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Bacteriophage Mu repressor as a target for the *Escherichia coli* ATP-dependent Clp protease

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Bacteriophage Mu repressor, which is stable in its wild-type form, can mutate to become sensitive to its *Escherichia coli* host ATP-dependent ClpXP protease. We further investigated the determinants of the mutant repressor's sensitivity to Clp. We show the crucial importance of a C-terminal, seven amino acid long sequence in which a single change is sufficient to decrease the rate of degradation of the protein. The sequence was fused at the C-terminal end of the CcdB and CcdA proteins encoded by plasmid F. CcdB, which is naturally stable, was unaffected, while CcdA, which is normally degraded by the Lon protease, became a substrate for ClpXP while remaining a substrate for Lon. In agreement with the current hypothesis on the mechanism of recognition of their substrates by energy-dependent proteases, these results support the existence, on the substrate polypeptides, of separate motifs responsible for recognition and cleavage by the protease.

Keywords: Clp protease/Lon protease/phage Mu/protease sensitivity/repressor

Introduction

Both in prokaryotic and eukaryotic cells, proteases play a crucial role by eliminating damaged or abnormal polypeptides, as well as by regulating the level and the availability of specific proteins, some of which are key regulatory polypeptides. It is thus obvious that proteases ought to be able to recognize the proteins they are to degrade. Despite their fundamental importance, the mechanisms which allow for selective recognition by proteases and the features which characterize the proteins which are targets for proteolytic degradation have yet to be understood [for a review, see Gottesman and Maurizi (1992), Maurizi (1992) and Peters (1994)].

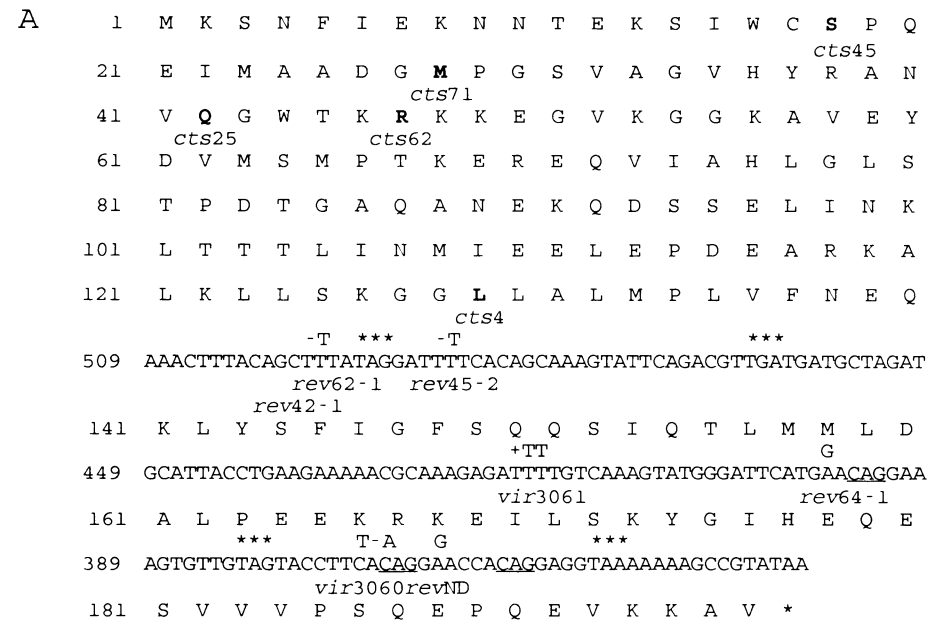
The RecA-dependent cleavage of the λ CI repressor, which leads to induction of *Escherichia coli* phage λ, was one of the first recognized examples of a specific proteolytic event involved in the regulation of gene expression in bacteria (Roberts and Roberts, 1975). Since then, several *E.coli* energy-dependent proteases have been shown to degrade regulatory proteins of bacterial phage and plasmid origin. The *E.coli* SulA, the phage λ N and the F plasmid CcdA proteins are substrates for the Lon (La) protease (Gottesman *et al.*, 1981; Mizusawa and Gottesman, 1983; Torres-Cabassa *et al.*, 1987; Van Melderen *et al.*, 1994), λ CII protein is a substrate for HflA (Cheng *et al.*, 1988) and the *E.coli* heat shock σ32 factor is degraded by HflB (Herman *et al.*, 1995).

The *E.coli* ATP-dependent Clp serine protease (also called Ti) was identified more recently (reviewed by Gottesman and Maurizi, 1992) and may be the prokaryotic equivalent of the eukaryotic proteasome (for a review, see Peters, 1994). It is a two-component enzyme consisting of one proteolytic subunit, ClpP, and at least two alternative regulatory ATPase subunits, ClpA (see, for instance, Singh and Maurizi, 1994) and ClpX (Yoo *et al.*, 1994). ClpX is encoded by the gene immediately 3′ of *clpP*, in the same operon which belongs to the heat shock regulon (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993). The ClpAP form is involved in the degradation of proteins with particular destabilizing N-terminal residues (Tobias *et al.*, 1991). The ClpXP form degrades the phage λ O protein *in vivo* and *in vitro* (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993), and the P1 plasmid Phd protein *in vivo* (which in conjunction with the Doc polypeptide participates in the P1 plasmid maintenance mechanisms; Lehnerr and Yarmolinsky, 1995).

Bacteriophage Mu repressor, a 196 amino acid, 22 kDa protein, is only slightly sensitive to ClpXP. Its degradation can only be detected (*in vivo*) upon overexpression of the protease (M.J.Gama, L.Desmet and J.Laachouch, unpublished results). However, some Mu repressor mutants are readily degraded by ClpXP [half-life of ~20 min versus >120 min for the wild-type (WT) protein; Geuskens *et al.*, 1992; Mhammedi-Alaoui *et al.*, 1994]. As shown in Figure 1, these mutant proteins (called *vir*) have a short modified C-terminal end resulting from a single frameshift mutation near the 3′-end of the *c* repressor gene (Geuskens *et al.*, 1991).

Mu phages which express such mutant repressors are unable to lysogenize because of their repressor instability. Indeed, they lysogenize normally on either a *clpP* or a *clpX* host strain in which the mutated repressor is stable (Geuskens *et al.*, 1992; Mhammedi-Alaoui *et al.*, 1994). In addition, Mu*vir* phages are virulent. They productively infect hosts lysogenic for a WT Mu and, when doing so, induce the resident prophage (van Vliet *et al.*, 1978). Geuskens *et al.* (1992) showed that this trans-dominant phenotype results from the ability of the mutated unstable repressor to target the active WT repressor synthesized by the prophage towards ClpP-dependent degradation.

Because the interaction of Mu repressor with its cognate operators on the viral genome is cooperative (Vogel *et al.*, 1991), and because the protein is known to form dimers and higher order oligomers in solution (V.Geuskens,



B.

	DNA changes	C-terminal protein changes
*vir*3060	-A at 372, C->T at 373	-11, +FRNHRR
*vir*3061	+2T at 418-421	-23, +FCQSMGFMNRKVL
*rev*62-1	-T at 495-497	-52, +L
*rev*45-2	-T at 485-488	-48, +HSKVFRR
*rev*64-1	A->G at 397	-11, +FCQSMGFM**D**RKVL
*rev*ND	*vir*3060 and A->G at 367	-11, +FR**D**HRR

C. Amino-acid sequence at the C-terminal end of the WT, *vir*, and *rev,vir* repressors

SFIGFSQQSIQTLMMLDALPEEKRKEILSKYGIHEQESVVVPSQEPQEVKKAV	Wild type
SFIGFSQQSIQTLMMLDALPEEKRKEILSKYGIHEQESVVVPFRNHRR	*vir*3060
SFIGFSQQSIQTLMMLDALPEEKRKEIFCQSMGFMNRKVL	*vir*3061(Sup^0)
SFIGFSQQSIQTLMMLDALPEEKRKEIFCQSMGFMNRKVLXYLHRNHRR	*vir*3061(*sup*)
SL	*rev*62-1(Sup^0)
SLXDFHSKVFRR	*rev*62-1(*sup*)
SFIGFHSKVFRR	*rev*45-2
SFIGFSQQSIQTLMMLDALPEEKRKEIFCQSMGFMDRKVL	*rev*64-1(Sup^0)
SFIGFSQQSIQTLMMLDALPEEKRKEIFCQSMGFMDRKVLXYLHRNHRR	*rev*64-1(*sup*)

Fig. 1. Nucleotide and amino acid sequence of mutant Mu repressor genes and proteins. (**A**) The positions of the different mutations discussed in the text are shown either at the amino acid or at the nucleotide level. Asterisks show the nonsense codons signalling a translational stop for the *rev* and *vir* frameshift mutations. Where the gene product ends at an *am* nonsense codon (*rev*62-1, *rev*42-1 and *vir*3061), in the presence of an *am* suppressor tRNA it is extended to the next stop codon TGA (in the case of the *rev* mutants) and TAA (in the case of *vir*3061). The TGA also marks the end of the *rev*45-2 truncated protein and the TAA is the stop codon for the *vir*3060 protein. (**B**) Summary of the consequences of the *vir* and *rev* mutations shown in (A). (**C**) Amino acid sequence at the C-terminal of the WT, *vir* and *rev,vir* repressors in *sup* and Sup^0 host backgrounds. Note that the *vir*3061 protein, if suppressed, carries the *vir*3060 terminal motif RNHRR hooked onto the *vir*3061 C-terminal motif FMNRKVL. The *rev*64-1 point mutation which displays the same translational properties as the *vir*3061 protein, if suppressed, carries the *vir*3060 RNHRR terminal motif hooked onto the modified FMDRKVL C-terminal motif.

unpublished results; J.E.Laachouch *et al.*, submitted for publication; Ph.Rousseau, submitted), trans-targeting of WT repressor towards Clp degradation by the mutant *vir* protein is most likely to occur through the formation of mixed oligomers.

Energy-dependent proteolytic degradation is only partially understood and for the Clp enzyme has only been analysed in detail for the ClpAP form. To be degraded, large peptides and proteins have to interact with a ClpA allosteric site. This activates ClpA ATPase activity. ATP promotes ClpA–ClpP interaction and this is sufficient to stimulate ClpP proteolytic activity. These steps do not require ATP hydrolysis, which is only necessary for the processive degradation of proteins by ClpP (see Thompson and Maurizi, 1994; Thompson *et al.*, 1994). If that scheme can be extended to ClpXP, *vir* repressor protomers could provide mixed repressor oligomers with a signal which is absent from WT repressor and allows for recognition by the ClpXP enzyme. In order to further evaluate the significance of the modified C-terminal amino acid sequence of the *vir* repressor mutants in specifying sensitivity to ClpXP, we characterized intragenic suppressors of the *vir* mutations. This revealed that the modified *vir* repressor C-terminal end is crucial for recognition by the

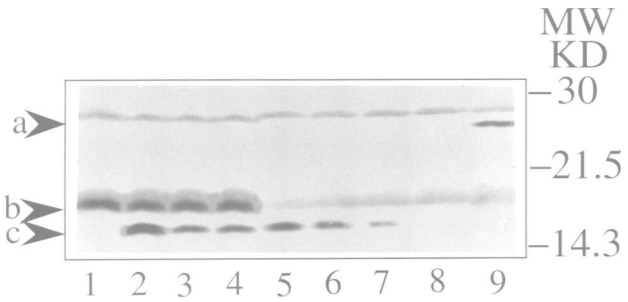


Fig. 2. Western blot analysis of repressors synthesized from *c* genes carrying the various *rev* mutations. Cells were grown at 30°C to middle exponential phase (3×10^8 bacteria/ml). Aliquots (1 ml) of the cultures were centrifuged and the pellets resuspended in 50 µl of lysis buffer. Six microlitres of extract were loaded on the gel. Lane 1, MC4100(λ*supE*)/pVG233 (*vir*3061*rev*45-2); lane 2, MC4100(λ*supE*)/pVG232 (*vir*3061*rev*62-1); lane 3, MC4100(λ*supE*)(Mu*vir*3060*rev*2-30); lane 4, MC4100(λ*supE*)(Mu*vir*3060*rev*42-1); lane 5, MC4100/pVG232; lane 6, MC4100(Mu*vir*3060*rev*2-30); lane 7, MC4100(Mu*vir*3060*rev*42-1); lane 8, MC4100/BluescribeM13+; lane 9, MC4100/pJV200 (WT repressor). The arrows point towards the different repressors (a, WT; b, *rev*45-2 or *rev*62-1 in the *supE* background; c, *rev*62-1 in the Sup^0 background).

Clp protease. A single amino acid change in that sequence is sufficient to stabilize the mutated repressor. We also looked at the effect of that short sequence on the stability of other proteins. We chose to use the two components of the gyrase poison/antidote Ccd system encoded by plasmid F (Bernard and Couturier, 1992). The poison polypeptide, CcdB, is stable, while the antidote, CcdA, is a natural target for the ATP-dependent Lon protease (van Melderen *et al.*, 1994).

Results

Characterization of the rev mutations

Geuskens *et al.* (1991) described the isolation from Mu*cts*-62*vir*3061pAp1 (a thermoinducible and virulent mutant of Mu unable to lysogenize unless the infected host is *clpP* or *clpX*) of revertants (*rev*) which recovered the ability to lysogenize at 30°C on Clp^+ strains. Most of the phages isolated synthesized truncated repressor proteins which led these authors to suggest that they had lost the C-terminal end typical of the *vir* mutant repressors.

We cloned and sequenced the repressor genes of three of these phages (Mu*rev*62-1, Mu*rev*45-2, Mu*rev*64-1; see Materials and methods for a detailed protocol). Figure 1 shows the nucleotide changes involved and their consequences on the corresponding translated repressor proteins. *rev*62-1 and *rev*45-2 are frameshift mutations located upstream of the *vir* mutation. As anticipated, they provoke the synthesis of a protein truncated from the modified C-terminal end of the parental *vir* repressor. The −1 frameshift in *rev*62-1 leads to the appearance of an *am* codon and hence, in a *sup* strain, the peptide should be elongated up to the opal codon (UGA) which also signals the translational stop of the truncated *rev*45-2 proteins. This is indeed what was observed upon Western blot analysis of the truncated proteins expressed in Sup^0 and *supE* strains carrying the pVG232 and pVG233 plasmids (see Figure 2). Two other *rev* mutations, *rev*2-30 and *rev*42-1, isolated from Mu*vir*3060, a Muc^+ derivative, display the exact same protein profile as *rev*62-1, showing that they are independent isolates of the same *rev* mutation (see Figure 2).

Consistent with the size of the *rev*64-1 protein which is the same as that of its parent, *vir*3061 (see Figure 4A), *rev*64-1 is a point mutation. An A→G transition substitutes an Asp (GAC) for an Asn (AAC) residue in the modified FMNRKVL C-terminal end of the *vir*3061 protein. Here again, in a *supE* host the peptide should be elongated up to the ochre codon (UAA) that signals translation arrest in the *vir*3060 protein. In such a host, the *rev*64-1 mutation should thus give rise to one repressor form with FMDRKVL as the C-terminus and another with, in addition, the *vir*3060 RNHRR typical C-terminal end (see Figure 1). In extracts prepared from *sup* strains expressing the *vir*3061*rev*64-1 *c* gene, we did indeed detect the two expected peptides (see Figure 4A).

All these results confirm that the modified C-terminal end of the *vir* proteins is essential for virulence and hence for recognition by Clp.

The modified C-terminal end of the vir repressors is a determinant of their sensitivity to the Clp protease

Being active in maintaining a Mu prophage repressed in a Clp^+ background *in vivo*, the *vir,rev* repressors should have recovered higher stability in that background. Stability of the *vir*3061*rev*62-1 and *vir*3061*rev*45-2 repressors was tested by Western blot analysis (see Materials and methods), at 30 and 42°C, using Clp^+ and *clpP* strains with and without a *supE* supressor and with the $BluescribeM13^+$ derived plasmids pVG232 and pVG233 which overexpress the mutated *c* gene from a *plac* promoter. For the *vir*3060*rev*42-1 and *vir*3060*rev*2-30 mutations, we used the same bacterial strains lysogenized by the corresponding phages. Figure 3A shows that at 30°C the *rev* proteins are more stable than their *vir* counterparts (they remain detectable up to 100 min after addition of spectinomycin versus >20 min for the *vir* proteins). At 42°C, the *rev* proteins are less stable (detected up to 60 min; see Figure 3B) and this remains unchanged in a *clpP* host (data not shown). Thus, the modified C-terminal end of the *vir* repressors is indeed a determinant of their sensitivity to the Clp protease. In addition, removal of 40–50 C-terminal residues at the repressor C-terminal end provokes a temperature-dependent, ClpP-independent destabilization. These results are consistent with the *in vivo* behaviour of Muc^+,*vir*,*rev* phages. They lysogenize Clp^+ and *clpP* hosts at 30°C with the same frequency as Muc^+, but at 42°C their lysogenization frequency is 1000-fold lower. Mu*cts*62,*vir*,*rev* phages form lysogens at normal frequency on both *clpP* and Clp^+ hosts, but only at 30°C (data not shown).

A single amino acid substitution in the vir3061 FMNRKVL C-terminal end is sufficient to avoid degradation by the Clp

Mu*rev*64-1 has a total of three mutations in its *c* gene: one frameshift (*vir*3061) and two point mutations (*cts*62, which makes the phage temperature inducible, and *rev*64-1). It was shown previously that the *vir*3061,*rev*64-1 repressor has recovered higher stability (half-life ~120 min) compared with its *vir*3061 parent (half-life ~20 min; Geuskens *et al.*, 1992; see also Figure 4A). Since the only

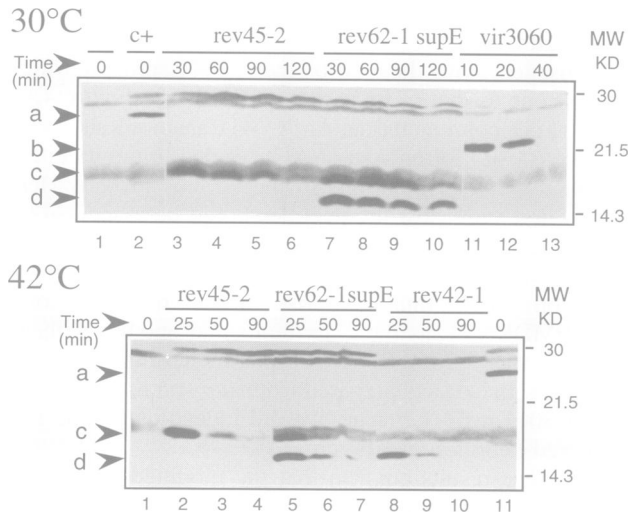


Fig. 3. Stability of the *rev45-2* and the *rev62-1* repressors. The stability assay using Western-blot analysis was performed as described in Materials and methods. Extracts (8 µl) were loaded on the gel. The arrows point towards the different repressor proteins (a, WT; b, *vir*3060; c, *rev*45-2 or *rev*62-1 in the *supE* background, and d, *rev*62-1 in the Sup^0 background). At 30°C, lane 1, MC4100/Bluescribe $M13^+$, at time 0 min; lane 2, MC4100(λ*supE*)/pJV200 (WT repressor), at time 0 min; lanes 3–6, MC4100(λ*supE*)/pVG233 (*vir*3061*rev*45-2), at times 30, 60, 90 and 120 min after the addition of spectinomycin; lanes 7–10, MC4100(λ*supE*)/pVG232 (*vir*3061*rev*62-1), at times 30, 60, 90 and 120 min after addition of spectinomycin; lanes 11–13, MC4100/pULB508 (*vir*3060), at times 10, 20 and 40 min after the addition of spectinomycin. At 42°C, lane 1, MC4100/Bluescribe $M13^+$, at time 0 min; lane 11, MC4100(λ*supE*)/pJV200 (WT repressor), at time 0 min; lanes 2–4, MC4100(λ*supE*)/pVG233 (*vir*3061*rev*45-2), at times 25, 50 and 90 min after the addition of spectinomycin; lanes 5–7, MC4100(l*supE*)/pVG232 (*vir*3061*rev*62-1), at times 25, 50 and 90 min after the addition of spectinomycin; lanes 8–10, MC4100(Mu*vir*3060*rev*42-1), at times 25, 50 and 90 min after the addition of spectinomycin. All the tests shown were performed in Clp^+ strains. Results obtained in *clpP* strains were the same (not shown).

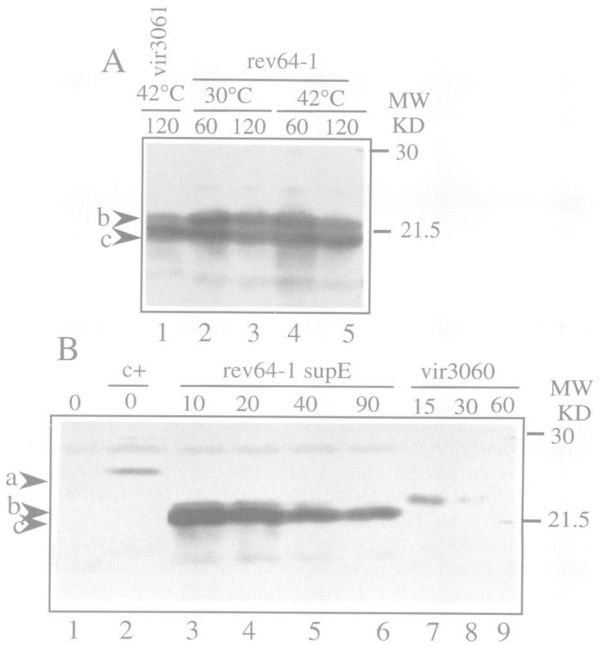


Fig. 4. Stability of the *rev*64-1 repressor. The stability assay using Western-blot analysis was performed as described in Materials and methods. The arrows point towards the different repressor proteins (a, WT; b, *vir*3060, *vir*3061 and *rev*64-1 in the *supE* background; c, *vir*3061 and *rev*64-1 in the Sup^0 background). Extracts (8 µl) were loaded on the gel. (**A**) lane 1, MC4100(λ*supE*)*clpP*::Cm/pULB507 (*vir*3061) at time 120 min after the addition of spectinomycin. This strain was used as a control for repressor size and stability; lanes 2–5, MC4100(λ*supE*)*clpP*::Cm/pVG234 (*vir*3061*rev*64-1), at times 60 and 120 min after the addition of spectinomycin, at 30°C (lanes 2 and 3) and 42°C (lanes 4 and 5). (**B**) lane 1, MC4100/Bluescribe $M13^+$, at time 0 min; lane 2, MC4100(λ*supE*)/pJV200 (WT repressor), at time 0 min; lanes 3–6, MC4100(λ*supE*)/pVG234 (*vir*3061*rev*64-1), at times 10, 20, 40 and 90 min after addition of spectinomycin, at 42°C; lanes 7–9, MC4100/pULB508 (*vir*3060), at times 15, 30 and 60 min after the addition of spectinomycin. The same experiment was performed at 30°C and gave the same results (not shown).

difference between the two proteins is an N to D change in the *vir*3061 FMNRKVL C-terminus, this sequence and the N residue in particular are clearly important for recognition by the Clp protease.

In a *supE* host, the *vir*3061*rev*64-1 *c* gene encodes two repressor forms, one with the FMDRKVL motif, the other with the *vir*3060 RNHRR motif at the C-terminus (see Figure 1). The latter should still be a destabilizing factor in the presence of Clp. As seen in Figure 4B, the larger form is barely detectable in the Clp^+ strain, with a half-life of ~40 min compared with 90 min for the shorter form. In the *clpP* strain, both forms are present in equivalent amounts and are similarly stable. This reconfirms the destabilizing role of the RNHRR motif which has the N residue in common with the *vir*3061 C-terminus. We therefore introduced a N→D mutation in the *vir*3060 *c* gene cloned in an M13 derived plasmid suitable for site-directed *in vitro* mutagenesis. The mutated gene was cloned in pUC19 under the control of the p*lac* promoter to generate plasmid pVG235. The stability of the mutated protein, expressed from the pVG235 plasmid, was compared with that of the parental *vir*3060 repressor. As shown in Figure 5, in a Clp^+ background, the N→D substitution does increase stability (half-life ~60 min compared with <30 min for *vir*3060), although not as much as it did for the *vir*3061*rev*64-1 protein. In a *clpP* strain, the *vir*3060ND and *vir*3060 are as stable as the WT protein (half-life >120 min).

The FMNRKVL motif is not sufficient to provide Clp sensitivity

The above experiment raised the question whether the *vir*3061 FMNRKVL C-terminal motif is sufficient for recognition by Clp. To test this, we hooked the *vir*3061 FMNRKVL terminus at the C-terminal end of two other proteins, one of which (CcdB) is naturally stable; the other (CcdA) is unstable and degraded by Lon (see Materials and methods for experimental details). The appropriate combination of *lon* and *clpP* mutations allowed for the detection of a possible change in recognition specificity by the two proteases of the CcdA derived fusion protein. Both fusion proteins retained their biological activity (see Materials and methods for more details). As shown in Figure 6C, no change in CcdB stability could be detected as a result of C-terminal addition of the FMNRKVL sequence. However, compared with CcdA, the CcdA derived fusion protein is less stable in a Clp^+ than in a *clpP* background (half-life ~20 min compared with 65 min; Figure 6A, B and F). In addition, the fusion protein has become more sensitive to Lon (half-

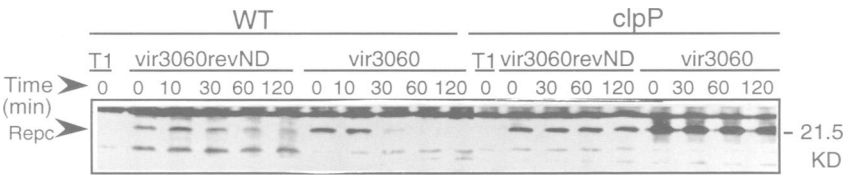


Fig. 5. Stability of the *vir*3060*rev*ND repressor. The protocol of the experiment was the same as in Figures 3 and 4. The arrow points towards the *vir*3060 or *vir*3060*rev*ND proteins. Extracts (5 µl) were loaded in each lane. The extracts were prepared from MC4100 (WT) or MC4100*clpP* (*clpP*) containing the pULB508 (*vir*3060 repressor) or the pVG235 (*vir*3060*rev*ND repressor) plasmid, prior to (0 min) or at the times indicated, after the addition of spectinomycin. T1 is the control strain MC4100/pUC19, at time 0 min.

life ~65 min compared with 80 min for the CcdA protein in the *clpP* background; see Figure 6A, B and F). Thus, the fusion protein gained sensitivity to Clp and retained its Lon sensitivity. To see whether Clp sensitivity was regulated by ClpX (as is the case for Mu*vir* repressor), the stability of the CcdA fusion polypeptide was measured in *clpX* and *clpA* genetic backgrounds. Figure 6D and E shows that only the *clpX* mutation increased the protein half-life (from ~45 to 80 min).

Discussion

The results presented above shed some new light on possible determinants of protein recognition and degradation by Clp.

In vivo experiments suggest that Mu WT repressor is degraded by the ClpXP protease [Shapiro (1993) and our unpublished results], although it is clearly an inefficient process. This and the fact that the WT repressor is readily degraded in conjunction with the *vir* repressor shows that the WT protein bears the proteolytic cleavage sites (if such sites exist), but is missing either the recognition determinant and/or the signal for the allosteric activation of ClpX (these two latter determinants could be different or the same, or linked in some way).

The behaviour of the *vir* repressor mutants shows, once again, that a slight change in the primary sequence of a stable protein can be sufficient to increase protease sensitivity. Since we have no insight into the three-dimensional structure of Mu repressor, it is of course difficult to assess the effect that this change in the primary sequence may have on the general conformation of the protein. However, *in vitro* DNA binding assays have shown that purified *vir* repressor binds Mu operators almost as efficiently as the WT protein (Vogel *et al.*, 1995), showing that the *vir* mutation does not substantially change the overall structure of the protein.

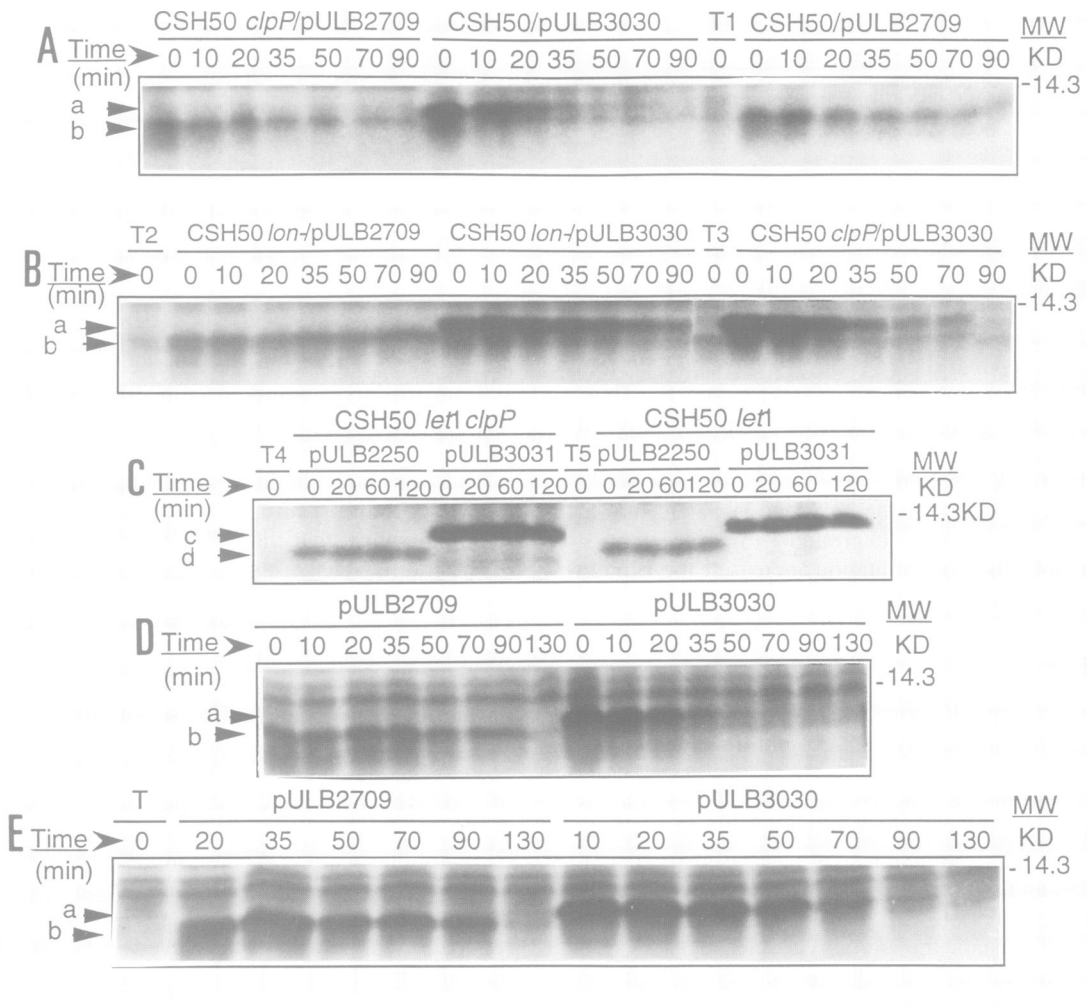
Two sets of data strongly suggest that Mu repressor bears an important intrinsic conformational flexibility. On the one hand, in the *cts*62 repressor, which is thermosensitive for DNA binding, removal of as little as eight amino acids at the C-terminal end suppresses the thermosensitivity (J.L.Vogel *et al.*, submitted for publication). On the other hand, results presented here show that removal of 50 residues at the C-terminal end of the WT repressor makes it less stable. This could be due to the overexpression at 42°C of a temperature-induced host protease (different from ClpP). Alternatively, it could reflect a temperature-induced conformational change of the truncated repressor which increases protease sensitivity. If this is the case, all repressors could bear the ClpX recognition determinant, but it could be masked for example on the WT protein and unmasked with different efficiencies in different mutants depending on their conformation and their conformational flexibility.

The fact that a single N→D change in the FMNRKVL C-terminal motif is sufficient to at least partially counteract its effect shows that there is some sequence requirement in the motif which triggers ClpXP sensitivity. It is intriguing that the λ O protein, which is a substrate for the ClpXP enzyme, bears a GRNHRD motif ~60 amino acids from its C-terminal end.

As could have been expected, the FMNRKVL motif is not sufficient to provide Clp sensitivity to any protein. When hooked at the C-terminal end of the stable CcdB protein, it does not alter its stability. However, addition of FMNRKVL at the C-terminal end of CcdA did make it Clp sensitive to a certain extent. Consistent with the current views that the ATPase moieties of ATP-dependent proteases bear the substrate recognition specificity (see, for instance, Thompson and Maurizi, 1994), it is the ClpX regulatory subunit that is involved in CcdA-FMNRKVL degradation. The *vir* C-terminal motif does thus bear some of the specificity determinants for recognition by ClpXP. CcdA already bears some features to be recognized by Lon and allosterically activates that protease, and these, in conjunction with the C-terminal motif, could be sufficient to bind and activate ClpXP. In addition, some of the determinants for Lon and Clp sensitivity seem to be similar since Lon substrates can be degraded by ClpAP *in vitro* (L.van Melderen and M.Maurizi, personal communication). This may, in some way, be related to the increased sensitivity of the CcdA-FMNRKVL peptide to Lon.

The insensitivity of CcdB to the addition of the C-terminal motif could result from the total absence of either the conformational information which in conjunction with FMNRKVL allows for recognition by ClpXP or, more trivially and less likely, of cleavage sites. Only a thorough *in vivo* and *in vitro* analysis of the interaction of these different substrates with Lon and Clp will allow that alternative to be resolved.

Clp appears more and more to be the bacterial equivalent of the eukaryotic 26S proteasome. They both display a multimeric organization. In addition, the 26S enzyme has a similar overall shape to the GroES chaperonin (Chen *et al.*, 1994; Peters, 1994). This certainly further supports a model according to which the ATPase moiety of ATP-dependent proteases, whether on a single polypeptide chain (Lon) or on a different subunit (Clp, RecA-dependent autocleavage of λ CI and LexA) as the protease catalytic site, interact with their substrates in a similar manner to the chaperons



F

	CSH50 and MC4100	CSH50 *clpP*::Cm	CSH50 *lon*::Tn10	MC4100 *clpA*::Kn	MC4100 *clpX*::Kn
pULB2709 (CcdA)	75	80	>90	85	90
pULB3030 (CcdA-GFMNRKVL)	20	65	90	42	80
pULB2250 (CcdB)	>120	>120	nd	nd	nd
pULB3031 (CcdB-GFMNRKVL)	>120	>120	nd	nd	nd

Fig. 6. Stability of CCdA and CCdB proteins with the *vir* C-terminal motif. Cells were pulse labelled with [^{35}S]methionine and chased for various times (see Materials and methods for details). After electrophoresis and autoradiography of the gels, the autoradiograms were scanned as described in the Materials and methods and the half-lives of the proteins [given in minutes in (**F**)] were estimated. Aliquots were analysed at the times indicated, after the addition of cold methionine. (**A**) and (**B**) Stability of CcdA (pULB2709) and CcdA–GFMNRKVL (pULB3030) proteins in CSH50, CSH50*clpP*::Cm, CSH50*lon*::Tn10. The two pULB plasmids express the CcdA and CcdA–GFMNRKVL proteins from the p*lac* promoter controlled, *in trans*, by the Lac repressor encoded by the pSC101*lacI*^{q} plasmid. (**C**) Stability of the CcdB (pULB2250) and CcdB–GFMNRKVL (pULB3031) proteins in CSH50*let*1 and CSH50*let1*,*clpP*::Cm. The chromosomal *let1* mutation (in the *gyrA* gene) prevents killing by the active CcdB proteins. The two pULB plasmids express the CcdB and CcdB–GFMNRKVL proteins from the p*lac* promoter controlled, *in trans*, as in (A) and (B). (**D**) Stability of CcdA and CcdA–GFMNRKVL proteins in MC4100*clpA*::Kn. Expression and control of the tested proteins is as in (A) and (B). (**E**) Stability of CcdA and CcdA–GFMNRKVL proteins in MC4100*clpX*::Kn. Expression and control of the tested proteins is as in (A) and (B). The arrows point towards the following proteins: a, CcdA–GFMNRKVL; b, CcdA; c, CcdB–GFMNRKVL; d, CcdB. Seven microlitres of extract were loaded in each lane. Controls were as follows: lane T1, extract from CSH50/pKK223/pSC101*lacI*^{q}, at time 0 min; lane T2, extract from CSH50*lon*::Tn10/pKK223/pSC101*lacI*^{q}, at time 0 min; lane T3, extract from CSH50*clpP*::Cm/pKK223/pSC101*lacI*^{q}, at time 0 min; lane T4, extract from CSH50*let1*,*clpP*::Cm/pKK223/pSC101*lacI*^{q}, at time 0 min; lane T5, extract from CSH50*let1*/pKK223/pSC101 *lacI*^{q}, at time 0 min.

(Gottesman and Maurizi, 1992; Squires and Squires, 1992; Wojtkowiak *et al.*, 1993). Several recent observations strongly support this view. On the one hand, Wickner *et al.* (1994) showed that, *in vitro*, ClpA can, in conjunction with DnaK, activate the P1 plasmid replication initiation protein RepA, which is normally activated by the DnaK/DnaJ/GrpE chaperon machine. On the other hand the ClpX protein was shown to dismantle aggregates of the phage λ O protein (Wawrzynow *et al.*, 1995) and the bacteriophage Mu transposase tetramer (Levchenko *et al.*, 1995).

Table I. Bacterial strains, phages and plasmids

Bacteria		
MC4100	*araD139,Δ(lacIPOZYA,argF)U169, fla,relA,rpsL*	Casadaban, 1976
MC4100*clpA*::Tc	same as above but *clpA*::Tc	From our laboratory
MC4100*clpX*::Kn	same as above but *clpX*::Kn	From our laboratory
MC4100*clpP*::Cm	same as above but *clpP*::Cm	From our laboratory
MC4100(λ*supE*)		This work
MC4100*clpP*::Cm(λ*supE*)		This work
C600	*thr,leu,tonA,lacY,thi,supE*	Campbell, 1961
CSH50	*Δpro,lzc, ara, rpsL, thi*	Miller, 1972
CSH50 *lon*::Tn*10*		From R.D'Ari
RH7999	CSH50*clpP*::Cm, P1 transduction of *clp*P::Cm from SG22030 into CSH50	
Phages		
Muc$^+$		Taylor, 1963
Mu*cts*62pAp1		Leach and Symonds, 1979
Mu*vir*3060pAp1		Geuskens *et al.*, 1991
Mu*cts*62*vir*3061pAp1		Geuskens *et al.*, 1991
Mu*cts*62*vir*3061*rev*45-2pAp1		Geuskens *et al.*, 1991
Mu*cts*62*vir*3061*rev*62-1pAp1		Geuskens *et al.*, 1991
Mu*cts*62*vir*3061*rev*64-1pAp1		Geuskens *et al.*, 1991
Mu*vir*3060*rev*42-1pAp1		Geuskens *et al.*, 1991
Mu*vir*3060*rev*2-30pAp1		Geuskens *et al.*, 1992
Plasmids		
pSC101::*lacI*^{q}	pSC101 derivative expressing LacI^q repressor	From M.Pato
pKK233-3	pBR322 with a ptac promoter	De Boer *et al.*, 1983
pJV200	pUC19 with*attL-Hin*dIII Muc$^+$	Vogel *et al.*, 1991
pULB508	BluescribeM13$^+$ with*attL-Hin*dIII Mu*vir*3060	Geuskens *et al.*, 1991
pULB507	BluescribeM13$^+$*attL-Hin*dIII Mu*cts*62*vir*3061	Geuskens *et al.*, 1991
pVG234	BluescribeM13$^+$*attL-Hin*dIII Mu*cts*62*vir*3061*rev*64-1	This work
pVG232	BluescribeM13$^+$*attL-Hin*dIII Mu*cts*62*vir*3061*rev*62-1	This work
pVG233	BluescribeM13$^+$*attL-Hin*dIII Mu*cts*62*vir*3061*rev*645-2	This work
pVG235	pUC19 with *Eco*RI–*Hin*dIII Mu*vir*3060*rev*ND	This work
pULB2705	pKK233-3 with the *ccdA,ccdB* operon under ptac control	Bernard *et al.*, 1993
pULB2250	pKK233-3 with the *ccdAB* gene under ptac control	
pULB2709	pKK233-3 with the *ccdA* gene under ptac control	This work
pULB3030	pKK223-3 with *Eco*RI–*Pst*I fragment carrying *ccd*A fused to *vir*3061 C-terminal end	This work
pULB3031	pKK223-3 with *Eco*RI–*Pst*I fragment carrying *ccd*B fused to *vir*3061 C-terminal end downstream of *ccd*A	This work

Materials and methods

Strains

Bacterial strains, phages and plasmids are listed in Table I. MupAp1 phages contain ~1 kb of Tn3 DNA conferring ampicillin resistance, which is substituted for Mu DNA in the Mu G region (Leach and Symonds, 1979).

Media

Bacteria were grown in LB (Miller, 1972) and titrated on LA plates (Miller, 1972) containing 1.2% Difco agar. Ampicillin (Ap, 25 μg/ml for selection of MupAp1 lysogens, 50 μg/ml for selection of plasmids), tetracycline (Tc, 12.5 μg/ml), spectinomycin (Spc, 100 μg/ml) and chloramphenicol (Cm, 25 μg/ml) were included when appropriate. Phage lysates were diluted in SM buffer (Weigle *et al.*, 1959) and titrated on lawns of sensitive bacteria (0.1 ml of an overnight culture in LB) poured with 2.5 ml of 0.7% LA agar on LA plates.

General procedures

Lysates of Mu*cts*62 and Mu*vir,rev* were prepared by thermal induction of a lysogen, and lysates of Muc+ and Mu*vir* mutants were prepared by infecting C600 on LA plates at 37°C, as described previously (Bukhari and Ljungquist, 1977).

Lysogenization frequencies

Lysogenization frequencies were measured by spotting aliquots of serial dilutions of lysates of MupAp1 derivatives on lawns of the bacteria to be tested on LA and LA plates supplemented with 25 μg/ml of ampicillin. The plates were incubated overnight at the desired temperatures. Lysogenization frequencies were calculated as the ratio between the number of Ap^R colonies on LA + Ap and the number of plaques on a nearly isogenic reference strain on LA plates.

DNA manipulations

Enzymes were purchased from Boehringer and used as recommended by the manufacturers. Isolation of the DNA and analysis of restriction fragments on 0.7% agarose gels in TAE buffer were essentially as described by Maniatis *et al.* (1982). DNA fragments for cloning were purified from agarose gels using a Gene Clean Kit (Stratagene).

Plasmid construction

Mu repressor is encoded by the left-most gene on the phage genome. Digestion of phage DNA with *Hin*dIII produces a 1 kb left-end fragment containing 50–150 bp of variable host DNA attached to Mu DNA. This fragment was isolated from purified DNA extracted from Mu*vir,rev* lysates as described previously (Bukhari and Ljungquist, 1977) and ligated to BluescribeM13+ (Vector Cloning Systems) digested with *Hin*dIII and *Sma*I as described previously (Vogel *et al.*, 1991). The resulting plasmids, pVG232, pVG233 and pVG234, carry the left end of Mu*vir*3061*rev*62-1, Mu*vir*3061*rev*45-2 and Mu*vir*3061*rev*64-1, respectively. The repressor genes are under *plac* control. Transformation of appropriate bacterial strains with plasmid DNA was as described by Maniatis *et al.* (1982).

DNA sequencing

DNA sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) according to the Pharmacia protocol.

In vitro mutagenesis

In vitro mutagenesis was performed with the Amersham oligonucleotide-directed *in vitro* mutagenesis kit, according to the manufacturer's

instructions. The GTAGTACCTTTCAGGGACCACAGG oligonucleotide was used to introduce the *vir*3060 mutation with an Asn to Asp change (see Figure 1). It was added to single-stranded DNA of M13mp18 phage with a cloned fragment containing the WT repressor gene (the *Eco*RI–*Hin*dIII fragment isolated from plasmid pJV200; see Table I). The mutagenized *Eco*RI–*Hin*dIII fragment was cloned into pUC19. The resulting plasmid, pVG235, carries the *vir*3060*rev*ND mutant repressor gene under p*lac* control.

Analysis of repressor sizes by Western blotting

This was performed as described by Geuskens *et al.* (1991).

Repressor stability

Repressor stability was assayed as described by Geuskens *et al.* (1992). Half-lives were estimated by computer scanning of the nitrocellulose membranes using a UMAX UC630 scanner connected to a Macintosh computer and the NIH Image software.

Construction of CcdB and CcdA fusion proteins

Using the polymerase chain reaction (PCR), the oligonucleotides TGACAGAATTCATGAAGCAGCGTATTACA and GTTCACTGCAGTTACAACACTTTCCTGTTCATGAATCCCCAGTCCCTGTTCTCGT were used to fuse the *vir*3061 GFMNRKVL tail sequence to the 3′ end of the *ccdA* gene from the F plasmid cloned in pULB2705, and the oligonucleotides GAAACAGAATTCATGCAGT and GTTCACTGCAGTTACAACACTTTCCTGTTCATGAATCCTATTCCCCAGAACATCA were used to fuse the same *vir*3061 GFMNRKVL tail sequence to the 3′ end of the *ccdB* gene from the F plasmid cloned in the pULB2250 (see Table I). The recombinant DNA obtained was digested with *Eco*RI and *Pst*I, and cloned in the pKK223–3 plasmid (see Table I) digested with the same restriction enzymes. The resulting plasmids, pULB3030 and pULB3031, express the CcdA–GFMNRKVL and CcdB–GFMNRKVL fusion proteins under the control of the ptac promoter. They were sequenced and then introduced into the appropriate strains which carried the pSC101::*lacI*^{q} plasmid (see Table I) to repress the ptac promoter. The biological activity of the proteins was tested: the CCdB–GFMNRKVL expressed from the above construct had a lethal effect counteracted by CcdA and the CcdA–GFMNRKVL fusion protein was still able to protect against CcdB-induced killing.

Pulse–chase labelling

The stability of the CcdA–GFMNRKVL and CcdB–GFMNRKVL fusion proteins was analysed using pulse–chase experiments.

Strains expressing one or the other protein were grown at 37°C in methionine assay medium (Difco) to an A_{530} of 0.2. IPTG (1 mM final concentration) was added 30 min before labelling which was carried out with [^{35}S]methionine for 3 min. Cells were then centrifuged at 4°C (8 min at 5000 r.p.m.) and the pellets resuspended in the same medium with an excess of cold methionine (1 mg/ml). Incubation was continued. Aliquots of 1 ml were removed at 10, 20, 35, 50, 70 and 90 min in the case of plasmids pULB2709 and pULB3030, and at 10, 20, 60 and 120 min in the case of pULB2250 and pULB3031. All samples were centrifuged. Proteins were extracted from the pellets by boiling at 100°C and separated by PAGE as described by Laemmli (1970). The gels were dried and autoradiographed (Kodak film). Relative amounts of CcdA–GFMNRKVL and CcdB–GFMNRKVL proteins were estimated after scanning the autoradiograms as described above for the immunoblots.

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