Trigger factor, one of the *Escherichia coli* chaperone proteins, is an original member of the FKBP family

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Received 31 July 1995

Abstract The trigger factor of *Escherichia coli* is known as a chaperone protein which forms soluble complexes with the precursor to outer membrane protein A and assists in the maintenance of translocation competence. Sequence analysis shows that trigger factor contains a domain belonging to the FK506-binding protein (FKBP) family and possessing all the amino acids necessary for FK506 binding and peptidyl-prolyl *cis-trans* isomerase (Ppiase) activity. Consequently, this protein could be directly involved in the unfolding/folding processes occurring during translocation across the *E. coli* plasma membrane and, more generally, in facilitating protein folding. The central position of the FKBP domain within the trigger factor sequence as well as several original features of the loops surrounding the FK506-binding pocket are not found in any other FKBPs, making it undetectable by the Fkbp-Ppiase signature patterns.

Key words: FKBP; Chaperone; Trigger factor; Sequence analysis; Hydrophobic cluster analysis

1. Introduction

FK506-binding proteins (FKBPs) form a large family of proteins possessing a minimum one hundred amino acid domain with peptidyl-prolyl *cis-trans* isomerase (Ppiase) activity [1]. This activity can be inhibited by the immunosuppressive drug FK506 which occupies the catalytic site. Due to this ability to bind immunosuppressants, shared with the cyclophilin family, these proteins are also called 'immunophilins'.

The archetype of this family, now named FKBP-12 to distinguish it from other FKBPs of higher molecular weight, was isolated in 1989 [2,3] and several atomic structures, complexed or not with immunosuppressants, have since been solved (see [1] for review). It consists of a five-stranded β-sheet wrapping with a right-handed twist around a short α-helix (Fig. 1). The complex FKBP-12/immunosuppressant (FK506 or rapamycin) interferes with signal transduction in T-cells, thereby inducing immunosuppression [5]. Rotamase inhibition is not however sufficient for immunosuppression since rapamycin and FK506, both of which inhibit FKBP rotamase activity, interfere with distinct signaling pathways [6]. Moreover, modified ligands which inhibit rotamase do not necessarily induce immunosuppression [7].

A growing number of other FKBPs of higher molecular weight have since been characterized (Fig. 2). They possess at least one FKBP-12-like domain harboring the main features necessary for rotamase and FK506-binding activities (*'FKBP domain'*). However, the events following FKBP-12 rotamase inhibition, such as interaction with calcineurin, do not seem to be conserved [17,18]. On the contrary, many FKBPs associate with proteins that play no role in T-cell mediated responses. Some of these FKBPs, like HBI (also known as p59 or FKBP-52), possess additional FKBP-12-like domains which have not conserved some of the hydrophobic residues that form the ligand binding pocket; we propose to term them *'FKBP-like domains'* in contrast to the strict *'FKBP domains'* with Ppiase activity [19]. The functions of these domains are not known although the FKBP-like domain of HBI has been predicted [19], and later shown [20], to bind nucleotides. The FKBP-like domains share around 30% sequence identity with FKBP-12 or with the FKBP domain of their host proteins while the FKBP domains are generally more conserved, from up to 90% sequence identity between human FKBP-12 and other mammalian FKBP-12s to about 35% between human FKBP-12 and the MIP proteins. A few proteins, such as the *E. coli* slyD [8,9] or the yaad gene product [14], possess only FKBP-like domains. Consequently, it seems that the FKBP structural family would not be limited to FK506-binding proteins in the strict sense but would include proteins which could be specific rotamases, bind modified ligands or have completely different functions.

In addition to these FKBP and FKBP-like domains, the high molecular weight FKBPs possess additional unrelated sequences either in the NH_2- or COOH termini. The functions of these domains appear to be various: involvement of histidine-rich peptides in metal binding [8,9], of acidic and basic domains in nuclear localization [10–12], or of tetratricopeptide repeats and calmodulin-binding domains in hsp90 binding [19,21], etc.

In this report, we show that the trigger factor, a protein originally described in *Escherichia coli* as belonging to a chaperone complex [22] and whose mode of action was heretofore unknown, possesses a domain which might be related to the FKBP family. However this new family member possesses special features that differentiate it from the previously described FKBP domains and which will be discussed below.

2. Materials and methods

Systematic searches of databanks (Swissprot, PIR) with representative sequences of a family or profiles generated from multiple alignments [23,24] allow detection of sequences which could belong to the same functional and/or structural family. However, at the low levels of sequence identity (15–30%) often observed, these automatic methods are not able to distinguish similarities due to structural relationships from background noise. The 'hydrophobic cluster analysis (HCA)' method is helpful in this regard in so far as it allows comparison of not

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Abbreviations: FKBP, FK506-binding protein; HCA, hydrophobic cluster analysis; Ppiase, peptidyl-prolyl *cis-trans* isomerase.

SSDI 0014-5793(95)01109-9

only the sequences but also the protein secondary structures statistically centered on hydrophobic clusters, as well as their distribution [25]. Similar plots could therefore indicate similar three-dimensional folds. Guidelines to the use of this method are given in [26,27].

The HCA score is proportional to the hydrophobic amino acids which are topologically conserved (often not chemically identical), and therefore reflects the degree of conservation of the hydrophobic core. High HCA scores are associated with low root mean squares values between three-dimensional structures [27].

The accuracy of the alignments can be assessed by computing identity or similarity Z scores; these represent differences between the considered alignment identity or similarity score and the mean score of a distribution computed for alignment of sequence 1 versus a large number of random shuffled versions of sequence 2. These differences are expressed relative to the standard deviation (S.D.) of the random distribution.

3. Results

Computer searches of sequence databanks reveal weak similarities between the sequences of some FKBP family members and trigger factor. This protein, first described in *E. coli* ([16], swissprot identifier: tig_ecoli), was also recently found in *Campylobacter jenuni* ([15], genbank identifier: cjdnatig). The highest identity percentages are observed for the comparison *E. coli* trigger factor/human FKBP-13 (28.0%) and *C. jujuni* trigger factor/*Neurospora crassa* FKBP-12 (29.7%) (in a 90 amino acid overlap). However, this level of similarity could reflect either a structural similarity or a chance occurrence. Consequently, we pursued the analysis by comparing the trigger factor HCA plots with those of the FKBP family (Fig. 3). HCA is a very sensitive method for the prediction of structural similarities, allowing confidence at amino acid sequence identity levels as low as 15% and good probabilities below that.

Interestingly, this comparison revealed that:

3.1. *All the regular secondary structures of the FKBP fold are also found in the trigger factor sequence*

Similarities in cluster shape, distribution and size between trigger factor and FKBPs are identified all along the HCA plots (Fig. 3), with indels occurring only in loop regions. Particularly well conserved are the β4, β5 and β3 strands and the α-helix, which are directly involved in the conformation of the ligand-binding pocket (Fig. 1). The β1 and β2 strands are less conserved in accordance with their variable character among the whole family (especially the β1 strand – Fig. 4).

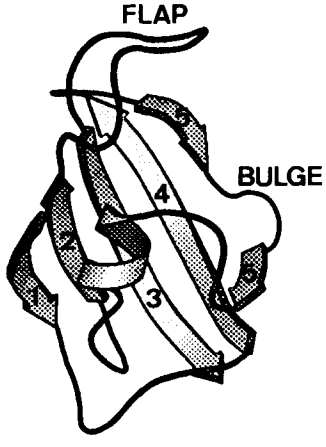

Fig. 1. Ribbon representation of the human FKBP-12 three-dimensional structure using MOLSCRIPT software [4].

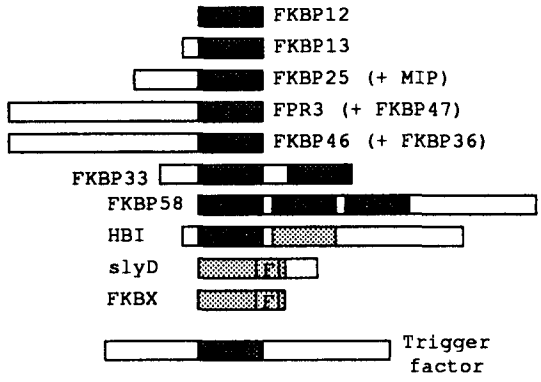

Fig. 2. The FKBP structural family. Organization into domains of several members of the FKBP structural family, including members with only 'FKBP-like' domains (light shaded) which do not possess all the residues needed for FK-506 binding. In contrast, strict FKBP domains are dark shaded: Ppiase activity and FK-506 binding have been demonstrated for many of them. Other non-FKBP domains are shown not shaded. F indicates large flap regions (>50 amino acids) within FKBP-like domains. References can be found in [1] and in [8,9] (slyD); [10,11] (yeast FPR3, FKBP-47); [12] (*Spodoptera frugiperda* FKBP46); [13] (FKBP-33); [14] (*Escherichia coli* and *Pseudomonas fluorescens* FKBX); ([15,16] and this study) (*Escherichia coli* and *Campylobacter jujuni* trigger factor).

For the comparison *E. coli* trigger factor/human FKBP-13 and *C. jujuni* trigger factor/*N. crassa* FKBP-12, the HCA scores, calculated on the whole domain except for the variable β1 strand, are 75.3% and 81.5%, respectively. These high values reflect overall conservation of the hydrophobic core and the regular secondary structures constituting it. They would correspond to root mean squares values less than 2 Å between the compared structures.

What appears original in the trigger factor sequences is the reduced length and different composition of the surface loop called the **80s flap**, which contains only 15 residues compared to 20 residues in FKBP-12 (Fig. 4). Moreover, its overall composition is quite different, except at the beginning of the flap which maintains an essential tyrosine (Y82 in FKBP-12, Y221 and Y225 in the *E. coli* and *C. jujuni* trigger factor sequences) whose hydroxyl group is observed, in the FKBP-12 structure, to form an atypical hydrogen bond with the amide carbonyl group of the bound ligand. This hydrogen bond is one of the two between the flap and the rest of the molecule and may be relevant to the natural function of FKBP-12, since this tyrosine residue is strictly conserved among the known FKBP domains (Fig. 4). The second hydrogen bond is between Y80-O and the N terminus of the α-helix (G58-N) but involves only atoms of the main chain. Consequently, this bond could be conserved in the trigger factor structure even though Y80 is not conserved (E219: *E. coli*; K222: *C. jujuni*).

The **40s bulge** which splits the β5 strand is somewhat shorter as compared to FKBP-12, with five amino acids instead of seven. This bulge is of variable length among the FKBP family: the longest, found in FKBP-25, contains 14 amino acids while the shortest, in the second FKBP-like domain of HBI, contains only two, probably leading to a continuous β5 strand [19].

Also, the **loop linking** β4 and β5 is shorter by one amino acid, as in the second FKBP-like domain of HBI [19]. This loop

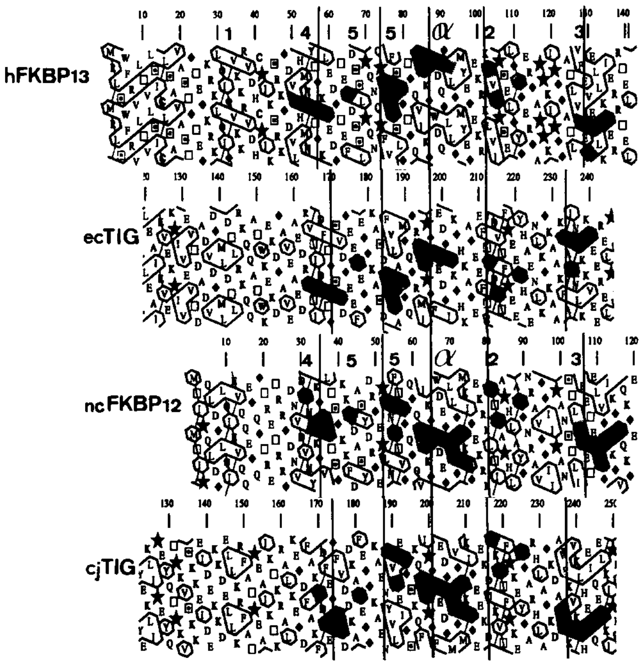

Fig. 3. Comparison of the HCA plots of human FKBP-13, *Neurospora crassa* FKBP-12 and trigger factor (tig) sequences. The standard one-letter code is used for all amino acids except proline (*), glycine (◇), serine (⊡) and threonine (□). Hydrophobic clusters are boxed. Correspondences between the sequences are indicated by vertical lines and similar hydrophobic clusters are shaded. Secondary structures, according to [28], are given above the HCA plots.

seems to be tolerant to indels, especially as lengthening is also observed, for example in the *Streptomyces chrysomallus* FKBP12 sequence [1].

3.2. *Most of the residues involved in the FKBP-12 FK506-binding pocket are identical (5/10) or similar (4/10) in the trigger factor sequences*

A total of 10 residues, highly conserved among FKBP sequences (Fig. 4; [37]), are involved in maintaining the hydrophobic core of the FKBP-12 structure that forms the ligand binding pocket (Table 1).

Five of them are strictly conserved in the trigger factor sequences (Table 1). The substitutions of four others are conservative. Some of these four residues are found identical in other FKBP sequences (for instance F198 of the *E. coli* trigger factor = F190 in the *Chlamydia trachomatis* MIP sequence). When amino acids involved in hydrogen bonding or C–O···H interactions with FK506 are only similar, the participating atoms are conserved: for example, the FKBP-12 Y26 interacts with the FK506 C9-carbonyl through its CεH; this atom is conserved when Y is substituted by F (*E. coli* F168; *C. jujuni* F172). However the ligand binding pocket should be slightly modified as the FKBP12 Y26-OH, forming an hydrogen bond with D37, is missing in trigger factor. The conservative substitution of D37 by E (*E. coli* E178, *C. jujuni* E182) could maintain the hydrogen bond between the involved oxygen (Oε instead of Oδ) and the ligand.

Only the FKBP-12 I91 is not conserved in trigger factor. However it can be seen that the I91-O maintains the flap conformation by forming an internal hydrogen bound with H87-N in the FKBP-12 structure. This region would probably be highly modified in trigger factor (the flap is shorter), therefore rendering isoleucine conservation nonessential.

Four additional amino acids have been shown to contact FK506 in the FKBP-12 complex: R42, Q53, E54, H87 [37]. They are not retrieved in the trigger factor sequence but they are also not conserved in most of the FKBP domains (Fig. 4).

3.3. *Amino acids required to maintain the hydrophobic core are conserved*

In addition to the hydrophobic residues which are often aromatic and directly involved in the ligand-binding pocket (see above), other hydrophobic amino acids are also conservatively substituted as they participate in the hydrophobic core structure (*E. coli* V172/*C. jujuni* V176 in strand β4 (L30 in FKBP-12), *E. coli* I235/*C. jujuni* I239 and *E. coli* V237/*C. jujuni* V241 in strand β2 (L74 and I76 in FKBP-12), *E. coli* I235/*C. jujuni* V239 and *E. coli* L237/*C. jujuni* L241 in strand β3 (V101 and L103 in FKBP-12)). Most of these residues are found identical in other FKBPs (Fig. 4).

The FKBP-12 G28 and G58 are strictly conserved, corre-

Table 1
Conservation of residues involved in the FK506-binding pocket (based on [37])

human FKBP-12	*E. coli* trigger factor	*C. jujuni* trigger factor	structure position	H bonds and C-H···O interactions with FK506
Y26	F168	F172	strand β4	Y-CεH with C9 carbonyl
G28	G170	G174	strand β4	
F36	F177	F181	strand β5	F-CεH with C9 carbonyl
D37	E178	E182	strand β5	D-Oδ with C10 hydroxyl
V55	M194	F198	β5–α loop	
I56	I195	I199	β5–α loop	I-N with C1-carbonyl
W59	F198	M206	α-helix	
Y82	Y221	Y225	β2–β3 flap	Y-OH with C8-carbonyl
I91	-	-	β2–β3 flap	
F99	F233	F237	strand β3	F-CεH with C9 carbonyl

Table 2
Correspondence of the human FKBP-12 and trigger factor (tig) sequences to the PROSITE Fkbp-Ppiase signature patterns [39,40]

Fkbp_Ppiase_1	L	X	Y	X	G	X (1,2)	L	X (2)	G	X (3)	D	S	S
	I		F		V		F				E	T	T
	V				L		T					A	N
	M											E	
	C											Q	
human FKBP-12	V	H	Y	T	G	M	L	ED	G	KKF	D	S	S
E. coli tig	I	D	F	T	G	S	**V**	.D	G	EEF	E	**G**	**G**
C. jujuni tig	**F**	D	F	E	G	F	**V**	.D	**D**	KAF	E	**G**	**G**

Fkbp_Ppiase_2	L	X (2)	G	X (3,4)	L	X (2)	L	X (2)	G	X (4)	L	X (3)	P	X (2)	A	F	G
	I		A		I		I				I		S		G	Y	
	V				V		V				V		G				
	M				F		F				F		A				
	C				M		M				M						
							H										
human FKBP-12	V	IR	G	WEEG	V	AQ	M	SV	G	QRAK	L	TIS	P	DY	A	Y	G
E. Coli tig	M	IP	G	FEDG	I	KG	H	KA	G	EEFT	I	DVT	**F**	PE	**E**	Y	**H**
C. jujuni tig	F	IP	G	FEDG	M	VG	M	KI	G	EEKD	I	KVT	**F**	PK	**E**	Y	G

These patterns include the end of strand β4, loop β4–β5 and strand β5 (Fkbp-Ppiase-1), the end of loop β5–α, the α-helix, loop α–β2, strand β2 and the beginning of the flap (Fkbp-Ppiase-2). Bold and underlined letters indicate mismatches.

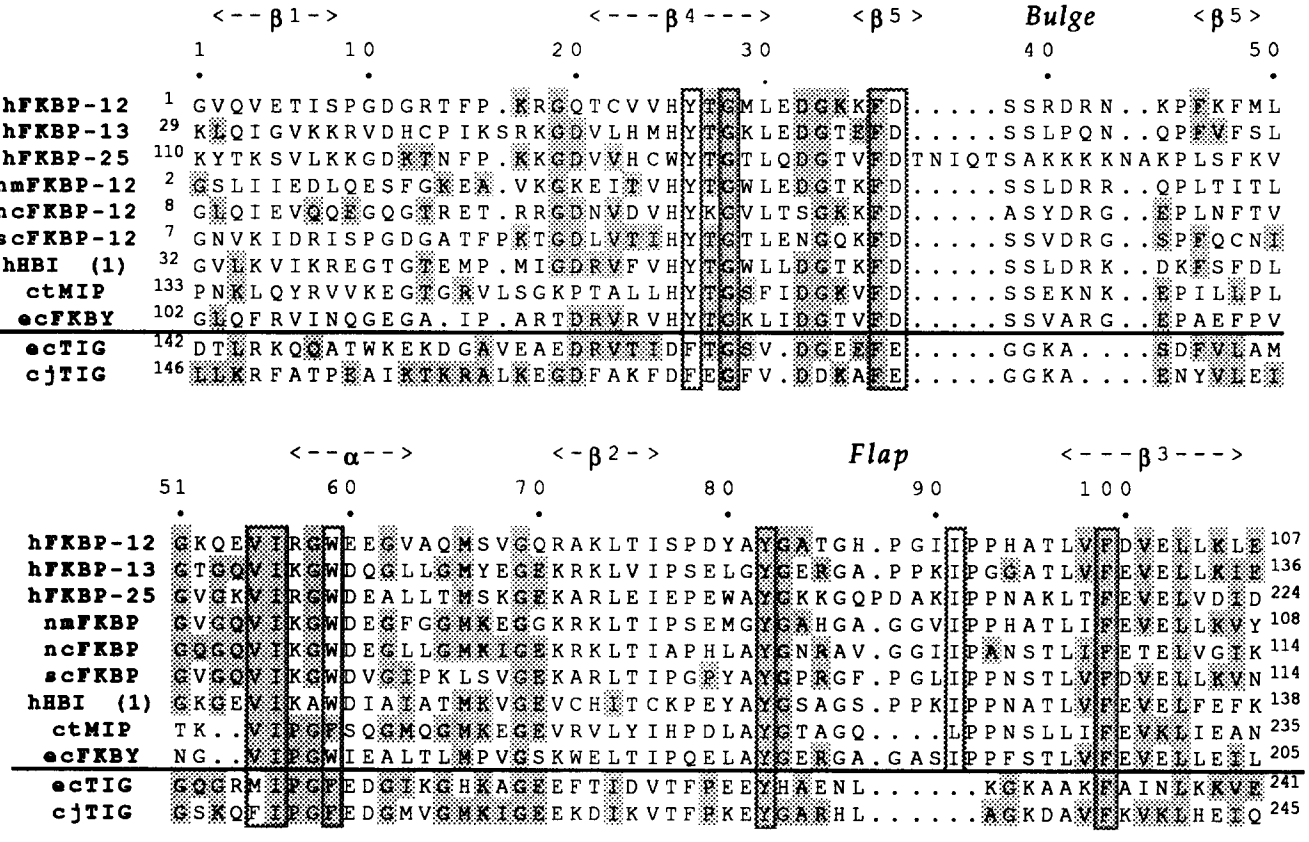

Fig. 4. Linear alignment of the trigger factor central domain with several FKBP domains. hFKBP-12 numbering and secondary structure (according to [28]) are given above its sequence. The first and last amino acids of each sequence are indicated. Identical amino acids are shaded and residues involved in ligand binding are boxed. hFKBP-12, human FKBP-12 [29]; hFKBP-13, human FKBP-13 [30]; hFKBP-25, human FKBP-25 [31]; nmFKBP-12, *Neisseria meningitidis* FKBP-12 [32], ncFKBP-12, *Neurospora crassa* FKBP-12 [33]; scFKBP-12, *Saccharomyces cerevisiae* FKBP-12 [34]; hHBI, human Hsp90-binding immunophilin (also known as FKBP52 or p59) [35]; ctMIP, *Chlamydia trachomatis* MIP [36]; ecFKBY, *Escherichia coli* probable FKBP-type 22kD ppiase -ytec- (swissprot identifier: fkby_ecoli): ecTIG, *Escherichia coli* trigger factor [16]; cjTIG, *Campylobacter jujuni* trigger factor [15].

sponding to G170/G174 and G197/G201 in the *E. coli* and *C. jujuni* trigger factor sequences. The absence of side chain is essential to allow FK506 binding (G28) or to avoid steric hindrance with side chains contributing to the hydrophobic core (G58). Another glycine is also conserved relative to FKBP12 and the other FKBPs (*E. coli* G208/*C. jujuni* G212 corresponding to G69 in FKBP-12): it seems to be important for the conformation of the α–β2 loop (the inner loop of a loop crossing) since it is highly conserved within the family.

3.4. Statistical scores assessing the validity of the prediction

An objective way to assess the validity of predictions is to calculate Z scores from the comparison of a real alignment with a large number of alignments (10,000) performed after randomization of one of the compared sequences. These values allow evaluation of how the signal emerges from background. They were calculated for the identity percentage, the similarity (using the Dayhoff matrix) and the HCA scores from the alignment between human FKBP-13 and *E. coli* trigger factor ($Z_{identity}$ = 7.0, $Z_{similarity}$ = 7.9, Z_{HCA} = 5.1) and between *Neurospora crassa* FKBP-12 and *C. jujuni* trigger factor ($Z_{identity}$ = 7.6, $Z_{similarity}$ = 8.9, Z_{HCA} = 6.0). Confidence is assumed for values exceeding 6, whereas values above 3 are often encountered for similar 3D structures sharing low level sequence identity.

4. Discussion

Escherichia coli trigger factor was originally identified as a molecular chaperone which maintains proOmpA in a membrane assembly-competent conformation [22]. Later, it was shown that trigger factor does not promote the synthesis and export of proOmpA (it may even function to slow it down) but may be needed as a chaperone for proteins involved in cell division [16]. However, regardless of the proteins with which trigger factor interacts, its mode of action remains unknown.

Here, we show that this chaperone possesses a domain related to the FKBP family and can probably function as a rotamase and also perhaps bind FK506. Indeed, all the amino acids involved in the ligand-binding pocket are identical or similar, with a conserved hydrogen bonding capacity.

However, because of the modification of the flap and bulge regions (both are shorter in trigger factor), it is possible that the function of this protein may be slightly different, for example by acting as a specific rotamase, specialized in the folding of particular proteins. On the other hand, the flap and bulge regions have been shown to be essential in FKBP-12 for its interaction with calcineurin [38]. Hence, their different nature in trigger factor would not influence its qualitative ability to have a ubiquitous Ppiase activity and bind FK506, as the in-

volved atoms are conserved, but rather would alter the events that follow this recognition. This hypothesis could be strengthened by the fact that FKBPs with modified bulge and/or flap regions (for example, human FKBP-25 and *Chlamydia trachomatis* MIP – Fig. 4) always have Ppiase activity and can bind FK-506. Further investigations on the exact role of trigger factor in a potential catalytic function may help to elucidate these hypotheses.

Little is known about the FKBP family in *E. coli*. (Fig. 2). Two probable FKBP-type Ppiases have been described, corresponding to the yaad and ytfc gene products (swissprot identifier fkbx_ecoli [14] and fkby_ecoli (unpublished data), respectively). However, only fkby contains a strict FKBP domain in its COOH-terminus. An FKBP-like domain has also been found in an *E. coli*. metal-binding protein, slyD, involved in phage lysis [8,9]. Like fkby, slyD is also a two domain protein but its FKBP-like domain is localized in its NH2-terminus. In contrast, one of the most striking features of trigger factor is its organization into three domains, the central position of the FKBP domain being unique in the FKBP family. It is worth noting that the sequence identity between the *E. coli* and *C. jujuni* trigger factor sequences is significantly greater on the predicted FKBP domains (48.9%) than on the whole length of the molecule (31.6%). Interestingly, similar bidimensional features could be observed between the trigger factor NH2 domain and the non-FKBP domain of the MIP protein (they have a high content in α helices and their lengths are similar) and between the trigger factor COOH domain and the non-FKBP COOH domain of HBI (or FKBP52). Searches to ascertain the role of these two regions flanking the central FKB domain are under way.

In conclusion, our study shows that the two FKBP signature patterns found within the PROSITE dictionary [39,40], although permissive, are not sufficient to describe all the members of the family (Table 2). Indeed, the trigger factor does not fulfill the first consensus sequence as three (*E. coli*) or five (*C. jujuni*) mismatches occur as well as gaps which are not tolerated between strands β4 and β5. Three (*E. coli*) or two (*C. jujuni*) mismatches also occur for the second pattern, nevertheless able to pick up the FKBP-like domains (hbi(2) for example). These mismatches are in the beginning of the 'flap' sequence which is highly modified in trigger factor. Revised signature patterns would better take into account the residues involved in the FKBP-binding pocket, for instance FKBP-12 F36 or I56, at least for strict FKBP domains.

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