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A Stationary-Phase-Dependent Viability Block Governed by Two Different Polypeptides from the *RhsA* Genetic Element of *Escherichia coli* K-12

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Multicopy plasmids bearing a small internal portion of the *RhsA* genetic element of *Escherichia coli* K-12 imparted a viability block on cultures grown to stationary phase in broth. Inclusion of the last 25 codons of the *RhsA* core open reading frame (called core-ORF) in the plasmid insert was crucial for eliciting this toxic effect. The toxic effect could be suppressed by including the adjacent *Rhs* component, dsORF-a1, on the multicopy plasmid. The toxic effect was enhanced in RpoS$^-$ strains.

RhsA is an accessory genetic element of *Escherichia coli* which is widely but not universally distributed among natural *E. coli* isolates (4, 9). Although members of this family generally share one or more discrete homologous structures, each element contains unique sequences specific for that element. The *RhsA* of *E. coli* K-12 is 8.2 kb long. It contains the 3.7-kb GC-rich core which is common to all *Rhs* elements, followed by a 1.3-kb AT-rich region which in *E. coli* K-12 is largely unique to *RhsA*. The core DNA sequence of *RhsA* encodes a long open reading frame (ORF), called core-DRF, which extends 417 bp into the AT-rich region. In addition to encoding this core extension (ext-a1) of the large core-ORF, the AT-rich region contains an 840-bp ORF called dsORF-a1. These components of *RhsA* are shown schematically in Fig. 1a. The function of *RhsA* is not known, and core-ORF is not expressed during routine laboratory cultivation (9). However, the DNA sequence of core-ORF predicts a protein which has significant homology to a wall-associated protein of *Bacillus subtilis* (5) and which shares with the *B. subtilis* protein a repetitive amino acid motif similar to certain carbohydrate-binding motifs of other bacterial species (23).

The toxic effect of a cloned *RhsA* fragment. *RhsA* was originally cloned as an 11-kb *Sal*I fragment (14), and the sequence of the entire insert has been determined (GenBank accession number L19044). As a part of the original characterization of the *RhsA* element (19), plasmid pJG1631 was created by inserting the 4,539-bp *Pvu*II-*Pvu*II fragment of *RhsA* into the *Sma*I site of pUC19 (16), with the left *Pvu*II site nearest the vector *Hin*dIII site. A nested set of deletion derivatives of pJG1631 was created by using exonuclease III (8) for the purpose of sequencing this part of *RhsA* (Fig. 1b). Plasmid pJG1631 and most of its deleted derivatives were observed to impart a toxic effect on non-wild-type strains used for routine cloning, in that broth cultures of such cell-plasmid combinations which had attained stationary phase exhibited viable titers reduced by 2 to 5 logarithms. The toxic effect was seen to become more severe as more of the pJG1631 insert was deleted, except that the toxicity disappeared if the deletion was sufficiently great. Plasmids with inserts ending at δD were most toxic, while those ending at δR were nontoxic. Early investigation into this toxicity suggested that the presence of the strong vector p_{lac} promoter in the plasmids might play a role in causing or complicating the toxic effect. Accordingly, a new vector, p18ΔZIP, was created out of pUC18 by sequentially deleting first the α-complementing *lacZ* fragment between *Nar*I and *Hin*dIII and then the p_{lac}-containing sequence between *Eco*RI and the remaining *Pvu*II site (regenerating *Eco*RI). The inserts from pJG1631 deletion derivatives were transferred into p18ΔZIP, and the resulting plasmids were designated by the left- and right-hand boundaries of their inserts, e.g., pPδR (insert *Pvu*II to δR, Fig. 1b), etc. The inserts retained the same relative toxicity when introduced into p18ΔZIP, but the toxic effect attributed to any individual insert was not as severe when the p18ΔZIP vector was used as it had been with either pUC19 or pUC18. All further work was done with the p18ΔZIP vector because of its presumed greater simplicity.

Viability loss coincides with onset of stationary phase. The insert of plasmid pSδD was the smallest of the inserts that elicited a toxic effect; it commences at the *Ssp*I site within ext-a1 and extends for 275 bp (Fig. 1). The presence of pSδD had two distinctive effects. First, the culture carrying pSδD exhibited a decrease in viable titer at the same time that the parallel control culture carrying only the p18ΔZIP vector approached a stationary titer (Fig. 2a). Second, when the pSδD culture was sampled and its titer was determined after achievement of stationary phase, its apparent viable titer became greater when the incubation of the titration plates was extended (Fig. 2b). In other words, new colonies continuously appeared on the plates during a prolonged incubation period, some colonies appearing only after 48 h of incubation. Importantly, fewer than 1% of the colonies arising from the sampling after 3.5 h of the pSδD culture (while it was still only approaching stationary phase [Fig. 2a]) were delayed. For the control (p18ΔZIP) culture, all colonies were visible at 12 h after plating, regardless of the time of sampling.

Standardized toxicity assay. On the basis of these results, a standard protocol for testing plasmid toxicity was developed. This protocol necessarily reflected the observation that cells needed to achieve stationary phase in order for toxicity to be exhibited: various cell-plasmid combinations would be grown in broth and held at saturation level for a specific amount of time before being diluted and plated to measure the extent and timing of viability. To begin an assay, all test and control plasmids to be included were freshly transformed into the *E.*

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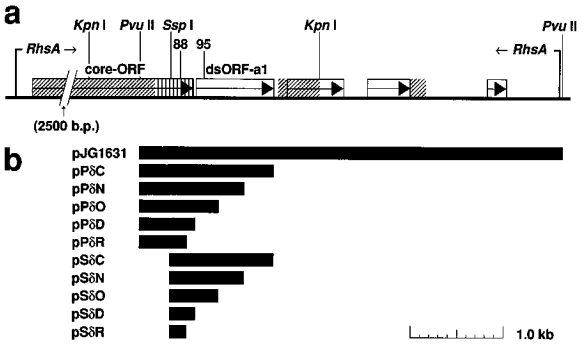

FIG. 1. Physical map of *RhsA*. (a) Full length of *RhsA* drawn to scale, with the exception of 2,500 bp of core-ORF. The location and orientation of ORFs are shown by boxed arrows. The GC-rich *RhsA* core sequence and its partial repetitions are highlighted by diagonal hatching, whereas ext-a1, the extension of core-ORF into an AT-rich sequence, is identified by vertical striping. The *Kpn*I sites above the map indicate the extent of chromosomal substitution with Km^r in the *RhsA*::Km^r disruption described in the text. The map locations of oligonucleotide-directed base pair substitutions are indicated by their oligonucleotide numbers: 88 and 95. (b) Map alignment of plasmid inserts. The bars represent the various plasmid inserts used in this study aligned with their sources on the physical map. The parental *Pvu*II-*Pvu*II insert portion of pJG1631 is indicated at the top. The remaining plasmid designations (pPδC, etc.) refer directly to the left boundary (P for *Pvu*II or S for *Ssp*I) and the right boundary (exonuclease III end point designation C, N, O, D, or R) of the plasmid insert as present in vector p18ΔZIP. There are actually two *Ssp*I sites at the location indicated in panel a, which are separated by 9 bp. All inserts deleted between *Pvu*II and *Ssp*I lacked *Ssp*I cleavage in the insert and were therefore bounded by the *Ssp*I site distal to the *Pvu*II site.

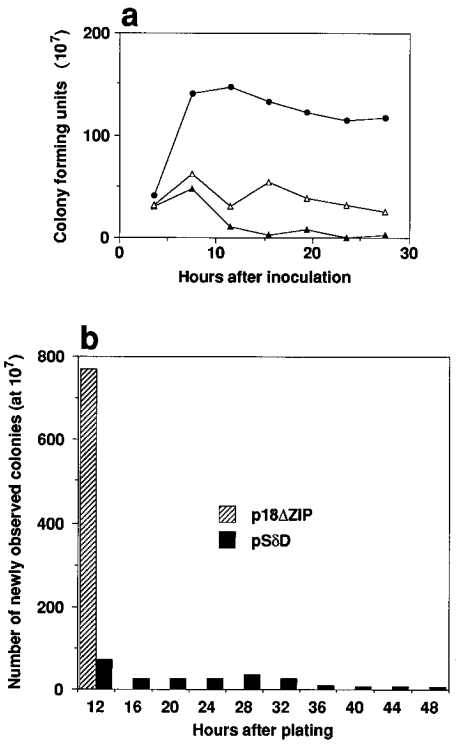

FIG. 2. Association of plasmid toxicity with entry into stationary phase. Plasmids p18ΔZIP and pSδD were freshly transformed into strain CH734. After 15.3 h of plate incubation, a small portion of a colony from each transformation was inoculated into culture tubes containing 5 ml of L broth with 50 mg of ampicillin per ml. At 3.5, 7.5, 11.5, 15.5, 19.5, 23.5, and 27.5 h after inoculation, a sample of each broth culture was diluted for immediate plating. Twelve hours after each plating, the colonies visible on each plate were counted for the first time; at 4-h intervals thereafter, each plate was again observed for colonies which had not previously been visible. The titration dilutions for the 3.5-h samples were 10^6; all other dilutions were 10^7. (a) Time course of CFU. Filled circles, total CFU observed with vector p18ΔZIP at 12 or at 60 h after spreading on titration plates; filled triangles, CFU with pSδD apparent at 12 h after spreading on titration plates; open triangles, total accumulated CFU with pSδD after 60 h of incubation of the same titration plates. (b) Temporal distribution of colony appearance. The stationary-phase (at 7.5 h and later) colony counts represented by the curves in panel a are accumulated here according to the time each colony appeared after the titration plate it was on began incubation.

coli strain(s) being used. Each of the various cell-plasmid combinations therefore started on the most equivalent basis possible, as suggested by the observations that (i) the transformation efficiencies of toxic and nontoxic plasmids were always equal and (ii) the resultant transformed colonies on each plate were uniformly normal in appearance: colonies carrying plasmids which would eventually elicit toxicity were not visibly distinguishable from colonies containing the control plasmid. Sixteen hours after transformation, a small portion of a colony representing each cell-plasmid combination was inoculated into 5 ml of broth (10 g of tryptone per liter, 5 g of yeast extract [Difco] per liter, 5 g of NaCl per liter, and 50 μg of ampicillin per ml) and incubated at 37°C with aeration by shaking. The optical density at 595 nm of each broth culture was followed until the culture approached stationary-phase density (3 to 4 h for control cultures). During the exponential growth phase, broth cultures containing plasmids which were capable of imparting a toxic effect grew with normal doubling times of 24 to 33 min (depending on the strain), just as the parallel control cultures did. Active, exponential growth therefore appeared to be unaffected by the toxic potential of a given plasmid both in broth and, as noted above, on plates following fresh transformation. However, the broth cultures inoculated from colonies containing toxic plasmids occasionally entered exponential growth only after a lag of up to 2 h or more, compared with the simultaneously inoculated control cultures, presumably because a toxic viability block already existed within an unknown percentage of cells in the colonies incubated for 16 h used for inoculation. The unpredictable lagging of some of the broth cultures inoculated from the transformation plates emphasized the advantage of measuring the toxic effect on cells grown in broth culture (where entry into stationary phase was relatively simultaneous for the entire culture) instead of on resuspended colonies. A simple growth curve standardization among all of the broth cultures was used in order to correct for any inequalities in both the numbers and the proportions of immediately viable cells among the individual plate-to-broth inocula. Since the amount of time between a culture's arrival at a mid-logarithmic optical density and its entry into stationary phase was the same for all cultures (of a given strain), the relative time at which each culture in a given experiment passed an optical density at 595 nm of 0.1 (well into logarithmic growth) was used to ensure that each culture was incubated for the same amount of time between its entry into stationary phase and its being diluted for titration. At 16 or 16.5 h after inoculation of the broth cultures (an insignificant experimental variation; to compare data, see Fig. 2a), the control cultures were diluted and plated for assay (same broth recipe with or without ampicillin, plus 15 g of agar [Sigma] per liter). All other broth cultures were diluted and plated at a time which was adjusted for their difference relative to the control broths in reaching an optical density at 595 nm of 0.1. Each titration plate was observed at 11 h after plating and at various times thereafter in order to determine the count and timing of colony appearance. It was eventually determined that extended curing (or drying) of the titration plates resulted in toxic effects that were quantitatively increasingly severe; precision between experiments was subsequently enhanced by controlling the degree to which plates were cured prior to use. The presence of ampicillin in

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
CH734	*trpA36 lysA xyl-4 ilvD130 argH rpoS*[a]	3
CH1678	Hfr PO2A *argH polA1 RhsA*::Km^r	This work[b]
CH4966	MG1655 *rpoS359*::Tn*10*	MG1655 × RH90[c]
CH4967	MG1655 *RhsA*::Km^r	MG1655 × CH1678[c]
CH4968	MG1655 *rpoS359*::Tn*10 RhsA*::Km^r	CH4966 × CH1678[c]
MG1655		6
RH90	MC4100 *rpoS359*::Tn*10*	12

[a] Glycogen synthesis not induced (17).
[b] The *RhsA*::Km^r construction has had the internal 2.7-kb *Kpn*I fragment of the chromosomal *RhsA* element replaced by a Km^r determinant. Its construction paralleled the *RhsC*::Km^r construction described elsewhere (19).
[c] Donor for P1 transduction.

the titration plates was not required for observation of the toxic effect, indicating that an inability to maintain plasmids was not the basis for the effect.

The *rpoS* sigma factor protects against toxic effects. Since RpoS$^+$ is critical in adjusting to stationary phase (7), we tested the effect of an RpoS deficiency by preparing an *rpoS359*::Tn*10* (13) derivative of MG1655 (Table 1). MG1655 is wild-type *E. coli* K-12 cured of the F$^+$ and lambda episomes (6). We verified its RpoS$^+$ status by testing for glycogen accumulation (17). The presence of pSδD in MG1655 *rpoS359* resulted in more than a 100-fold reduction in final titer compared with that for p18ΔZIP in the same strain, with a relatively high proportion, 91%, of delayed colonies (Table 2). In the RpoS$^+$ control strain, the small effect of pSδD was seen primarily in the late appearance of 12% of the colonies. The toxic effect of pPδD on MG1655 was similarly mild, and the enhancement of the toxic effect by *rpoS359* was manifested primarily in the increased proportion of delayed colonies. When compared with that obtained with wild-type MG1655, the smaller number of CFU obtained with MG1655 *rpoS359* transformed with vector was consistent with the finding that *rpoS* mutation prevents the cells from accomplishing a final reduction in size (and thereby a final increase in viable cell number) as stationary phase is entered (13). It is not known whether RpoS$^+$ itself or an RpoS-dependent function is involved in (partial) protection from the toxicity, but its involvement fully implicates the conversion from exponential phase to stationary phase as playing a role in the *RhsA*-derived viability block.

Deletion of a portion of *RhsA* from the host cell genome enhances toxicity. Possible explanations for pSδD toxicity can be divided into two general categories. In the first case, a product that interferes with colony formation by stationary-phase cells might be synthesized from the plasmid insert. Alternatively, the small fragment of *RhsA* present in pSδD may be toxic at a high copy number because it binds and titrates a regulatory factor, leading to the misregulation of chromosomal information. A specific hypothesis of this second category holds that the toxic product is produced from the chromosomal *RhsA* element itself. To investigate this possibility, we constructed an *E. coli* mutant in which the *RhsA* segment between the *Kpn*I sites shown in Fig. 1a was replaced by a gene for kanamycin resistance (Table 1). The effect of this chromosomal *RhsA* disruption on cell sensitivity to plasmid toxicity was tested in a variety of backgrounds, and contrary to the preliminary hypothesis, it did not make cells resistant to plasmid toxicity (Table 2). In fact, the *RhsA*::Km^r derivative was actually slightly more sensitive to pSδD toxicity than was its wild-type parent (Table 2). This enhancement of toxicity by *RhsA*::Km^r substitution is even more pronounced in other strains than it is in MG1655 (data not shown). A parallel Km^r substitution at *RhsC* (19) was found to have no effect on toxicity in any strain (data not shown).

The cloned carboxy-terminal portion of core-ORF is the source of toxicity. Both pPδD and pSδD contained a single ATG codon (and no GTG) which was in frame with the portion of *RhsA* core-ORF present on either plasmid: the ATG which initiates ORF-ex (Fig. 3). It was possible that this 72-codon ORF was the source of the toxic effect for both plasmids. In order to investigate the relationship between core-ORF integrity and the stationary-phase viability block, we introduced a stop codon into the reading frame by site-directed mutagenesis. To do this, a DNA fragment derived from *RhsA* was subcloned into vector pALTER-1 (Promega) and a mutagenic oligonucleotide was incorporated into the fragment by using the Promega Altered Sites II system. Mutagenesis was verified by DNA sequencing, and the mutated insert was recloned into the toxicity test vector p18ΔZIP for assay. Procedures for the extraction, digestion, electrophoretic gel analysis, and ligation of plasmid DNA, as well as the comparative sequencing of cloned DNA, have been previously reported (24). Incorporation of mutagenic oligonucleotide 88 into pPδD created pPδD88, converting an arginine codon (AGA) into a stop codon (TGA) in core-ORF (Fig. 3). This single-base-pair substitution completely abolished toxicity (Table 3). The artificial stop codon in pPδD88 would be expected to prevent the translation of the last 49 codons of either core-ORF or the in-frame ORF-ex. The fact that preventing the translation of these 49 carboxy-terminal codons simultaneously eliminated toxicity was consistent with the fact that deletion of the final 25 codons from the insert (as in pPδR or pSδR) also eliminated plasmid toxicity (Table 4, experiment 1, and data not shown). Together, these results clearly implicate a polypeptide that includes the last 25 amino acids of core-ORF as the crucial source of the toxic effect. The toxic effect of pSδD, which lacked the *Pvu*II-

TABLE 2. Sensitivity of MG1655-derived strains disrupted at *rpoS* and/or *RhsA* to plasmid-mediated toxicity

Strain or mutation[a]	Viability of strain with plasmid[b]:					
	p18ΔZIP		pSδD		pPδD	
	Final titer	Colonies (%) delayed	Final titer	Colonies (%) delayed	Final titer	Colonies (%) delayed
MG1655	3.8×10^9	0	3.2×10^9	12	3.0×10^9	13
RhsA::Km^r	4.3×10^9	0	1.1×10^9	55	2.3×10^9	10
rpoS359	6.6×10^8	36	5.4×10^6	91	1.5×10^9	89
RhsA::Km^r *rpoS359*	7.1×10^8	46	4.0×10^6	94	1.1×10^9	89

[a] MG1655 derivatives are described in Table 1.
[b] Toxicity assays for each plasmid were performed with strain MG1655 and its derivatives as described in the text. The final titers were determined after ≥55 h of incubation. Colonies delayed refers to the proportion (percent) of colonies which were not observable 11 h after plating.

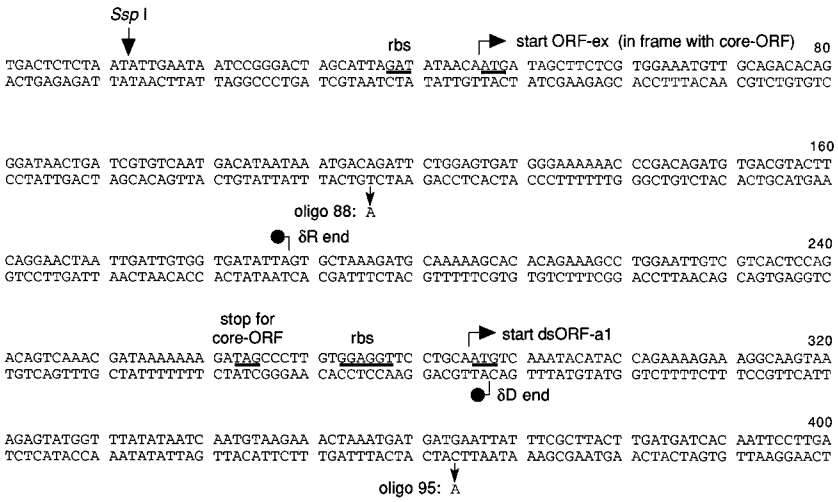

FIG. 3. DNA sequence of the region of *RhsA* associated with plasmid toxicity. A portion of the DNA sequence of *RhsA* (GenBank accession number L19044 [4]) is shown; base 1 here is equivalent to base 4821 in the complete sequence, which commences with an *Mlu*I site upstream of *RhsA*. The stop codon (TAG) for core-ORF as well as the ATG codons for ORF-ex and dsORF-a1 are labeled and underlined. The deletion (right-hand) termini of δR and δD (filled circles) are also indicated. Base changes introduced by incorporation of oligonucleotides (oligo) 88 and 95 (arrows) are shown for the DNA strand homologous to each of them. Apparent ribosome binding sites (rbs) are labeled. The *Ssp*I site is the same one indicated in Fig. 1; the *RhsA*-derived inserts of plasmids pSδR and pSδD are therefore included in the sequence in their entirety.

*Ssp*I portion of the PδD insert (Fig. 1), was quantitatively more severe than that of pPδD (Table 2). A consequence of this *Pvu*II-*Ssp*I deletion was that the ORF-ex start codon in pSδD was located only 34 bp away from the upstream vector-insert junction. The greater toxicity of pSδD may therefore reflect an increase in the number and/or the survival rate of transcripts, initiated at unknown vector transcription start sites, which actually contained the ORF-ex sequence.

Intact dsORF-a1 suppresses toxicity. Three lines of evidence led us to the conclusion that an intact, translatable dsORF-a1 accomplished suppression of plasmid toxicity. First, as noted above, cells in which a kanamycin resistance gene had been substituted for the central portion of *RhsA* (including dsORF-a1) exhibited an increased susceptibility to the toxic effect of pSδD. Among 10 strains into which the *RhsA*::Km^r substitution was introduced, the increase in the degree of susceptibility varied from small (viability reduced by twofold or less [Table 2]) to substantial, with the greatest increase in susceptibility (a 4-log reduction in viability) being obtained when the *RhsA*::Km^r derivative of strain GC4780 (10) was tested for pSδD toxicity (data not shown). Second, when dsORF-a1 or a significant portion of it was present on the test plasmid (but absent from the cell chromosome), toxicity was reduced (Table 4, experiment 1). Plasmids pPδC and pSδC, which had no toxic effect at all, contained dsORF-a1 intact, with the δC insert extending 8 bp beyond the dsORF-a1 stop codon. Plasmid inserts δN and δO contained progressively less of dsORF-a1 (Fig. 1), and the δD deletion, which produced the most toxic plasmids, deleted all of dsORF-a1 but retained all of the carboxy-terminal sequence of core-ORF (Fig. 3). An increase in toxicity was not simply the result of a decreased insert size, since pPδN, which exhibited some toxicity, was actually slightly larger than pSδC, which showed no toxicity but did retain a complete dsORF-a1. The reduction in toxicity afforded by plasmid inclusion of less than full-length dsORF-a1 is puzzling, but it might reflect a partial activity of the amino-terminal portion of the dsORF-a1 protein in alleviating toxicity.

The strongest indication of the role of a dsORF-a1 product

TABLE 3. Elimination of plasmid toxicity by nonsense mutation in plasmid-borne ORF-ex

Plasmid	Viability of strain[a]			
	MG1655		MG1655 *rpoS359*	
	Final titer	Colonies (%) delayed	Final titer	Colonies (%) delayed
pPδD	1.1×10^9	54	2.5×10^8	100
pPδD88	3.8×10^9	0	1.5×10^9	0
p18ΔZIP	3.0×10^9	0	1.7×10^9	0

[a] Toxicity assays were performed with strain MG1655 and its *rpoS359*::Tn*10* derivative (Table 1). Other procedures were performed as specified in Table 2, footnote *b*.

TABLE 4. Inverse relationship of plasmid toxicity and the integrity of plasmid-borne dsORF-a1

Expt and plasmid	Viability of strain[a]			
	MG1655 *RhsA*::Km^r		MG1655 *RhsA*::Km^r *rpoS359*	
	Final titer	Colonies (%) delayed	Final titer	Colonies (%) delayed
Expt 1				
pSδC	3.3×10^9	0	2.3×10^9	0.2
pSδN	3.1×10^9	10	2.0×10^9	83
pSδO	2.5×10^9	57	1.3×10^9	99
pSδD	8.9×10^8	97	2.5×10^7	100
pSδR	3.9×10^9	0.5	2.2×10^9	0
p18ΔZIP	4.3×10^9	0.2	1.8×10^9	0.8
Expt 2				
pSδC	3.6×10^9	0	1.4×10^9	0
pSδC95	3.2×10^9	9	2.5×10^7	99
pSδD	6.3×10^8	78	3.1×10^6	100
p18ΔZIP	3.2×10^9	0	1.3×10^9	0

[a] Toxicity assays were performed with the *RhsA*::Km^r and *RhsA*::Km^r *rpoS359*::Tn*10* derivatives of MG1655 (Table 1) as described in the text. All plates used for titration were made with nonselective (ampicillin-free) medium. Other procedures were performed as specified in Table 2, footnote *b*.

in alleviating the stationary-phase viability block was obtained by introducing a stop codon into the dsORF-a1 of pSδC at the position indicated in Fig. 1 and 3, creating plasmid pSδC95. The protocol described above for the incorporation of oligonucleotide 88 was used to introduce the oligonucleotide 95 stop codon into dsORF-a1, except that the final male recipient of mutation-bearing f1 phage single strands was a *recA56* F′ derivative of strain M182 (1), which was relatively insensitive to the *RhsA* viability block. As shown in Table 4, experiment 2, the single-base-pair change in pSδC95 resulted in significant toxicity from a plasmid otherwise identical to the nontoxic pSδC. From this and the preceding evidence, we conclude that a translation product of the dsORF-a1 sequence counteracts the establishment of a viability block by the ORF-ex product.

Other experimental systems have linked the activity of one or more genes with the loss or retention of viability in stationary phase (11, 15, 18, 20). Many of these genes follow the pattern of *surA*, which is required for cells to retain viability when held for several days in stationary phase (22). Similarly to the *RhsA* viability block described here, the *surA* mutation has no effect on exponentially growing cultures. Unlike the *RhsA* block, however, the *surA* mutation is characterized by a cumulative and nearly total loss of viability over a period of 6 to 7 days in culture. As we have shown, the *RhsA* block takes maximal effect as cells enter stationary phase, at which point individual cells appear to belong to one of three categories: (i) those cells which experience little or no difficulty in reentering exponential growth phase, (ii) those cells which are delayed in returning to exponential growth, and (iii) those cells which will not multiply. The *RhsA* viability block, then, seems most to resemble the *surB* mutation (21) in restricting a cell's ability to reenter growth phase.

Another experimental system in which *E. coli* cells are seen to lose viability in stationary phase involves the overexpression of the HSP70 class protein DnaK, which functions both as a regulator and as a chaperone (2). Plasmid-encoded overproduction of DnaK affects cell growth in ways not observed in this study, but a loss of viability occurs only when DnaK-overproducing cells are allowed to enter stationary phase, as with the *RhsA* viability block. Further in parallel with the *RhsA* core-ORF–dsORF-a1 establishment and alleviation of stationary-phase toxicity is the fact that the deleterious effects of overproducing DnaK are at least partly alleviated by the cooverproduction of DnaJ, which is normally cotranscribed from the chromosome along with the immediately upstream DnaK. The presumption concerning this protective effect of DnaJ is that DnaJ either titrates much of the excess DnaK in the cell, qualitatively alters the activity of DnaK, and/or masks the molecule(s) through which the excess DnaK affects the cell negatively. One of these relationships may ultimately hold true for the interactions between the polypeptide products of ORF-ex dsORF-a1 and their targeted cell component(s).

General features of *Rhs* element structure are a GC-rich core (>60% G+C) and an adjacent AT-rich region (<40% G+C). While the cores of various elements are highly conserved, each AT-rich region is different. Each AT-rich region encodes both a core extension (e.g., ext-a1 of *RhsA*) and its closely linked downstream ORF (e.g., dsORF-a1 of *RhsA*). On the basis of evolutionary considerations, we have proposed elsewhere that the C-terminal polypeptide encoded by each core extension interacts directly with the product of its adjacent downstream ORF (9). It would be of considerable interest if the interaction implied by the results described here reflected a specific recognition between ext-a1 and dsORF-a1 that is essential for their function in *Rhs* biology.

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