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Efficiency of the pTF-FC2 *pas* Poison-Antidote Stability System in *Escherichia coli* Is Affected by the Host Strain, and Antidote Degradation Requires the Lon Protease

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The stabilization of a test plasmid by the proteic, poison-antidote plasmid addiction system (*pas*) of plasmid pTF-FC2 was host strain dependent, with a 100-fold increase in stability in *Escherichia coli* CSH50, a 2.5-fold increase in *E. coli* JM105, and no detectable stabilization in *E. coli* strains JM107 and JM109. The lethality of the PasB toxin was far higher in the *E. coli* strains in which the *pas* was most effective. Models for the way in which poison-antidote systems stabilize plasmids require that the antidote have a much higher rate of turnover than that of the toxin. A decrease in host cell death following plasmid loss from an *E. coli lon* mutant and a decrease in plasmid stability suggested that the Lon protease plays a role in the rate of turnover of PasA antidote.

Proteic plasmid stabilization systems have been discovered on a number of plasmids and include the *ccd* system of plasmid F (7), the identical *parD*/*pem* and *kis*/*kid* systems of plasmids R1 and R100 (1, 16, 19), the *parDE* system of plasmids RP4/ RK2 (14) and the *phd*/*doc* system of phage P1 (10). These systems consist of a long-lived toxin which is expressed at low levels and a short-lived, highly expressed antidote (8). On cell division, if a progeny cell fails to inherit the plasmid, it loses the ability to make the shorter-lived and more abundantly produced antidote and is unable to counter the toxic effects of the poison. As a result, plasmid-free cells are killed or their cell division is inhibited, depending on the type of poison-antidote system.

The 12.2-kb mobilizable, broad-host-range plasmid pTF-FC2 (GenBank accession nos. M64981 and M35249 [13]) was originally isolated from *Thiobacillus ferrooxidans*. This natural hybrid plasmid has a replicon clearly related to those of the IncQ plasmids (e.g., R1162 and RSF1010) (4) and a mobilization region with low but clear similarity to those of the IncP plasmids (e.g., R751 and RK2/R68/RP4) (15). Situated within the IncQ-like replicon and between the *repB* and *repA* genes (Fig. 1) is a proteic poison-antidote system named *pas* (for plasmid addiction system). This system is unusual in that it consists of three genes; *pasA* encodes an antidote, *pasB* encodes a toxin (which is bacteriocidal rather than bacteriostatic), and *pasC* encodes a protein that appears to enhance the neutralizing effect of the antidote (17).

Efficiency of the *pas* stability system in *Escherichia coli* is strain dependent. The ability of the pTF-FC2 *pasABC* system to stabilize a heterologous plasmid in *E. coli* JM105 had previously been shown (17) by cloning the *pasABC* genes into the unstable, low-copy-number, test plasmid pOU82 (6). We repeated the stability assays in *E. coli* CSH50 to compare the efficiency of the pTF-FC2 *pas* to those of other poison-antidote systems in a host strain background identical to that used by other workers (9). It was observed that *pas* varied in its ability to act as a plasmid stabilization system depending upon which strain (Table 1) was used as host. Plasmid stability was determined by growing plasmid-containing *E. coli* cells in batch culture for 100 generations without selection in TB (24 g of yeast extract, 12 g of tryptone, and 4 ml of glycerol per 900 ml with 100 ml of sterile 0.17 M KH_2PO_4–0.72 M K_2HPO_4 added immediately before use). Aliquots were taken at 20-generation intervals and grown at 37°C overnight in the absence of selection. One hundred colonies were transferred to Luria agar (LA) plates with plasmid selection (ampicillin [100 μg ml^{-1}], chloramphenicol [30 μg ml^{-1}], or kanamycin [50 μg ml^{-1}], as required), and the percentage survival was used to calculate plasmid loss. The stability assay for pOU82 and derivatives was performed as described above, except that aliquots were plated on LA containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. Plasmid-containing cells form blue colonies, whereas plasmid-free cells are white. At least three stability tests were performed for each strain, and the loss frequency was calculated by the method of Gerdes et al. (6). After 100 generations, a test plasmid containing the *pasABC* genes (pOU-pasABC [Table 1]) was stabilized approximately 2.5-fold in an *E. coli* JM105 host (Table 2). However, in an *E. coli* CSH50 host, the *pas* enhanced plasmid stability about 100-fold. In contrast, the *pas* was ineffective in enhancing the stability of the test plasmid in an *E. coli* JM107 host strain or its *recA* derivative, *E. coli* JM109. Surprisingly, the base level of pOU82 stability in *E. coli* JM109 was 10-fold higher than in strain JM107 (Table 2). The fact that *E. coli* strains JM107 and JM109 are isogenic except for the *recA* gene implies that the *recA* system has an effect on the stability of the pOU82 test plasmid. The reason for this increased stability is unknown, but the finding is similar to the finding that mini-RK2 plasmids were threefold more stable in *E. coli* JM109 than in JM107 (14).

In previous work (17), we showed that inactivation of PasC (through the introduction of a frameshift mutation in the *pasC* gene) resulted in an increase in the toxicity of the PasA-PasB poison-antidote complex (see also Fig. 2). We therefore examined how inactivation of PasC affects the ability of the *pas* to stabilize the test plasmid in different *E. coli* hosts. In *E. coli* JM105 (pOU-pasABC*), plasmid stability was about the same (loss frequency of 3×10^{-2}) as that of the pOU82 test plasmid and considerably less than that of pOU-pasABC (loss fre-

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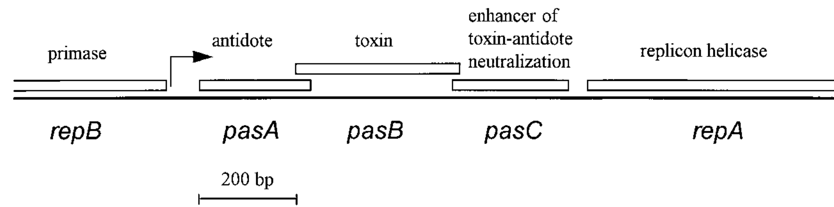

FIG. 1. Layout of the pTF-FC2 *pas* showing its location within the plasmid replicon. The positions of the genes are relative to the *Cla*I site of pTF-FC2 (5).

quency of 9×10^{-3}), whereas in *E. coli* CSH50, inactivation of *pasC* reduced the ability of *pas* to stabilize the test plasmid from about 100- to 2.5-fold. Inactivation of *pasC* had little effect in *E. coli* JM107 or JM109.

Pas toxicity varies between *E. coli* host strains. To investigate the reason for host-strain dependent variation in stability, combinations of the *pas* genes were cloned behind the *tac* promoter of a pKK223-3 vector (Table 1). In *lacI*^{q} strains containing these constructs, *tac*-controlled expression of the *pas* genes is induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Since *E. coli* CSH50 does not contain *lacI*^{q}, the F′ *lacI*^{q}-containing episome was transferred from *E. coli* 71/18 to CSH50 by conjugation. Conjugation was carried out overnight on the surface of a LA plate followed by plating on minimal medium plus streptomycin (100 μg/ml) to select for streptomycin resistance and proline independence. The stability of the test plasmid in this *E. coli* CSH50-I^q strain was indistinguishable from that of the test plasmid in CSH50. The effect of IPTG-induced *pas* gene expression on the growth of *E. coli* host strains JM105, JM107, JM109, and CSH50-I^q is shown in Fig. 2. Not all of the strains were equally sensitive to the PasB toxin. Growth of strain CSH50-I^q was the most severely inhibited by induction of pTac-pasB, with JM105 less inhibited and strains JM107 and JM109 the least inhibited. Expression of the *pasA* gene (encoding the antidote) relieved the toxic effect of *pasB*, although in *E. coli* strain CSH50-I^q, growth inhibition was relieved only slightly. When all three *pasABC* genes were expressed, the growth rates of all strains increased further, although in no strain did the growth rate reach that of the vector control. IPTG-induced expression of the *pasABC* system was toxic to all strains, but toxicity was most severe in *E. coli* CSH50-I^q, which was also the strain in which the *pas* plasmid stabilization system was most effective.

The effects of combinations of *pas* genes expressed under the control of a *tac* promoter on the host strains provided some insight into why *pas*-mediated plasmid stability varied in strains. The lower growth rate and cell density of *E. coli* CSH50-I^q containing different combinations of *pas* genes indicated that in this strain the PasA antidote did not effectively neutralize the toxic effect of PasB toxin even in the presence of PasC. It may be that the greater toxicity of the *pas* in *E. coli* CSH50-I^q was why the test plasmid was best stabilized by *pas* in this strain. Possible reasons for increased PasB toxicity in this strain are that the as yet unidentified cytoplasmic target may be more susceptible to the PasB toxin and that the protease which degrades the antidote may be particularly effective in *E. coli* CSH50-I^q. Variations in the levels of *pas* gene expression between strains may also play a role in the efficiency of plasmid stability, but this is less likely. Since the *pas* is autoregulated (18), differences in *pas* promoter strength between strains

TABLE 1. Bacteria, plasmid vectors, and *pas* constructs used in this study

Strain, plasmid, or construct	Genotype or description[a]	Source or reference
E. coli strains		
JM105	*thi rpsL endA sbcB15 hspR4* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	20
JM107	*thi endA gyrA96 hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	20
JM109	*recA1 thi endA gyrA96 hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	20
71/18	*thi supE* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	20
CSH50	*rpsL* Δ(*lac-pro*)	12
CSH50-I^q	*rpsL* Δ(*lac-pro*) [F′ *traD36 proAB lacI*^{q}ΔM15]	This work
SG22025	Δ*lac rcsA166*::mini-*kan* parent of SG22093 and SG22095	S. Gottesman (11)
SG22093	Δ*lac rcsA166*::mini-*kan clpP1*::*cat*	S. Gottesman (11)
SG22095	Δ*lac rcsA166*::mini-*kan lon-146*::mini-Tn*10*	S. Gottesman (11)
Plasmids		
pACYC184	p15a replicon, Cm^r Tc^r	3
pKK223-3	ColE1 replicon, Ap^r *tac*	2
pOU82	R1 replicon, Ap^r *lacZYA*	6
pKG399	pSC101 replicon, Tc^r (see Fig. 3)	9
pKGCm	pACYC184 replicon, Cm^r (see Fig. 3)	This work
pas-containing constructs		
pTac-pasB	pKK223-3 replicon, Ap^r, *pas* region 1518–1816[b]	17
pTac-pasAB	pKK223-3 replicon, Ap^r *pas* region 1316–1816	17
pTac-pasABC	pKK223-3 replicon, Ap^r *pas* region 1316–2028	17
pOU-pasABC	pOU82 replicon (R1), Ap^r, *pas* region 1158–2027	17
pOU-pasABC*[c]	pOU82 replicon (R1), Ap^r, *pas* region 1158–2027	17

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; *tac*, *trp-lac* hybrid promoter.
[b] The *pas* region numbers are the nucleotide positions relative to those of the *Cla*I-*Pst*I fragment of pTV100 (5).
[c] C* indicates a T insertion at 1833 bp to inactivate the *pasC* product (17).

TABLE 2. Loss frequency of plasmids from *E. coli* host strains after 100 generations of growth

Strain	Loss frequency		Approximate fold increase in stability
	No stability genes present (pOU82)	Stability genes present (pOU-pasABC)	
JM105	2×10^{-2}	9×10^{-3}	2.5
JM107	5×10^{-2}	5×10^{-2}	1
JM109	5×10^{-3}	4×10^{-3}	1
CSH50	2×10^{-2}	3×10^{-4}	100

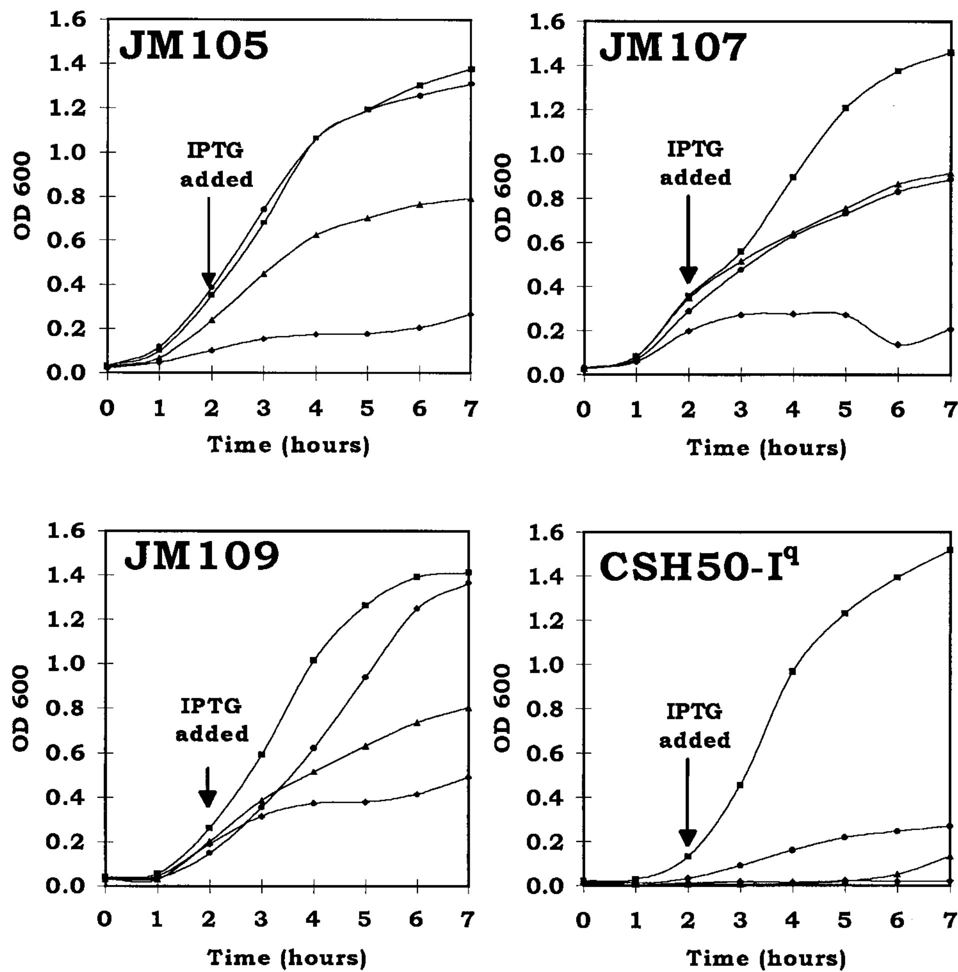

FIG. 2. Growth curves of *E. coli* strains overexpressing the *pas* genes. Each graph shows pKK223-3 (control) (■), pTac-pasB (◆), pTac-pasAB (▲), and pTac-pasABC (●). Datum points are the means of three separate experiments. OD 600, optical density at 600 nm.

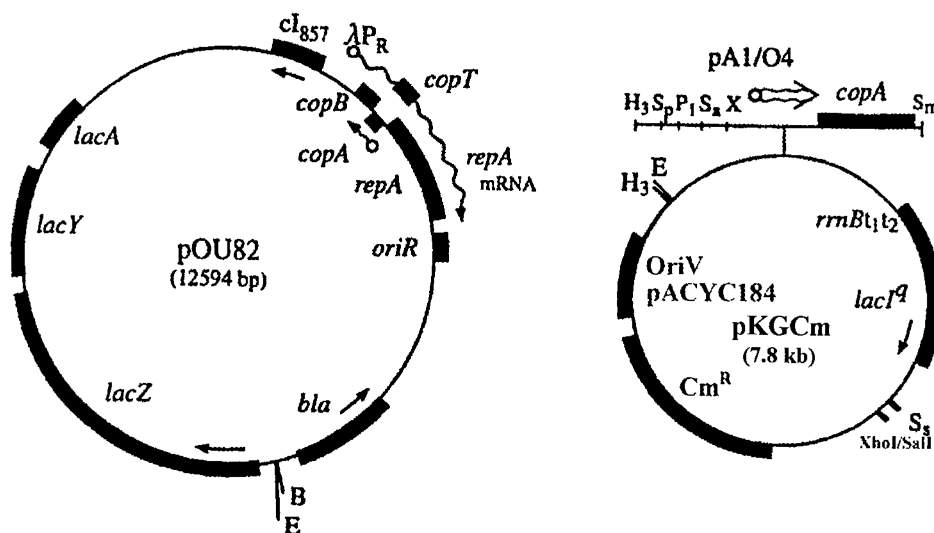

FIG. 3. pOU82-pKGCm conditional replication system based on the pOU82-pKG339 system (modified from reference 9 with permission of the publisher). The pACYC replicon and chloramphenicol resistance markers of pKGCm have replaced the pSC101 replicon and tetracycline resistance marker of pKG339. Addition of IPTG results in expression of *copA* from the pA1/O4 promoter, and CopA inhibits replication of the pOU82 R1 replicon. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; H_3, *Hin*dIII; Sp, *Sph*I; P_1, *Pst*I; Sa, *Sal*I; X, *Xha*I; Sm, *Sma*I; Ss, *Ssp*I.

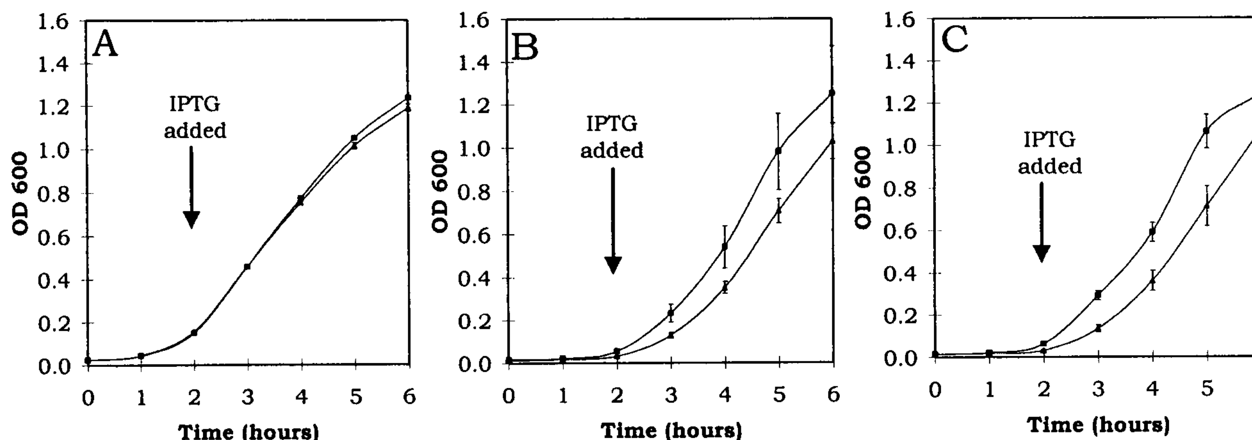

FIG. 4. Growth curves of protease mutants with different plasmids. Growth curves of *E. coli lon* mutant SG22095 (A), *E. coli clpP1* mutant SG22093 (B), and *E. coli* protease mutant parent strain SG22025 (C) containing pOU82 and pKGCm (control) (■) or pOU-pasABC and pKGCm (▲) are shown. OD 600, optical density at 600 nm.

would not be expected to be as important as in a non-self-regulated system. Variations in the rate of transcription would alter the time required to reach self-regulating levels of antidote and toxin but probably not the actual levels reached.

Role of Lon protease in plasmid stabilization. Plasmid proteic stabilization systems operate on the principle that the antidote is subject to a much higher rate of turnover than the toxin (8). A pOU82-pKG339-based conditional replication system (9) was used to determine which *E. coli* protease is involved in the selective degradation of the PasA antidote. Since one of the *E. coli* protease mutants was resistant to tetracycline (Table 1), the tetracycline resistance marker and pSC101 replicon of pKG339 were replaced by the chloramphenicol resistance marker and the p15a replicon of pACYC184 to produce plasmid pKGCm. When the *copA* gene of pKGCm is provided in *trans* to pOU82, replication of pOU82 can be halted by IPTG induction of the pA1/O4 promoter (Fig. 3). Therefore, addition of IPTG to *E. coli* (pOU-pasABC) cells will result in arrest of plasmid replication, and plasmid-free cells containing the toxin-antidote complex will result. The protease responsible for selective degradation of the antidote will digest the antidote, which will no longer inhibit the toxin, resulting in cell death. Functional antidote will persist in *E. coli* mutants deficient in the antidote-degrading protease, and cell death will not occur. Plasmid pOU82 and the *pas*-containing pOU82-based plasmid (pOU-pasABC) were transformed into *E. coli* SG22093 *clpP1* and SG22095 *lon* mutants and into *E. coli* SG22025, a protease-proficient parental strain of these mutants, each of which contained a coresident pKGCm plasmid. The growth of all strains following the addition of 2 mM IPTG is shown in Fig. 4. Growth of the *E. coli* (pOU-pasABC) *lon* mutant strain was similar to that of the same strain containing pOU82 (Fig. 4A), suggesting that in this strain the antidote was long-lived. In contrast, growth of the *E. coli* (pOU-pasABC) *clpP* mutant and *E. coli* (pOU-pasABC) protease-proficient strains was reduced relative to that of the same strains containing the pOU82 control plasmid (Fig. 4B and C). The observation that growth was not reduced by forced plasmid loss in an *E. coli lon* mutant, whereas there was a decrease in growth following the loss of plasmids expressing the *pasABC* genes in *lon*-proficient strains, suggests that the antidote protein was stable in the *lon* mutant and that the Lon protease is involved in the degradation of the PasA antidote.

To obtain additional evidence for the involvement of the Lon protease, the stability of pOU-pasABC was tested in each strain in the absence of pKGCm. Strains in which the protease required for selective degradation of the antidote is not present would cause pOU-pasABC to be less stably inherited. In the *E. coli clpP* mutant and *lon*-proficient parental strains, the pOU-pasABC loss frequencies were 1.5×10^{-2} and 1.4×10^{-2}, respectively, whereas the loss frequency was increased to 3.5×10^{-2} in the *E. coli lon* mutant. This 2.5-fold decrease in plasmid stability seen in the *E. coli lon* mutant strain supports the notion that the Lon protease plays a role in the degradation of PasA. The level of stabilization by the *pas* in the *E. coli lon* parental strain was comparable to that in *E. coli* JM105 but less than that found in *E. coli* CSH50-I^q. This is additional evidence that the Lon protease plays a role in *pas*-mediated plasmid stability.

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