in region 4, three were located at the invariant amino acids of the FliA-like sigma factors (L199, E209 and V213) (Figure 5). Because the *rflD*-mutant FliA proteins retained sigma activity, these invariant amino acids probably are not essential for sigma function. They may have regulatory roles. This also supports our hypothesis that the FlgM-binding domain may be located in region 4.

In a previous report, we showed that binding of FlgM to FliA prevents its association with RNA polymerase core enzyme (Ohnishi *et al.*, 1992). Therefore, the results presented here suggest that binding of FlgM to region 4 must affect the ability of region 2.1 to interact with the core enzyme. Recently, Dombroski *et al.*, (1993) demonstrated that, unlike other sigma factors, the FliA protein can bind to a flagellum-specific promoter in the absence of the core enzyme. This suggests that FliA may bind first to the promoter and then to the core enzyme in the process of transcription initiation. If this is the case, it is also possible that the repression by FlgM is achieved by an alternative mechanism in which binding of FlgM to FliA prevents its association with flagellum-specific promoters. Two of the *rflD* mutation sites (E209K and V213E) are located within the first helix of the HTH motif in subregion 4.2 of FliA (Figure 5). The second helix of the HTH motif of sigma 70 has been believed to be involved in the direct interaction with the −35 sequence of the promoter, whereas the first helix has been suggested to facilitate this interaction (Helmann and Chamberlin, 1988; Gardella *et al.*, 1989; Siegele *et al.*, 1989). This information led to a model proposing that the amino acids in the first helix of the HTH motif of FliA may be involved in direct interaction with FlgM and that the conformational change caused by binding of FlgM to the first helix may be transduced to the second helix, resulting in the latter being unable to contact the −35 sequence of the flagellum-specific promoters.

An increasing amount of evidence suggests the importance of region 4 of sigma 70 in interaction between RNA polymerase and activator proteins (Ishihama, 1993; Kumar *et al.*, 1994). Makino *et al.* (1993) reported that the *rpoD* mutants of *Escherichia coli* in which amino acid substitutions occurred within or near the first helix of the HTH motif of sigma 70 could not activate a set of genes whose transcription was dependent on PhoB, the activator protein of the phosphate regulon. Based on this result, they postulated that the first helix of the HTH motif of sigma 70 may make direct contact with PhoB. One such mutation (*rpoD75*) occurred at amino acid residue E575 of sigma 70. Interestingly, this amino acid corresponds to E209 of FliA at which one of the *rflD* mutations occurred, and these two mutations caused the same amino acid substitution, namely glutamate to lysine (Figure 5). Therefore, the first helix of the HTH motif in region 4 of the

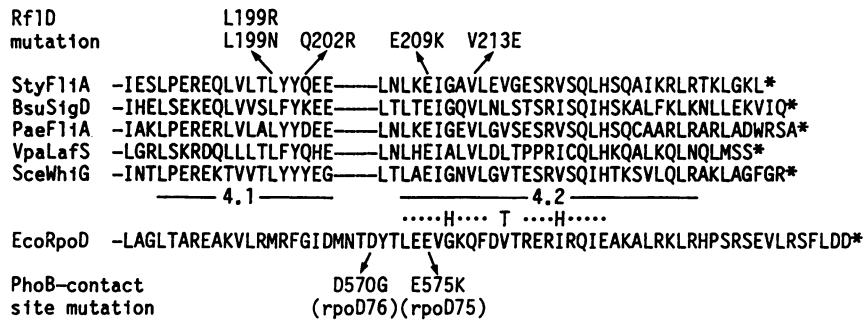

Fig. 5. Comparison of amino acid sequences of region 4 of FliA-like sigma factors and sigma 70. Data on the mutations which affect the contact ability with FlgM (*rflD*) or PhoB (*rpoD*) were adopted from Figure 1 or Makino *et al.* (1993), respectively. HTH and asterisk indicate the extent of the helix–turn–helix motif and the C-terminus of each sigma factor, respectively. Six-letter codes for the sigma factors are as follows: StyFliA, *Salmonella typhimurium* FliA (Ohnishi *et al.*, 1990); BsuSigD, *Bacillus subtilis* SigD (sigma 28) (Helmann *et al.*, 1988); PaeFliA, *Pseudomonas aeruginosa* FliA (Starnbach and Lory, 1992); VpaLafS, *Vibrio parahaemolyticus* LafS (McCarter and Wright, 1993); SceWhiG, *Streptomyces coelicolor* WhiG (Chater *et al.*, 1989); EcoRpoD, *Escherichia coli* RpoD (sigma 70) (Helmann and Chamberlin, 1988).

sigma factors should act as the contact sites with both positive and negative transcription factors.

Our truncation analysis of the FliA protein (Table III) revealed the regions essential for sigma activity in addition to the FlgM-binding domain. Loss of the 30 N-terminal amino acids was shown to eliminate sigma activity (pSIIA2). This result was expected, because these amino acids correspond to region 2.1 which is believed to be responsible for core binding. It was found that loss of the the 12 C-terminal amino acids eliminated sigma activity (pSIIA6), indicating that the C-terminal region is also essential for sigma activity. Recently, we presented evidence suggesting that FliA may also act as an activator in the transcription of class 2 flagellar operons (Kutsukake and Iino, 1994). The ability of the truncated FliA proteins to activate the class 2 operons will be the subject of future research.

Some of the derepression mutants isolated in the present study had defects unlinked to known flagellar gene clusters. Because their defects are closely linked to one another, they may define a single genetic locus, *rflC*. All the *rflC* mutations were shown to confer weakly constitutive expression of the *tar–lac* fusion gene in the hook–basal body mutant background (data not shown). This suggests that FlgM may require the *rflC* gene product to exert maximal repression of the class 3 operons. This is an unexpected result, because our previous study on *in vitro* transcription with purified FliA and FlgM proteins and RNA polymerase core enzyme demonstrated that FlgM can by itself inhibit FliA-dependent transcription. This difference should be solved by molecular characterization of the *rflC* locus.

The existence of an anti-sigma factor that binds to a specific sigma factor to inhibit its activity is not unique to the FliA sigma system. For example, it has been reported that *Bacillus subtilis* cells have at least two additional secondary sigma systems in which anti-sigma factors are involved in the modulation of sigma activity (Benson and Haldenwang, 1993; Duncan and Losick, 1993). Because the reported amino acid sequences of the anti-sigma factors show no significant homology, the mechanism of interaction between sigma and anti-sigma factors is not necessarily the same among the various systems. However, we believe that the data presented here can help in understanding the mechanism for controlling the activity of secondary sigma factors.

Materials and methods

Bacterial strains, plasmids and phages

Bacterial strains and plasmids used in the present study are listed in Table V. KK1105 carried a transposition-deficient derivative of Mud1 (Ap *lac*) inserted in the *tar* operon (Kutsukake and Iino, 1994). P22 phages used for transduction and Tn*10* mutagenesis were P22HT*int* (Schmieger, 1972) and P22 (*12*amN11 *13*amH101 *c2*ts29 *int3* Tn*10*), respectively.

Media

Ordinary culture media including L broth, L agar plates and motility agar plates were made as described previously (Kutsukake *et al.*, 1988). Minimal lactose plates were prepared according to Berkowitz *et al.* (1968). MacConkey lactose plates were prepared from MacConkey agar (Difco). In order to induce genes under the control of the *tac* promoter on high-expression plasmids, IPTG was added to a final concentration of 1 mM. Ampicillin, tetracycline and chloramphenicol were ordinarily used at final concentrations of 50, 20 and 5 μg/ml, respectively.

Transduction

Transductional crosses were performed using P22HT*int* as described previously (Kutsukake *et al.*, 1988).

Construction of a strain carrying Tn10 inserted near γflC

A pool of tetracycline-resistant clones from, KK1004 infected with P22 (*12*amN11 *13*amH101 *c2*ts29 *int3* Tn*10*) was obtained according to the method described previously (Kutsukake *et al.*, 1980). P22HT*int* lysate propagated on the pool was used to transduce one of the *rflC* mutants (*rflC192*) and tetracycline-resistant Lac$^-$ transductants were selected. One such transductant was designated KK1192. The Tn*10* insertion in this strain was shown by P22-mediated transduction to be 32% linked to the *rflC192* mutation.

Gene manipulation

DNA manipulation and transformation techniques were as described previously (Kutsukake *et al.*, 1985). All the enzymes used in the present study were purchased from Toyobo Co. Ltd or Nippon Gene Co. Ltd. When the ampicillin-resistant *tar–lac* fusion strains (KK1505GM, KK1505HB and KK1505IA) had been transformed with multi-copy plasmids encoding ampicillin resistance, the transformation mixtures were plated onto L agar plates containing 250 μg/ml ampicillin and 1.25 mg/ml methicillin to prevent untransformed cells from forming colonies.

Cloning and sequencing of the fliA gene from the rflD mutants

Chromosomal DNA was prepared from the *rflD* mutants according to the method described previously (Kutsukake *et al.*, 1985). Chromosomal DNA and pBR322 were digested simultaneously with *Hin*dIII and *Eco*RI and ligated. EKK23 (*fliA*) was transformed with this mixture and motile

Table V. Bacterial strains and plasmids

Strain/Plasmid	Relevant characteristics	Source/Reference
E.coli strain		
EKK23	*hsdR fliA*	Ohnishi *et al.* (1990)
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′*traD proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	Yanisch-Perron *et al.* (1985)
S.typhimurium strain		
JR501	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilv452 rpsL120 xyl-404 galE719* Fels2$^-$ *fliC*-b *fljB*-e,n,x *fliB fla-66*	Tsai *et al.* (1989)
KK1004	LT2 Δ(*flj*)	Kutsukake *et al.* (1988)
KK1105	KK1004 *tar–lac* Ap^r	Kutsukake and Iino (1994)
KK1311	KK1004 *flgM*::*cat*	Kutsukake (1994)
KK1355	KK1004 Δ(*flhB–flhA*)	This study
KK1355T	KK1355 *tar–lac* Ap^r	P22 (KK1105)×KK1355
KK1361	KK1004 Δ(*fliA*)	Kutsukake (1994)
KK2019	KK1004 *flhB*::Tn*10*	Kutsukake *et al.* (1988)
KK2085	KK1004 *flgB*::Tn*10*	Kutsukake *et al.* (1988)
KK2091	KK1004 *fliA*::Tn*10*	Kutsukake *et al.* (1988)
KK2604	KK1004 *fliC*::Tn*10*	Kutsukake *et al.* (1988)
KK1501	JR501 *fla*$^+$	P22(KK1004)×JR501
KK1505	KK1501 *tar–lac* Ap^r	P22(KK1105)×KK1501
KK1505GM	KK1505 *flgM*::*cat*	P22(KK1311)×KK1505
KK1505HB	KK1505 *flhB*::Tn*10*	P22(KK2019)×KK1505
KK1505IA	KK1505 *fliA*::Tn*10*	P22(KK2091)×KK1505
NK337	*leu515* sup19 P22(*12*amN11 *13*amH101 *c2*ts29 *int*3 Tn*10*)	Noel and Ames (1978)
Plasmid		
pBR322	*amp tet*	Bolivar *et al.* (1977)
pTrc99A,B,C	*amp lacI*^{q} *tac*	Amann *et al.* (1988)
pUC118, 119	*amp*	Vieira and Messing (1987)
pKK1064-2	pBR322 *fliA*$^+$	Ohnishi *et al.* (1990)
pKKOPB99A	pTrc99A *flgM*$^+$	Kutsukake (1994)

transformants were selected on motility agar plates containing ampicillin. From one such transformant, a hybrid plasmid carrying the *fliA* gene was purified. The *Eco*RI–*Pvu*II fragment was excised from the plasmid and recloned into the *Eco*RI and *Sma*I sites of pUC118 and pUC119. The DNA sequences of the inserted fragments in the resulting plasmids were determined according to the method of Vieira and Messing (1987) using various synthetic primers.

Plasmid construction

Plasmid pKK1064-2 possesses a unique *Pvu*II site in the 3′ non-coding region of *fliA* (Figure 1). This plasmid was digested with *Pvu*II and ligated with *Hin*dIII linkers to obtain pKK1064-2Z. Various portions of the *fliA* gene were excised from pKK1064-2 or pKK1064-2Z and inserted in-frame into one of the pTrc99 plasmids at the polylinker site which was preceded by the *tac* promoter and the *lacZ* ribosome-binding site (Amann *et al.*, 1988). Because these plasmid vectors contained the *lacI*^{q} gene, polypeptides corresponding to various portions of FliA could be synthesized in excess within the cell after induction with IPTG. Restriction sites used in the present study are listed in Figure 1. FliA amino acid residues in the respective polypeptides are shown in Table III. A set of the plasmids which directed the synthesis of intact or truncated FliA proteins was constructed as follows. An *Eco*RI–*Pvu*II fragment containing the entire *fliA* gene was excised from pKK1064-2 and inserted into pTrc99C at the *Eco*RI and *Sma*I sites to obtain pSIIA1. The *Eco*RI–*Hae*III and *Eco*RI–*Hpa*I fragments were excised from pKK1064-2Z and inserted into pTrc99C at the *Eco*RI and *Sma*I sites to obtain pSIIA6 and pSIIA8, respectively. The *Sac*I fragment from pKK1064-2Z was inserted into pTrc99C at the *Eco*RI and *Sac*I sites to obtain pSIIA7. The *Pst*I–*Hin*dIII and *Sal*I–*Hin*dIII fragments were excised from pKK1064-2Z and inserted into pTrc99A at the *Pst*I/*Hin*dIII and *Sal*I/*Hin*dIII sites to obtain pSIIA2 and pSIIA3, respectively. Plasmid pSIIA3 was digested with *Kpn*I and *Sal*I and treated with exonuclease III. After treatment with mung bean nuclease and Klenow enzyme, the DNA was self-ligated to obtain pSIIA4. DNA sequencing indicated that this plasmid could direct synthesis of polypeptide corresponding to amino acids 154–239 of FliA. The *Hpa*I–*Hin*dIII fragment was excised from pKK1064-2Z and inserted into pTrc99B at the *Sma*I and *Hin*dIII sites to obtain pSIIA5. The *Rsa*I fragment was excised from pKK1064-2Z and inserted into pTrc99B at the *Sma*I site to yield pSIIA9. The *Eco*RI–*Hae*III fragment was excised from pSIIA4 and inserted into the *Eco*RI and *Sma*I sites of pTrc99A to obtain pSIIIA10.

A set of the plasmids carrying the truncated *fliA* genes with the *rflD* mutations was constructed as follows. *Eco*RI–*Hae*III or *Sal*I–*Hin*dIII fragments were excised from pUC118 carrying the *rflD*-mutant *fliA* genes constructed above and inserted into pTrc99C at the *Eco*RI and *Sma*I sites or into pTrc99A at the *Sal*I and *Hin*dIII sites respectively. The former corresponds to the *rflD*-mutant form of pSIIA6 and the latter to that of pSIIA3.

Enzyme assay

The activity of β-galactosidase was assayed with the cells grown in L broth by the method of Miller (Platt *et al.*, 1972) as described previously (Kutsukake *et al.*, 1990). The enzyme units (Miller units) reported here were the average of at least three independent assays.

Western blotting

Cells harbouring the appropriate plasmids were grown at 37°C in L broth containing ampicillin and IPTG. Proteins from a constant number of cells were separated by SDS–PAGE and transferred onto nitrocellulose membrane as described previously (Kutsukake, 1994). The membrane was incubated with polyclonal rabbit antibody against FliA prepared previously (Ohnishi *et al.*, 1990) and developed with a chemiluminescence Western blotting kit (Smilight) according to the manufacturer's recommendation.

Cross-linking of proteins synthesized in vitro

FliA and FlgM proteins were synthesized *in vitro* in the presence of [^{35}S]methionine (American Radiolabeled Chemicals, Inc.) using an *E.coli* S30 coupled transcription–translation system (Promega) in accordance with the manufacturer's recommendations. The proteins were cross-linked by DSP (Sigma) as described previously (Ohnishi *et al.*, 1992). Cross-linked products were separated immunologically using both anti-FliA and anti-FlgM antibodies (Ohnishi *et al.*, 1990, 1992) and analysed by SDS–PAGE in the absence of 2-ME. The gels were fixed with a fixing solution containing 10% acetic acid and 40% methanol, soaked in Enlightning (New England Nuclear) and dried. The dried gels were exposed to X-ray films at −80°C.

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