The EMBO Journal vol.11 no.13 pp.5121–5127, 1992

Virulence in bacteriophage Mu: a case of *trans*-dominant proteolysis by the *Escherichia coli* Clp serine protease

Vincent Geuskens, Amina Mhammedi-Alaoui, Lucie Desmet and Ariane Toussaint[1]

Unité Transposition Bactérienne, Laboratoire de Génétique, Université Libre de Bruxelles, B1640 Rhode-St-Genèse, Belgium
[1]Corresponding author

Communicated by A.Toussaint

The importance of proteases in gene regulation is well documented in both prokaryotic and eukaryotic systems. Here we describe the first example of genetic regulation controlled by the *Escherichia coli* Clp ATP-dependent serine protease. Virulent mutants of bacteriophage Mu, which carry a particular mutation in their repressor gene (*vir* mutation), successfully infect Mu lysogens and induce the resident Mu prophage. We show that the mutated repressors have an abnormally short half-life due to an increased susceptibility to Clp-dependent degradation. This susceptibility is communicated to the wild type repressor present in the same cell, which provides the Mu*vir* phages with their *trans*-dominant phenotype. To our knowledge this is the first case where the instability of a mutant protein is shown to trigger the degradation of its wild type parent.

Key words: ATP-dependent protease/bacteriophage Mu/induced proteolysis/virulence

Introduction

Virulent mutants of temperate bacteriophages escape immunity and grow on strains lysogenic for their parental wild type phage. For those phages that carry a single immunity region, virulent mutants typically carry multiple operator mutations, which prevent repressor binding, and as a result are *cis*-dominant (Jacob and Wollman, 1954; Gussin *et al.*, 1983); they multiply in the lysogen without disturbing prophage repression. Other temperate phages carry two immunity regions, one of which (ImmC) behaves as just described. The second immunity region (ImmI) encodes an anti-repressor that inactivates the ImmC repressor. In lysogens, anti-repressor synthesis is itself blocked by a second repressor. Inactivation by mutation of that second repressor recognition site results in virulent mutants that express the anti-repressor constitutively and therefore not only multiply in the lysogen they infect, but also induce the resident prophage due to a complete inactivation of all the ImmC repressor present in the cell (for reviews, see Poteete, 1987; Yarmolinsky and Sternberg, 1987).

We recently characterized virulent mutants of the transposable phage Mu. Unexpectedly, they were found to be repressor mutants. As a result of a frameshift mutation (either −2 or +1 combined with a substitution) in the 3′ end of the repressor coding gene (*c*), they synthesize shortened proteins with six or 13 altered amino acids at their C-terminal end (Geuskens *et al.*, 1991; Figure 1). This finding was difficult to reconcile with the fact that Mu*vir* phages efficiently induce the resident prophage upon infection of a Mu lysogen (van Vliet *et al.*, 1978).

In the present paper we characterize further the *vir* mutant repressors. We show that contrary to the wild type protein, *vir* repressors are naturally unstable. This led us to investigate whether instability was responsible for the Mu*vir* phenotype and to identify the protease reponsible for the degradation. Among the *Escherichia coli* proteases we tested was Hfl. Hfl plays a key role in the decision between lysogeny and lytic development in phage λ (Hoyt *et al.*, 1982) and is involved in the degradation of Mu transposase (Gama *et al.*, 1990). The ATP-dependent Lon protease catalyses the

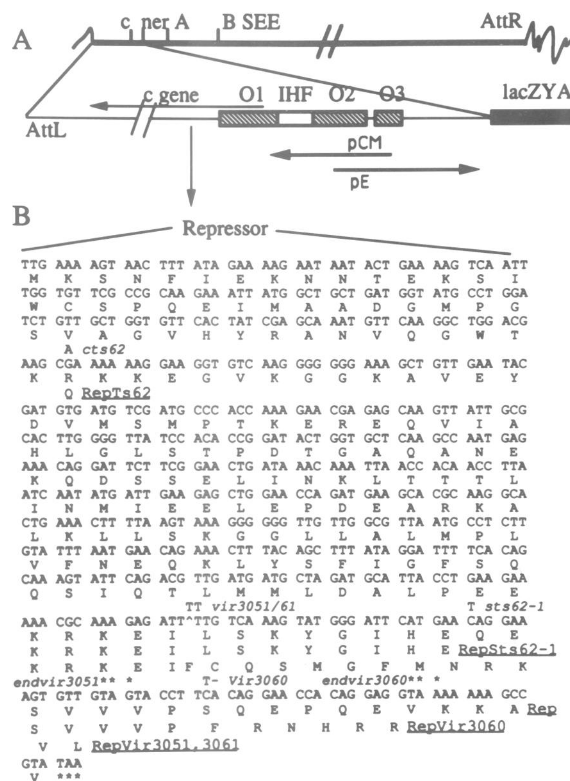

Fig. 1. Mu repressor gene and protein. **A.** Schematic representation of the Mu genome and sequence detail of the left-end region carried by the pRS551-derived plasmids and λRS45-derived phages. In these constructs *lacZ* transcription is driven from the phage early promoter pE and hence controlled by Mu repressor binding to operators O1 and O2. pCM is the repressor promoter. Arrows indicate the directions of transcription (bold) and translation (narrow). **B.** Repressor gene and protein with indications of the mutations discussed in the text. Note that the *vir*3051 and 3061 mutations, which were isolated from Muc[+] and Mu*cts*62 respectively, are independent isolates of the same mutation. A Mu*cts*62*vir*3051pAp1 phage was isolated by recombination between Mu*cts*62pAp1 and λJV320. It was considered identical to the Mu*cts*62*vir*3061pAp1 phage.

© Oxford University Press

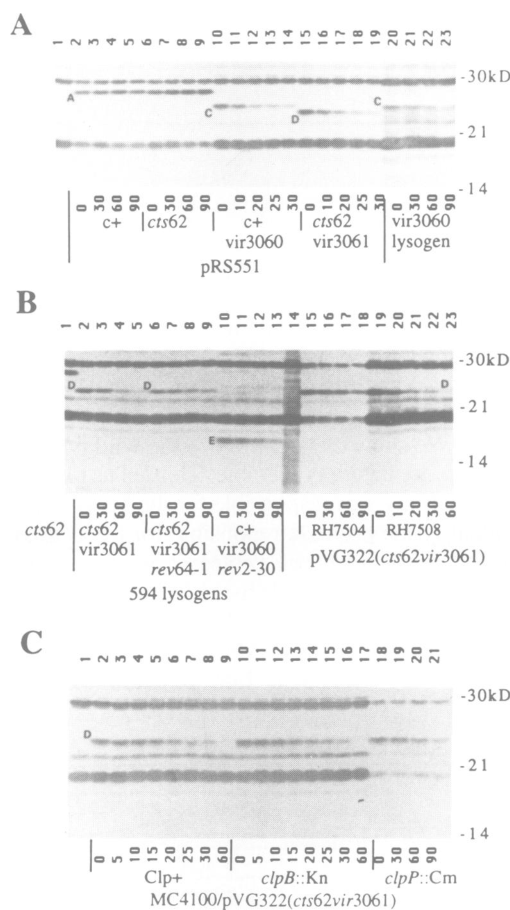

Fig. 2. Stability of different forms of Mu repressor. Western blot analysis was performed as described in Materials and methods. The different repressor proteins are identified by capital letters (A, c^+ and *cts*62; C, *vir*3060; D, *vir*3061; E, *vir*3060*rev*2−30) the relative sizes of which can be seen in Figure 1. **A.** Lane 1, 3 μl of 594/pRS551 extract; lanes 2−5, 1 μl 594/pJV300 (c^+ repressor) at times 0, 30, 60 and 90 min after addition of spectinomycin; lanes 6−9, 1 μl of 594/pJV304 (*cts*62 repressor) extract at times 0, 30, 60 and 90 min after Spc addition; lanes 10−14, 3 μl of 594/pVG321 (*vir*3060 repressor) extract at times 0, 10, 20, 25 and 30 min after Spc addition; lanes 15−19, 3 μl of 594/pVG322 (*cts*62*vir*3061 repressor) extract at times 0, 10, 20, 25 and 30 min after Spc addition; lanes 20−23, 15 μl of 594−2−42(Mu*vir*3060pAp1) extract at times 0, 30, 60 and 90 min after Spc addition. **B.** Lane 1, 15 μl of 594(Mu*cts*62pAp1) extract; lanes 2−5, 5 μl of 594−52−3(Mu*cts*62*vir*3061pAp1) at times 0, 30, 60 and 90 min after addition of Spc; lanes 6−9, 5 μl of 594(Mu*cts*62*vir*3061*rev*64−1pAp1) extract at times 0, 30, 60 and 90 min after addition of Spc; lanes 10−13, 5 μl 594(Mu*vir*3060*rev*2−30pAp1) extract at times 0, 30, 60 and 90 min after addition of Spc; slot 14, 15 μl of RH7504/pRS551 extract; lanes 15−18, 1.5 μl of RH7504/pVG322 (*cts*62*vir*3061 repressor) at times 0, 30, 60 and 90 min after addition of Spc; lanes 19−23, 15 μl of RH7508/pVG322 extract at times 0, 10, 20, 30 and 60 min after Spc addition. **C.** Lane 1, 15 μl of MC4100/pRS551 extract; Lanes 2−9, 15 μl of MC4100/pVG322 extract at times 0, 5, 10, 15, 20, 25, 30 and 60 min after Spc addition; lanes 10−17, 15 μl of RH7183/pVG322 (*clpB::Kn*) extract at times 0, 5, 10, 15, 20, 25, 30 and 60 min after Spc addition; lanes 18−21, 1.5 μl of RH7182/pVG322 (*clpP::Cm*) extract at times 0, 30, 60 and 90 min after Spc addition. Note that for stable proteins, sampling times ran from 0−90 min after the arrest of translation while for unstable proteins it ran from 0 to only 30 or 60 min after antibiotic addition. The higher backgrounds in lanes 20−23 in panel A and lanes 14 and 19−23 in panel B, is due to the larger amount of total extract loaded on the gels to allow for the visualization of the unstable repressor.

degradation of the *E.coli* proteins SulA and RcsA (Mizusawa and Gottesman, 1983; Torres-Cabassa and Gottesman, 1987) as well as that of phage λ N protein (Maurizi, 1987). The Prc (or Tsp) protease has been reported to cleave the C-terminal end of the penicillin-binding protein PBP3 (Hara *et al.*, 1991) and to be involved in the rapid degradation of a bacteriophage λ repressor variant (Silber *et al.*, 1992). We also looked at the effect of the heat shock-induced proteases whose expression is controlled by σ32 (HtpR), the heat shock-specific sigma factor, and of the ATP-dependent Clp protease. The latter (also called Ti) comprises at least two components, the 21.5 kDa ClpP catalytic peptide, which has the proteolytic activity, and the 81 kDa ClpA ATPase regulatory peptide (Hwang *et al.*, 1987, 1988; Katayama-Fujimura *et al.*, 1987; Katayama *et al.*, 1988). Both of these peptides have homologues in eukaryotic cells, suggesting that this type of protease plays an important role in all organisms (Gottesman *et al.*, 1990; Maurizi *et al.*, 1990b). Like Lon (for a review see Gottesman, 1989), ClpP is a heat shock protein (Kroh and Simon, 1990). Moreover ClpA has a HS homologue in *E.coli*, ClpB (Kitagawa *et al.*, 1991; Squires *et al.*, 1991), which is also the homologue of the heat shock eukaryotic chaperonin protein Hsp104 (Parsell *et al.*, 1991; for a review, see Squires and Squires, 1992).

Results

A vir repressor induces a resident Mu prophage

Mu*vir* mutant phages synthesize repressors with a modified C-terminal end as a result of a frameshift mutation in the repressor gene. They grow well in Muc^+ lysogens and efficiently induce the resident prophage. Only the repressor mutation could be detected in Mu*vir* mutants, raising the possibility that expression of the mutated RepVir protein is necessary and sufficient to provide *vir* phages with their dominant phenotype. We tested this hypothesis by infecting Mu lysogens, also immune to infection by the lambdoid phage λi21, with λJV320, λVG321 and λVG322 phages. These λRS45 derivatives respectively carry a cloned copy of the Mu *vir*3051, *vir*3060 and *cts*62*vir*3061 gene under the control of the genuine *c* gene promoter pCM and no other Mu gene (Geuskens *et al.*, 1991). Under the conditions used, these superinfecting phages synthesize only the λi21 repressor and one or the other Mu RepVir protein. Induction of the resident Mu prophage was assessed by the amount of Mu phages produced titrated on lawns of Mu-sensitive bacteria lysogenic for λi21. Mu lysogens produced $\sim 10^{-3}$ Muc^+ phage/bacterium by spontaneous induction and after infection with either λRS45 or the same phage with a wild type repressor gene (λJV300). Cultures infected with λJV320, λVG321 and λVG322 (which synthesize *vir*3051, *vir*3060 and *cts*62*vir*3061 repressor respectively), produced 10 000-fold more phage (10−20 Muc^+ phage/bacterium). Thus RepVir protein induces a resident Mu prophage.

RepVir proteins are unstable in vivo

We tested the stability of the RepVir3060 and RepVir3061 proteins in strains containing the pVG321 or pVG322 plasmid, which synthesize repressor from the genuine repressor promoter pCM. Spectinomycin (Spc) was added to exponential phase cultures to block translation and aliquots were withdrawn from the cultures at various times thereafter.

Total proteins were extracted, separated by PAGE and blotted onto nitrocellulose membranes. Repressor was identified by reaction with a polyclonal antibody raised against the purified wild type protein (see Materials and methods for details). As shown in Figure 2, RepVir3060 and RepVir3061 rapidly disappeared, while the amount of wild type and Ts62 proteins detected was unchanged up to 90 min after the addition of Spc. The half-life of RepVir proteins, as measured from this type of experiment, was between 10 and 20 min.

Is virulence correlated with repressor instability?

Mu*vir* phages containing a selective marker such as ampicillin resistance (Ap^R), were used to select for rare lysogens. These fell into two classes (i) those that contain and produce *vir* phages and thus most probably carry a host mutation that suppresses the *vir* mutation and (ii) those that contain and produce non-virulent pseudorevertant phages that synthesize active repressors. Almost all of the second class make repressors shorter than the *vir* protein they derive from (Geuskens *et al.*, 1991; L.Desmet and R.Grimaud, unpublished observations). The fact that stable lysogens for Mu*vir* can be isolated shows that the RepVir proteins can bind operator DNA. We wondered whether the mutant *vir* repressor had recovered stability in such lysogens. The repressor stability was measured for two independently isolated Mu*vir* lysogens and two strains lysogenic for Mu*vir* pseudorevertants (Mu*virrev*). In all the strains tested the repressor had largely regained stability (see Figure 2A, lanes 20–23 and 2B, lanes 2–13). This result supports a causal relationship between the instability of the RepVir protein and the virulent phenotype of the Mu*vir* phage. RepVir protein synthesized upon infection of a Muc^+ lysogen may induce the degradation of the WT repressor present in the infected lysogen, resulting in the observed induction of the resident prophage. The data also suggest that Mu*vir* phage may lysogenize bacteria that are defective in the protease that degrades the RepVir proteins, and hence that the host mutants of the first class mentioned above could be deficient for that particular protease.

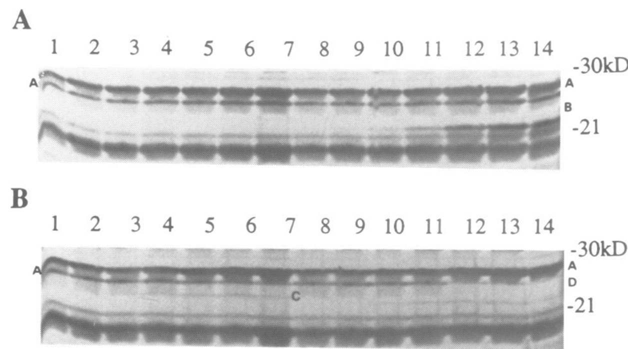

Fig. 3. *Trans*-dominance of *vir* repressor instability. Infections with λRS45 derivatives, protein extracts preparations and separation by PAGE as well as Western blot analysis were performed as described in Materials and methods and in the text. **A.** Lanes 1–7, 594(λJV300) (wild type repressor) infected with the parental phage λRS45, at 0, 5, 10, 15, 30, 45 and 60 min after infection; lanes 8–14, 594(λJV300) (wild type repressor) infected with λJV313 (*cts62sts62–1* repressor), at 0, 5, 10, 15, 30, 45 and 60 min after infection. **B.** Lanes 1–7, 594(λJV300) (wild type repressor) infected with λVG321 (*vir3060* repressor), at 0, 5, 10, 15, 30, 45 and 60 min after infection; lanes 8–14, 594(λJV300) infected with λVG322 (*cts62vir3061* repressor), at 0, 5, 10, 15, 30, 45 and 60 min after infection. As in Figure 2, the different repressors are identified by capital letters (A, c^+; B, *cts62sts62–1*; C, *vir3060*; D, *vir3061*).

The instability of the RepVir proteins is trans-dominant

To look at the effect of RepVir synthesis on the stability of wild type repressor, a strain lysogenic for a λRS45 derived phage carrying the wild type Mu *c* gene was superinfected at a multiplicity of infection (m.o.i) of 3–5 with equivalent phage carrying either the *vir*3060, the *vir*3051 or the *cts*62*vir*3061 gene. The parent vector and phages with the *cts*62*sts*62–1 gene, which allows for a clear distinction between repressors synthesized by the prophage and superinfecting phage due to the smaller size of the Sts protein, were used as controls. Aliquots were removed at various times and treated as described above. As shown in Figure 3, the superinfecting phages synthesized Mu repressor despite the presence of the WT repressor in the infected lysogen. Because infected cells continued to grow after infection, the amount of wild type and Sts repressor synthesized by the prophage and the superinfecting phage increased with time (Figure 3A, lanes 1–7 and 8–14). However, by 30 min after infection with λVG322 (*cts*62*vir*3061; Figure 3B, lanes 8–14) and λJV320

Table I. Lysogenization of Mu*vir* phages on different *E.coli clp* mutant strains

	Frequencies of lysogenization				Repvir3061 stability at 30°C
	Mu*cts*62pAp1		Mu*cts*62*vir*3051pAp1		
	30°C	42°C	30°C	42°C	
MC4100	1.8×10^{-2}	$<4.6\times10^{-7}$	$<3.2\times10^{-6}$	$<3.2\times10^{-6}$	–
RH7189 (*clpA*)	8.5×10^{-3}	$<4.3\times10^{-7}$	$<2.2\times10^{-6}$	$<2.2\times10^{-6}$	–
RH7183 (*clpB*)	2.1×10^{-2}	$<3.3\times10^{-7}$	$<2.4\times10^{-6}$	$<2.4\times10^{-6}$	–
RH7190 (*clpA,clpB*)	1.2×10^{-2}	$<4.0\times10^{-7}$	$<2.5\times10^{-6}$	$<2.5\times10^{-6}$	–
RH7182 (*clpP*)	2.2×10^{-2}	3.1×10^{-5}	4.9×10^{-2}	1.8×10^{-5}	+
RH7508	8.9×10^{-2}	$<5.3\times10^{-7}$	$<2.0\times10^{-6}$	$<2.0\times10^{-6}$	–
RH7504 (*clpP*)	2.5×10^{-2}	3.0×10^{-5}	7.2×10^{-2}	1.9×10^{-5}	+

Lysogenization frequencies were measured as described in Materials and methods. <, no colonies found on LB/Amp; –, half-life ~20 min (see Figure 2); +, stable in the conditions used.

Table II. Bacterial strains, phages and plasmids

Strain	Genotype	Reference or source
Bacteria		
N99	*rpsL,galK,lacZ*	Gottesman and Yarmolinsky (1968)
MP1492	N99,*hflA*::Tn*5*	M.Pato
AB1157	*thr-1,ara-14,leuB6,*Δ*(gpt-proA)62,lacY1,tsx-33, supE44,GalK2,*λ^-*,rac,hisG4(Oc),rfbD1,mgl5-1, rpsL31,kdgK51,xyl-5,mtl-1,argE3,thi-1*	Howard-Flanders (1964)
GC4364	AB1157,*lon*	Howard-Flanders (1964)
SG12049	MC4100,*clpA*182::ΔTn*10*	S.Gottesman
SG22030	MC4100,Δ*lon*,Δ*clpA*,Δ*gal*,*clpB*::Δkn,*clpP*::*Cm*	S.Gottesman
MC4100	*araD139*,Δ*(lacIPOZYA,argF)*U169,*fla,relA,rpsL*	Casadaban (1976)
RH7182	*clpP::Cm* from SG22030 transduced by P1 in MC4100	this work
RH7183	*clpB::Kn* from SG22030 transduced by P1 in MC4100	this work
RH7189	*clpA*::ΔTn*10* from SG12049 transduced by P1 in MC4100	this work
RH7190	*clpA*::ΔTn10 from SG12049,*clpB::Kn* from SG22030 transduced by P1 in MC4100	this work
KY1431	MC4100,*rpoH*15,*zhf*50::Tn*10*	Tobe *et al.* (1984)
CSH41	Δ(pro,lac),*galE,thi,*F*lacI,P,proA,*$^+B^+$	Miller (1972)
RH7508	CSH41 cured from F′	
RH7504	RH7508 *clpP*, spontaneous mutant of RH7508	this work
UGM247	MC4100,*hupB*::kn	this laboratory
HfrH	*azi-7,relA1,rpsL100,spoT1,metB1*	Hayes (1953)
BW6158	HfrC*leu63*::Tn*10,relA1,tonA22,ompF627*	Wanner (1986)
JE7924	W3110	Hara *et al.* (1991)
JE7925	JE7924,*prc*7304	Hara *et al.* (1991)
594	*rpsL,galK,galT,lacZ*	Appleyard (1954)
594−2−42 (Mu*vir*3060pAp1)	selection of ApR colonies after infection of 594 with Mu*vir*3060pAp1	Geuskens *et al.* (1991)
594−52−3 (Mu*cts*62*vir*3061pAp1)	selection of ApR colonies after infection of 594 with Mucts62vir3061pAp1	Geuskens *et al.* (1991)
C600	*thr,leu,tonA,lacY,thi,supE*	Campbell (1961)
C600(λi21*c*$^+$)(Mu*c*$^+$)		this work
C600(Mu*c*$^+$Gov3251)		van Vliet *et al.* (1978)
Phages		
λc^+		Thomas (1966)
λ3B6	λEMBL4 containing *clpP, lon* and *hupB*	Kohara *et al.* (1987)
λRS45	λi21*c*$^+$derivative	Simons *et al.* (1987)
λJV300	λRS45 with *c*$^+$pE::*lacZ* fusion	Vogel *et al.* (1991)
λJV304	λRS45 with *cts* 62pE::*lacZ* fusion	Vogel *et al.* (1991)
λJV313	λRS45 with *cts*62,*sts62-1*,pE::*lacZ* fusion	J.L.Vogel and N.P.Higgins
λJV320	λRS45 with *vir*3051,pE::*lacZ* fusion	Geuskens *et al.* (1991)
λVG321	λRS45 with *vir*3060,pE::*lacZ* fusion	Geuskens *et al.* (1991)
λVG322	λRS45 with *cts*62*vir*3061,pE::*lacZ* fusion	Geuskens *et al.* (1991)
Mu*c*$^+$		Taylor (1963)
Mu*cts*62pAp1		Leach and Symonds (1979)
Mu*vir*3051		van Vliet *et al.* (1978)
Mu*cts*62*vir*3051pAp1	Recombinant between λJV320 and Mu*cts*62pAp1	this work
Mu*vir*3060		Geuskens *et al.* (1991)
Mu*vir*3060pAp1		Geuskens *et al.* (1991)
Mu*cts*62*vir*3061		Geuskens *et al.* (1991)
Mu*cts*62*vir*3061pAp1		Geuskens *et al.* (1991)
Mu*vir*3060*rev*2−30pAp1		Geuskens *et al.* (1991)
Mu*cts*62*vir*3061*rev*64−1pAp1		Geuskens *et al.* (1991)
Plasmids		
pRS551	pBR322 derivative	Simons *et al.* (1987)
pJV320	pRS551*attL-Hin*dIII Mu*vir*3051	Geuskens *et al.* (1991)
pVG321	pRS551*attL-Hin*dIII Mu*vir*3060	Geuskens *et al.* (1991)
pVG322	pRS551*attL-Hin*dIII Mu*cts*62*vir*3061	Geuskens *et al.* (1991)
pWPC21	*Nru*I *clpP* fragment inserted in pBR322	W.P.Clark and S.Gottesman

(*vir*3051; data not shown), the WT repressor was almost undetectable as was RepVir. Infection with λVG321 (*vir*3060; Figure 3B, lanes 1−7) also resulted in the destabilization of the resident WT repressor, but to a lesser extent: it only prevented its accumulation. The same level of destabilization of wild type repressor was found after superinfection of Muc$^+$ lysogens with Mu*vir* phages (data not shown).

Clp protease degrades RepVir repressor

To characterize the protease involved in RepVir degradation, several well characterized protease deficient *E.coli* mutants were tested for RepVir stability and the ability of Mu*vir* phages to lysogenize. In addition, a spontaneous mutation that allows an *E.coli* strain to be efficiently lysogenized by Mu*vir* phages was mapped.

RepVir3060 protein stability was unaltered in *lon*, *hflA*, *prc* or *htpR* (at 42°C) strains and these could not be lysogenized with Mu*cts*62*vir*3051pAp1 (data not shown; for detailed genotype of the strains used, see Table II). In contrast, RepVir proteins were stable in RH7182 (MC4100*clpP*::*Cm*; see Figure 2C, lanes 18–21), but not in *clpA*, *clpB* or *clpA,clpB* isogenic strains (RH7189, RH7183, and RH7190; Figure 2C, lanes 10–17 for the *clpB* strain; other data not shown). As shown in Table I, at 30°C, Mu*cts*62*vir*3051pAp1 lysogenized the *clpP* strain and the frequency of appearance of lysogens was the same as that obtained with a Mu*cts*62pAp1 phage at the same temperature. On *clpA*, *clpB* or *clpA,clpB* hosts the *vir* mutant did not lysogenize even at 30°C while Mu*cts*62pAp1 did. The Mu*cts*62*vir*3051pAp1 lysogens obtained were inducible at 42°C where they produced normal burst size (50–100 phage/bacterium) consisting of *vir* phages only.

At 42°C, after infection of the *clpP* host with either Mu*cts*62*vir*3051pAp1 or Mu*cts*62pAp1, the Ap^R colonies were more abundant than expected. This may have resulted from an increased stability of the *cts62* repressor at 42°C in the absence of the ClpP peptide. In an experiment similar to that presented in Figure 2, where the proteins analysed were overproduced, the *cts*62 protein was as stable at 42°C than at 30°C, even in a Clp^+ strain. However, when repression by the *cts*62 repressor was measured at the same temperature by assaying β-galactosidase activity in a Clp^+ and a *clpP* strain lysogenic for λJV304 (in which the Mu pE promoter is fused to the *lacZ* gene), repression was somewhat increased in the *clp* background. Thus, the *cts*62 repressor may itself be a poor substrate for the Clp protease (data not shown). The fact that mini-Mu phages carrying the *cts*62 gene show a reduced transposition frequency in *clp* hosts (J.Shapiro, submitted for publication) is consistent with such an assumption. Muc^+*vir*3051pAp1 behaved as its *cts*62 counterpart except that it lysogenized at the same frequency at 30°C and 42°C and that the lysogens obtained were not temperature inducible (data not shown).

The involvement of the ClpP protease in RepVir degradation was confirmed through the characterization of RH7504, a spontaneous mutant isolated from RH7508. RH7504 is lysogenized by Mu*vir* phages at a frequency of ~1% (see Table I) and RepVir proteins are stable in that strain (see Figure 2B, lanes 15–18).

When a Nal^R derivative of RH7504 (*Δpro,lac*) was used as a recipient in matings with an HfrH and an HfrC*leu*::Tn*10* as donors, 15/36 (i.e. 42%) of the Nal^R,Pro^+ transconjugants resulting from a mating with HfrH had lost the ability to be lysogenized by Mu*vir* as did 30/36 (i.e. 84%) of the Nal^R,Tc^R transconjugants resulting from the mating with HfrC. This located the RH7504 mutation to a point between the HfrH and HfrC origins of transfer, i.e. near the *clpP*, *lon* and *hupB* genes, around 10 min on the *E.coli* map (Figure 4). To refine the mapping, a P1 lysate grown on MC4100*hupB*::*Kn* (UGM247) was used to transduce the Kn^R marker in RH7504. The Kn^R transductants were tested for the ability to be lysogenized by Mu*vir*. 63% had lost

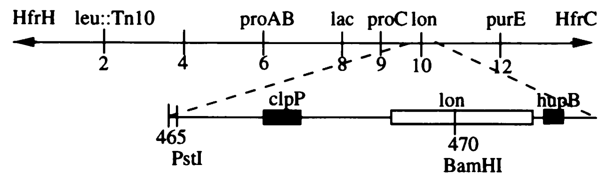

Fig. 4. *E.coli* chromosomal map in the *leu-purE* region. The upper part shows the region of the *E.coli* chromosome included between the HfrH and HfrC origins of transfer (which are indicated by arrows) calibrated in min. The lower part shows details of the *clpP–hupB* region calibrated in kilobase pairs with the positions of one *Pst*I and one *Bam*HI restriction sites. Only the markers discussed in the text are indicated.

that property confirming strong linkage between the RH7504 mutation and *hupB*. Using a helper λc^+ phage, RH7504 was lysogenized with the Kohara phage λ3B6 (which covers the *clpP-hupB* region; Maurizi *et al.*, 1990a). For that purpose a *hupB*::*Kn* mutation was recombined on λ3B6 so that Kn^R could be used as a selective marker for the isolation of the lysogens. These recombinants could not be lysogenized by Mu*vir*, showing that the mutation was recessive and could be complemented. Transformation of RH7504 with the pWPC21 plasmid, which carries a cloned copy of only the *clpP* gene, confirmed the location of the RH7504 mutation in *clpP* since the transformants had lost the ability to be lysogenized by Mu*vir* (data not shown).

Lysogens derived from RH7508 and RH7504, which carried a single Muc^+ prophage in the *malB* region were isolated and superinfected with Mu*vir*. As expected, the superinfecting phage grew and induced the prophage in RH7508, but did not grow in RH7504 (data not shown). Thus virulence is abolished in a *clpP* background.

Discussion

The experiments described above show that the mutant *vir* repressors of phage Mu are naturally unstable (half-life of <20 min versus >90 min for the wild type protein) and, more importantly, can trigger the degradation of wild type repressor. The loss of virulence by Mu*vir* phages is, in all cases analysed, correlated with a significant recovery of repressor stability. Pseudorevertants of Mu*vir* phages selected for their ability to lysogenize (i.e. to produce an active repressor) usually synthesize shortened repressors (Geuskens *et al.*, 1991). They were now shown to be stable. Moreover, bacterial mutations in the *clpP* protease suppressed virulence and stabilized Vir repressors. Therefore ClpP-induced degradation of Vir repressor and the triggering of wild type repressor degradation by these mutant proteins should constitute at least one crucial step of the molecular mechanism through which Mu*vir* phages escape Mu immunity and induce the resident prophage upon infection of a Mu lysogen. Mu virulence is thus a post-translational regulatory mechanism that involves two forms of the same protein, demonstrating that mutant proteins which become hyper-sensitive to proteolysis can destabilize a wild type homologue and confer a *trans*-dominant phenotype.

Although the active oligomeric form of Mu repressor is not yet known, repressor–operator interactions are very cooperative (Vogel *et al.*, 1991). The *trans*-effect of Vir repressors could be accounted for if Vir or mixed Vir–wild type and Vir–Ts oligomers were good substrates for the Clp protease, while pure wild type and Ts oligomers were

not. Further characterization of ClpP specificity and an *in vitro* proteolytic assay on various oligomeric forms of mutant and wild type Mu repressor should allow confirmation for that hypothesis.

The present results raise interesting questions about the activity of the Clp protease. As far as we can tell, ClpA and its HS homologue ClpB are not involved in Vir repressor degradation (see Table II and Figure 2C). In the case described here, either ClpP acts alone or it associates with another yet unidentified subunit which may or may not be similar to ClpA and ClpB. We isolated several independent *E.coli* K12 mutant strains that can be lysogenized by Mu*vir*. Preliminary complementation tests indicate that they are all mutants in the ClpP region (data not shown). Thus we have no evidence for a new Clp subunit but we cannot exclude the possibility that a ClpA/B analogue could be essential for growth or have escaped detection. *In vitro* proteolytic assay may show whether ClpP is sufficient for degradation.

Vir repressors differ from the wild-type and Ts at their C-terminal end, but should have the same N-terminal residue, the nature of which is an open question. Purified repressor, which was isolated from an overproducing strain, has an N-terminal Met (K.Mizuuchi, unpublished result). This however could result from the saturation of the protease that normally removes it. The second residue that would be exposed upon cleavage of the N-terminal Met is a Lys. The Clp protease has been implicated in the rapid degradation of proteins that have Arg, Lys, Phe, Leu, Trp or Tyr at their N-terminus (Tobias *et al.*, 1991; Varshavsky, 1992). Whether having Met or Lys, the N-terminal residue is most probably the same in *vir* and WT repressors so that in any case one of the two proteins would not simply conform to that rule. Vir proteins can trigger wild type protein degradation, so some particular conformation of the oligomers is most likely to be the important recognition signal for proteolysis. Mu repressor proteins should obviously be good tools to find the rules of recognition and activation of the Clp protease.

Materials and methods

Strains

Bacterial strains, phages and plasmids are listed in Table II. MupAp1 phages contain ~1 kb of Tn*3* 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.

Kanamycin (Kn, 20 μg/ml), ampicillin (Ap, 25 μg/ml for selection of MupAp1 lysogens, 50 μg/ml for selection of plasmids), tetracyclin (Tc, 12.5 μg/ml) and chloramphenicol (Cm, 25 μg/ml) were included when appropriate. Minimal medium was 132 (Glansdorff, 1965) supplemented with 0.2% glucose. 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 0.7% LA agar on LA plates.

General procedures

Lysates of Mu*cts*62 were prepared by thermal induction of a lysogen. Lysates of Mu*vir* mutants were prepared by infecting C600 on LA plates at 37°C, as described (Bukhari and Ljungquist, 1977). Lysates of λRS45 and its derivatives were prepared as described by Arber *et al.* (1983). The Hfr matings were as described by Miller (1972) as well as P1 transductions (Miller, 1972). Transformation of bacterial strains with plasmid DNA was as described by Maniatis *et al.* (1982).

Infections with Mu

1 ml of bacterial culture in LB, in exponential phase ($2-3 \times 10^8$ bacteria/ml), was mixed with phages at an m.o.i. of 3–5 in the presence of 10 mM $CaCl_2$. After 15 min adsorbtion at 37°C (or 30°C), the unadsorbed phages were removed by dilution with 5 ml 10 mM $MgSO_4$ and centrifugation. The bacterial pellet was resuspended in 1 ml LB, diluted 100-fold in LB and the dilutions were incubated at 37°C for 90 min or at 30°C for 120 min. The phage suspensions were sterilized by adding a few drops of $CHCl_3$ and titrated on C600 to titrate total phage and C600(Muc$^+$Gov3251) to titrate *vir* phage.

Infections with λ

Infections with λRS45 and its derivatives were performed in the same way except that bacteria were grown in LB supplemented with 1% maltose and the step of elimination of unadsorbed phages was omitted. The time at which phages were added to the bacterial culture was considered as time 0. 1 ml aliquots were withdrawn at 0, 5, 10, 15, 30, 45 and 60 min after infection and treated as described below.

Lysogenization frequencies

Lysogenization by different MupAp1 derivatives was assayed by spotting 10 μl of serial dilutions of phage lysates on LA plates with and without Ap and seeded with appropriate lawns. After overnight incubation at 30°C or 42°C, the frequency of lysogenization was calculated as the ratio between the titre of Ap^R colonies obtained on the LA + Ap plates incubated at 30°C or 42°C and the phage titres obtained on the same strain on LA plates at 42°C.

Tests for repressor stability

Bacteria were grown in LB at 30°C up to a concentration of 3×10^8 bacteria/ml. Spectinomycin was added at a final concentration of 100 μg/ml. Crude extracts were prepared from 1 ml aliquots withdrawn from the culture at various times thereafter and analysed by Western blotting with a polyclonal antibody raised against purified wild type Mu repressor (Vogel *et al.*, 1991) as described by Geuskens *et al.* (1991). Superinfections of strains lysogenic for λRS45 derivatives with λRS45 derivatives were performed as described in the previous section. 1 ml aliquots were withdrawn at different times after infection and crude extracts prepared and analysed following exactly the same procedure. Repressor half-life was estimated by scanning the Western blots using a Videoscope CCD-200E camera and the NIH-Image software on a MacintoshII computer.

Acknowledgements

We most specially thank S.Gottesman who provided us with all the *clp* mutant strains. Discussions with N.P.Higgins, M.Bétermier, M.Chandler, R.Kahmann and D.Sherratt were very stimulatory. H.Hara kindly sent us the *prc* strain. The present work was supported by the EEC Stimulation Program (SCI-0143-C) and by a collaborative grant from the Belgian Fonds de la Recherche Scientifique Medicale to AT and from NSF to N.P.Higgins and M.Pato. V.G. was a fellow from the 'Institut de Recherche Scientifique pour l'Industrie et l'Agriculture' and AMA from the Government of Morocco. A.T. is 'Maître de Recherche' from the Fonds National de la Recherche Scientifique (Belgium).

References

Appleyard,R.K. (1954) *Genetics*, **39**, 429–439.

Arber,W., Enquist,L., Hohn,B., Murray,N.E. and Murray,K. (1983) In Hendrix,R.H., Roberts,J.W., Stahl,F.W. and Weisberg,R.A. (eds), *Lambda II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 433–466.

Bukhari,A.I. and Ljungquist,E. (1977) In Bukhari,A.I., Shapiro,J.A. and Adhya,S.L. (eds), *DNA Insertion Elements, Plasmids and Episomes*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 749–756.

Campbell,A. (1961) *Virology*, **14**, 22–32.

Casadaban,M.J. (1976) *J. Mol. Biol.*, **104**, 541–555.

Gama,M.J. and Toussaint,A. and Pato,M.L. (1990) *Mol. Microbiol.*, **4**, 1891–1897.

Geuskens,V., Vogel,J.L., Grimaud,R., Desmet,L., Higgins,N.P. and Toussaint,A. (1991) *J. Bacteriol.*, **173**, 6578–6585.

Glansdorff,N. (1965) *Genetics*, **51**, 167–179.

Gottesman,M. and Yarmolinsky,M. (1968) *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 735–741.

Gottesman,S. (1989) *Annu. Rev. Genet.*, **23**, 163–198.

Gottesman,S. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3513–3517.

Gussin,G.N., Johnson,A.D., Pabo,C.O. and Sauer,R.T. (1983) In Hendrix,R.H., Roberts,J.W., Stahl,F.W. and Weisberg,R.A. (eds), *Lambda II*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 93–121.
Hara,H., Yamamoto,Y., Higashitani,A., Suzuki,H. and Nishimura,Y. (1991) *J. Bacteriol.*, **173**, 4799–4813.
Hayes,W. (1953) *Cold Spring Harbor Symp. Quant. Biol.*, **18**, 75–93.
Howard-Flanders,P. (1964) *Genetics*, **49**, 237–246.
Hoyt,M.A., Knight,D.M., Das,A., Miller,H.I. and Echols,H. (1982) *Cell*, **31**, 565–573.
Hwang,B.J., Park,W.J., Chung,C.H. and Goldberg,A.L. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5550–5554.
Hwang,B.J., Woo,K.M., Goldberg,A.L. and Chung,C.H. (1988) *J. Biol. Chem.*, **263**, 8727–8734.
Jacob,F. and Wollman,E.L. (1954) *Ann. Inst. Pasteur*, **87**, 653–657.
Katayama,Y., Gottesman,S., Pumphrey,J., Rudikoff,S., Clark,W.P. and Maurizi,M.R. (1988) *J. Biol. Chem.*, **263**, 15226–15236.
Katayama-Fujimura,Y., Gottesman,S. and Maurizi,M.R. (1987) *J. Biol. Chem.*, **262**, 4477–4485.
Kitagawa,M., Wada,C., Yoshida,S. and Yura,T. (1991) *J. Bacteriol.*, **173**, 4247–4253.
Kohara,Y., Akiyama,K and Isono,K. (1987) *Cell*, **50**, 495–708.
Kroh,H.E. and Simon,L.D. (1990) *J. Bacteriol.*, **172**, 6026–6034.
Leach,D. and Symonds,N. (1979) *Mol. Gen. Genet.*, **172**, 179–184.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Maurizi,M.R. (1987) *J. Biol. Chem.*, **262**, 2696–2703.
Maurizi,M.R., Clark,W.P., Katayama,Y., Rudikoff,Z.S., Pumphrey,J., Bowers,B. and Gottesman,S. (1990a) *J. Biol. Chem.*, **265**, 12536–12545.
Maurizi,M.R., Clark,W.P., Kim,S.-H. and Gottesman,S. (1990b) *J. Biol. Chem.*, **265**, 12546–12552.
Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mizusawa,S. and Gottesman,S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 358–362.
Parsell,D.A., Sanchez,Y., Stitzel,J.D. and Lindquist,S. (1991) *Nature*, **353**, 270–273.
Poteete,A.R. (1987) In Calendar,R. (ed.), *The Bacteriophages*. Plenum Press, pp. 647–682.
Silber,K.R., Keiler,K.C. and Sauer,R.T. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 295–299.
Simons,R.W., Houman,F., and Kleckner,N. (1987) *Gene*, **53**, 85–96.
Squires,C. and Squires,C.L. (1992) *J. Bacteriol.*, **174**, 1081–1085.
Squires,C.L., Pedersen,S., Ross,B.M. and Squires,C. (1991) *J. Bacteriol.*, **173**, 4254–4262.
Taylor,A.L. (1963) *Proc. Natl. Acad. Sci. USA*, **50**, 1043–1051.
Thomas,R. (1966) *J. Mol. Biol.*, **22**, 79–95.
Tobe,T., Ito,K. and Yura,T. (1984) *Mol. Gen. Genet.*, **195**, 10-16.
Tobias,J.W., Schrader,T.E., Rocap,G. and Varshavsky,A. (1991) *Science*, **254**, 1374–1376.
Torres-Cabassa,A.S. and Gottesman,S. (1987) *J. Bacteriol.*, **169**, 981–989.
van Vliet,F., Couturier,M., Desmet,L., Faelen,M. and Toussaint,A. (1978) *Mol. Gen. Genet.*, **160**, 195–202.
Varshavsky,A. (1992) *Cell*, **69**, 725–735.
Vogel,J.L., Zhu,J.-L., Howe,M.M., Toussaint,A. and Higgins,N.P. (1991) *J. Bacteriol.*, **173**, 6568–6577.
Wanner,B.L. (1986) *J. Mol. Biol.*, **191**, 39–58.
Weigle,J.M., Meselson,M. and Paigen,K. (1959) *J. Mol. Biol.*, **1**, 379–386.
Yarmolinsky,M.B. and Sternberg,N. (1987) In Calendar,R. (ed.), *The Bacteriophages*. Plenum Press, pp. 291–438.

Received on June 1, 1992; revised on August 31, 1992