Autoregulation of the pTF-FC2 Proteic Poison-Antidote Plasmid Addiction System (*pas*) Is Essential for Plasmid Stabilization

Anthony S. G. Smith and Douglas E. Rawlings
***J. Bacteriol.* 1998, 180(20):5463.**

JOURNAL OF BACTERIOLOGY, Oct. 1998, p. 5463–5465 Vol. 180, No. 20
0021-9193/98/$04.00+0

Autoregulation of the pTF-FC2 Proteic Poison-Antidote Plasmid Addiction System (*pas*) Is Essential for Plasmid Stabilization

ANTHONY S. G. SMITH AND DOUGLAS E. RAWLINGS*

Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

Received 13 February 1998/Accepted 22 June 1998

The *pasABC* genes of the proteic plasmid addiction system of broad-host-range plasmid pTF-FC2 were autoregulated. The PasA antidote was able to repress the operon 25-fold on its own, and repression was increased to 100-fold when the PasB toxin was also present. Autoregulation appears to be an essential requirement for *pas*-mediated plasmid stabilization because when the *pas* genes were placed behind the isopropyl-β-D-thiogalactopyranoside (IPTG)-regulated *tac* promoter, they were unable to stabilize a heterologous test plasmid.

Plasmid pTF-FC2 is a 12.2-kb, mobilizable, broad-host-range plasmid that was originally isolated from the biomining bacterium *Thiobacillus ferrooxidans* (9). The plasmid contains a proteic poison-antidote plasmid addiction system (*pas*) located between the *repB* and *repA* genes (Fig. 1) of its IncQ-like replicon (4). This stability system is unusual in that it consists of three genes rather than the two-gene systems identified in other plasmids (7). The *pasA* gene encodes an antidote, *pasB* encodes a toxin, and *pasC* encodes a protein that appears to enhance the neutralizing effect of the antidote (12). Autoregulation is a general property of proteic stabilization systems in which regulation has been studied. For example, the *ccd* system of plasmid F is autoregulated by a 69-kDa complex of CcdA and CcdB (14), and neither CcdA nor CcdB alone is capable of autorepression. In contrast, the *parDE* system of plasmid RK2 is autoregulated solely by the ParD antidote protein (10). The *parD* locus of plasmid R1 is repressed only 30 to 40% by Kis on its own, and the complete Kis-Kid complex is required for maximal repression (11).

We investigated whether the *pas* of pTF-FC2 is autoregulated and whether the third component of the *pas*, PasC, plays a role in regulation. Furthermore, we investigated whether autoregulation is a necessary requirement for *pas* stabilization. It is conceivable that differences in the half-lives of the antidote and toxin proteins together with differences in the levels of toxin and antidote translation may by themselves be sufficient to increase plasmid stability. The bacteria, plasmids, and constructs we used in this study are given in Table 1.

Autoregulation of *pasABC*. Regulation of the *pasABC* genes was investigated by the construction of an in-frame translational fusion of *pasA* to a *lacZ* reporter gene. A translational fusion would indicate the cumulative effect of transcriptional and translational regulation. An in-frame translational fusion of *pasA* to a *lacZ* reporter gene was constructed by cloning a PCR amplification product which extended for 124 bp upstream of the *pasA* start into the vector pMC1403. The primers 1212F (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and FP2 (5′-AGTAGGGATCCACTTCGGCGGGCAGTCGG-3′) (shown in Fig. 1), were used to amplify the *pasA* promoter from pTV400 (4). The PCR was carried out by using DynazymeII (Finnenzymes Oy) in a JDI2500 thermocycler (denaturation step of 2 min at 95°C and then 30 cycles, with 1 cycle consisting of 30 s at 95°C, 30 s at 52°C, and 60 s at 72°C). Primer FP2 introduced a *Bam*HI site which allowed in-frame cloning of the fragment into vector pMC1403 to create construct pP2H. DNA sequencing with a Pharmacia ALF express automated DNA sequencer was used to confirm the integrity of the construct. β-Galactosidase assays were performed by the method of Miller (8) on log-phase cultures grown with the appropriate antibiotic selection. The *pas-lacZ* fusion, pP2H, when placed in *Escherichia coli* CSH50-I^q gave moderate levels of β-galactosidase activity (252 Miller units) (Table 2). When plasmid pKmM0, which has the PasABC system situated within its natural, broad-host-range, pTF-FC2 replicon, was placed in *trans* to pP2H, expression of β-galactosidase activity was reduced to 12 Miller units. To identify the repressor of gene expression, β-galactosidase activity was measured in strains in which pKmM0 was replaced by the pKmM0-based *pas* mutant plasmids pKmM1 (*pasA*), pKmM2 (*pasB*), and pKmM3 (*pasC*) (12). However, the pKmM1 *pasA* mutant was lethal to *Escherichia coli* CSH50-I^q, and inactivation of *pasB* or *pasC* relieved the repression of *lacZ* reporter gene expression to a small extent (from 12 to 31 and 14 Miller units, respectively). When two spontaneous pKmM0 *pas* deletion mutants in which the *pasA* promoter region (pKmM1del1) or most of *pasABC* (pKmM1del2) had been deleted (Fig. 1) (12) were placed in *trans* to pP2H, reporter gene expression was restored to near unrepressed levels. This indicated that PasA was the primary repressor.

To confirm regulation by the *pas* gene products, constructs of each of the *pas* genes cloned individually and in combination behind the non-*pas*-regulated *tac* promoter of vector pKK223-3 were used. Since both the pMC1403 reporter gene vector and pKK223-3 use ColE1 origins of replication and both are ampicillin resistant, the *tac*-regulated *pas* genes were subcloned into the pACYC184 vector. *tac-pas* fusions were excised from their respective pKK223-3 constructs as *Pvu*I (blunted)-*Bam*HI fragments and cloned into pACYC184 which had been cut with *Bam*HI and *Cla*I (blunted). These constructs, pTac-pasA-pACYC, pTac-pasB-pACYC, pTac-pasC-pACYC, pTac-pasAB-pACYC, and pTac-pasABC-pACYC were transformed into *E. coli* CSH50-I^q cells containing pP2H. The level of β-

* Corresponding author. Present address: Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa. Phone: 27 21 808 4866. Fax: 27 21 808 3611. E-mail: der@maties.sun.ac.za.

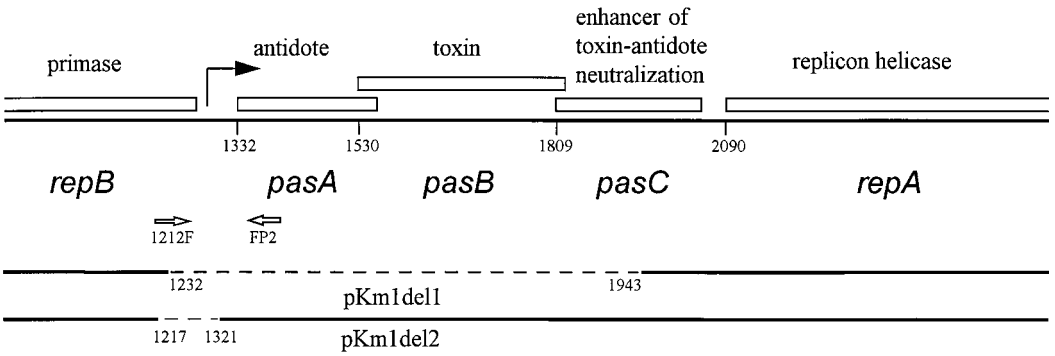

FIG. 1. Layout of the pTF-FC2 *pas* showing its location within the plasmid replicon. Numbers below the thick line indicate the positions of the genes relative to that of the *Cla*I site of pTF-FC2 (5). The positions of the PCR primers used to amplify the *pas* promoter region and the regions missing from the spontaneous deletions following PasA antidote inactivation (12) (broken lines) are shown below the layout.

galactosidase expression decreased from 252 to 10 Miller units when only *pasA* was provided in *trans* (Table 2). This repression was enhanced when *pasAB* (2 Miller units) or *pasABC* (3 Miller units) was present. The *pasABC* promoter therefore appears to be autorepressed (25-fold) by PasA, and this repression increased when both PasA and PasB were present (100-fold). Regulation by *pasB* or *pasBC* could not be tested due to the lethal effects of PasB in the absence of PasA. PasC alone had little effect on the expression of the *pasA* promoter.

Levels of reporter gene activity varied substantially between strains and experiments. Only the data for *E. coli* CSH50-I^q, in which reporter gene activity was less variable than in some of the other strains, are presented. Similar experiments with *E. coli* JM105 showed the same trends, although these data were unreliable because of the greater variability in reporter gene expression (data not shown). PasA was clearly the primary repressor, and this negative regulation was enhanced in the presence of PasB. The pTF-FC2 *pas* is therefore similar to the *par*D locus of R1 in that *parD* is only partially repressed by Kis (30 to 40%) and the complete Kis-Kid complex is required for maximal repression (11).

Effect of expression from a heterologous promoter on *pas* function. To investigate whether autoregulation is important for the proper functioning of the *pas* proteic plasmid stability system, we examined the stability of the pOU82 test plasmid containing the *pasABC* genes under the control of a *tac* promoter (pOU-tac-pasABC) in *E. coli* CSH50-I^q. The *tac-pas* fusions in pOU82 were constructed by excising the *pasABC* genes

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

Strain, plasmid, or construct	Genotype or description[a]	Reference
E. coli strains		
JM105	*thi rpsL endA sbcB15 hspR4* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	15
CSH50-I^q	*rpsL* Δ(*lac-pro*) [F′ *traD36 proAB lacI*^{q}ΔM15]	13
Plasmids		
pMC1403	ColE1 replicon, Ap^r *lacZYA**	2
pACYC184	p15a replicon, Cm^r Tc^r	3
pKK223-3	ColE1 replicon, Ap^r *tac*	1
pOU82	R1 replicon, Ap^r *lacZYA*	6
Constructs containing *pas* genes		
pP2H	pMC1403 vector, Ap^r, *pas* region 1240–1362[b]	This work
pTac-pasA-pACYC	pACYC184 vector, Cm^r, *pas* region 1316–1559	This work
pTac-pasB-pACYC	pACYC184 vector, Cm^r, *pas* region 1518–1816	This work
pTac-pasC-pACYC	pACYC184 vector, Cm^r, *pas* region 1789–2028	This work
pTac-pasAB-pACYC	pACYC184 vector, Cm^r, *pas* region 1316–1816	This work
pTac-pasABC-pACYC	pACYC184 vector, Cm^r, *pas* region 1316–2028	This work
pKmM0	pTF-FC2 replicon, Km^r, 1–4910[c]	12
pKmM1	pTF-FC2 replicon, Km^r, 1–4911 *pasA*	12
pKmM2	pTF-FC2 replicon, Km^r, 1–4911 *pasB*	12
pKmM3	pTF-FC2 replicon, Km^r, 1–4911 *pasC*	12
pKmM1del1[d]	pTF-FC2 replicon, Km^r, 1–4911, Δ1217–1321[e]	12
pKmM1del2[d]	pTF-FC2 replicon, Km^r, 1–4911, Δ1232–1943[e]	12
pOU-pasABC	pOU82 R1 replicon, Ap^r, *pas* region 1158–2027	12
pOU-tac-pasABC	pOU82 R1 replicon, Ap^r, *tac*, *pas* region 1158–2027	This work

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; *lacZYA**, *lacZYA* genes with deletion of *lacZ* promoter and ATG; *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] Entire pTF-FC2 replicon from the *Cla*I site to the *Pst*I site of pTV100 including the *pas* region (5).
[d] Spontaneous deletions of the toxic plasmid pKmM1 (12).
[e] Regions missing from the spontaneous deletions after PasA antidote inactivation (12).

TABLE 2. Regulation of the *pasABC* genes in *E. coli* CSH50-I^q containing the *pas-lacZ* reporter construct, pP2H

Coresident plasmid	Avg β-galactosidase activity[a] (Miller units) ± SD	% Activity
pACYC184 (control)	252 ± 6	100
pKmM0	12 ± 1	5
pKmM2	31 ± 4	12
pKmM3	14 ± 1	6
pKmM1del1	185 ± 70	73
pKmM1del2	206 ± 9	82
pTac-pasA-pACYC	10 ± 1	4
pTac-pasB-pACYC	NA[b]	NA
pTac-pasC-pACYC	226 ± 37	90
pTac-pasAB-pACYC	2 ± 1	1
pTac-pasABC-pACYC	3 ± 2	1

[a] β-Galactosidase activity was measured three times on each of three independently selected colonies.
[b] No assay possible because of the lethality of the PasB toxin in CSH50-I^q.

linked to the *tac* promoter from the construct pTac-pasABC on a *Bam*HI-*Pvu*I (blunted) fragment and ligating them into pOU82 which had been cut with *Eco*RI (blunted) and *Bam*HI. Stability of the pOU-tac-pasABC construct was tested in the presence and absence of *pas* induction by 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 2). This was compared with the stability of the pOU82 control and pOU82-pasABC containing the *pas* genes under the control of the natural promoter. The pOU82-tac-pasABC construct was less stable than the pOU82 control even without induction of the *pas* genes from the *tac* promoter. On IPTG induction of the *pas* genes, the pOU82-tac-pasABC construct was even less stable than without induction. Lower levels of IPTG (0.5 and 1.0 mM) were also used, but the result was similar to that for 2 mM (data not shown). Autoregulatory feedback by PasA-PasB would therefore appear to be an essential feature of the proteic poison-antidote *pas* for it to stabilize a heterologous test plasmid in *E. coli*.

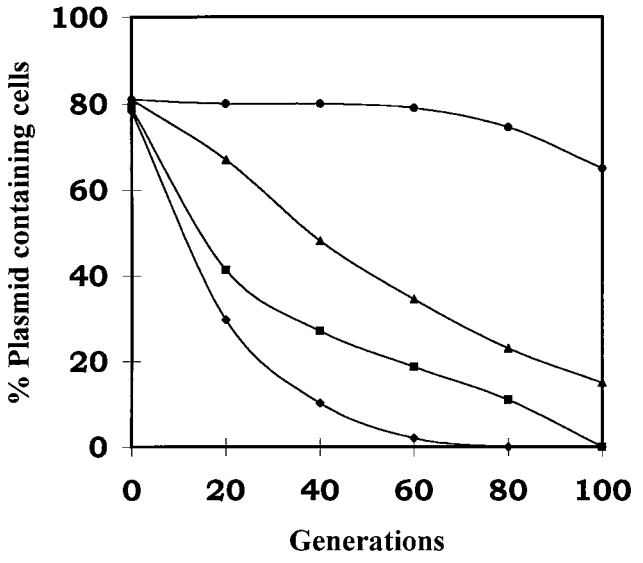

FIG. 2. Stability of plasmids in *E. coli* CSH50-I^q host cells. Cells containing pOU82 (▲), pOU82-pasABC (●), pOU-tac-pasABC without IPTG induction (■), or pOU-tac-pasABC with 2 mM IPTG (◆) are shown.

We are most grateful to Kenn Gerdes for the gift of plasmid pOU82.
This work was supported by grants from Gencor (now Billiton) Process Research (Randburg, South Africa), the Foundation for Research Development (Pretoria, South Africa) and the THRIP programme of the Department of Trade and Industry.

REFERENCES

1. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA **81:**6929–6933.
2. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **142:**971–980.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
4. **Dorrington, R. A., and D. E. Rawlings.** 1990. Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. **172:**5697–5705.
5. **Dorrington, R. A., S. Bardien, and D. E. Rawlings.** 1991. The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli.* Gene **108:**7–14.
6. **Gerdes, K., J. E. L. Larsen, and S. Molin.** 1985. Stable inheritance of plasmid R1 requires two different loci. J. Bacteriol. **161:**292–298.
7. **Jensen, R. B., and K. Gerdes.** 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. **17:**205–210.
8. **Miller, J. H.** 1983. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
9. **Rawlings, D. E., and T. Kusano.** 1994. Molecular genetics of *Thiobacillus ferrooxidans*. Microbiol. Rev. **58:**39–55.
10. **Roberts, R. C., C. Spangler, and D. R. Helinski.** 1993. Characteristics and significance of the DNA binding activity of plasmid stabilization system protein ParD from the broad-host-range plasmid RK2. J. Biol. Chem. **268:** 27109–27117.
11. **Ruiz-Echevarrìa, M. J., A. Berzal-Herranz, K. Gerdes, and R. Díaz-Orejas.** 1991. The *kis* and *kid* genes of the *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the coordinated action of the Kis and Kid proteins. Mol. Microbiol. **5:**2685–2693.
12. **Smith, A. S. G., and D. E. Rawlings.** 1997. The poison antidote stability system of the broad-host range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol. **26:**261–270.
13. **Smith, A. S. G., and D. E. Rawlings.** 1998. 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. J. Bacteriol. **180:**187–98.
14. **Tam, J. E., and B. C. Kline.** 1989. The F plasmid ccd autorepressor is a complex of CcdA and CcdB proteins. Mol. Gen. Genet. **219:**26–32.
15. **Yannisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.