J. Genet., Vol. 74, Numbers 1 & 2, April & August 1995, pp. 1–17. © Printed in India.

An unusual suicidal interaction in *Escherichia coli* involving nucleoid protein H-NS

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MS received 11 July 1995

Abstract. A conditional-lethal mutation (*rpoB364*) mapping to the gene that encodes the β-subunit of RNA polymerase was obtained in *Escherichia coli*. This mutation caused cell filamentation at the restrictive growth temperature and partial derepression of the osmotically regulated *proU* operon at the permissive growth temperature. Even under the latter condition, transformants of the *rpoB364* mutant strain carrying the plasmid vector pACYC184, but not those carrying other *polA*-dependent multicopy plasmids such as pACYC177 or pBR322, were killed in early stationary phase; one class of suppressor mutants isolated as survivors within these transformant colonies were further derepressed for *proU–lac* expression, and the mutation in each of several independent clones of this class was mapped to *hns*, the gene that encodes the protein H-NS of the *E. coli* nucleoid. The *hns* mutations did not suppress the conditional-lethal growth phenotype of the *rpoB364* mutant itself. On the other hand, intracellular overproduction of guanosine 3′,5′-bispyrophosphate (ppGpp) in the *rpoB364* strain alleviated both the growth inhibition at the restrictive temperature and the pACYC184-mediated stationary-phase lethality. Upon subcloning into pUC19 or into pACYC177, a 105-bp *Xba*I–*Hin*dIII fragment from pACYC184 was shown to be sufficient to confer the *rpoB364 hns*$^+$-dependent lethal phenotype. We suggest that the level in stationary-phase cultures of a gene product(s) that interacts with the pACYC184 DNA fragment is altered in the *rpoB364 hns*$^+$ derivative (compared to that in *rpoB*$^+$ or *rpoB364 hns* strains) and that this results in cell suicide.

Keywords. *rpoB* mutation; pACYC184; H-NS; ppGpp; programmed cell death; stationary phase.

1. Introduction

> Nothing in his life
> Became him like the leaving it. He died
> As one that had been studied in his death . . .
> (*Macbeth*, I.iv.7–9)

Three abundant proteins, HU, IHF and H-NS, have been identified as major constituents of the chromatin or nucleoid in *Escherichia coli*. HU and IHF are basic heterodimeric proteins whereas H-NS is a neutral protein that exists as a homodimer. Although the organization and structure of the bacterial nucleoid is still incompletely understood, clues to the pleiotropic roles played by each of the three nucleoid proteins in *E. coli* physiology, including gene regulation, replication, recombination and maintenance of DNA topology, have emerged from a variety of genetic and biochemical studies (see Drlica and Rouviere-Yaniv 1987; Pettijohn

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1988; Schmid 1990; Higgins *et al.* 1990 for reviews). Mutants simultaneously defective in any two of the three nucleoid proteins are sick but still viable, whereas triple mutants cannot be constructed and are presumably nonviable (Yasuzawa *et al.* 1992).

The H-NS protein (also called H1 or Hla) is encoded by the *hns* gene located at 27 min on the *E. coli* chromosome. Genetic studies have shown that H-NS is involved in regulation (in most instances, repression) of the transcription of a large number of unlinked and unrelated genes, in addition to modulation of the frequencies of homologous and illegitimate recombination and of phage Mu transposition (Bertin *et al.* 1990; Higgins *et al.* 1990; Falconi *et al.* 1991; Yamada *et al.* 1991; Dri *et al.* 1992; Kano *et al.* 1993). Overproduction of a small RNA species (DsrA) antagonizes the repression mediated by H-NS in the case of at least some of the target genes (Sledjeski and Gottesman 1995).

Several reports have suggested a role, as yet not completely defined, for H-NS in stationary-phase physiology. Synthesis of H-NS is elevated in cultures as they approach stationary phase (Spassky *et al.* 1984; Dersch *et al.* 1993), and Spassky *et al.* (1984) had also suggested that the protein exists in multiple isoforms whose relative proportions are altered in the various phases of growth. H-NS has recently been shown to reduce intracellular accumulation of σ^s, the stationary-phase sigma factor of RNA polymerase encoded by the *rpoS* gene (Yamashino *et al.* 1995). The σ^s dependence of transcription from certain promoters, such as those of the *csgBA* and *hdeAB* operons, is relieved in *hns* mutants so that these promoters can now also be recognized by the σ^{70}-bearing RNA polymerase holoenzyme (Arnqvist *et al.* 1994).

In this paper we report the identification of a novel and extremely unusual phenotype associated with the *hns* gene, namely suicide in stationary phase of derivatives of a particular RNA polymerase mutant strain carrying the commonly used multicopy plasmid vector pACYC184. The relevant mutation (*rpoB364*) was itself obtained in the course of our studies on the mechanisms of osmotic regulation of transcription of the *E. coli proU* operon (Gowrishankar 1985). A defined 105-bp fragment of pACYC184 was shown to be sufficient for its participation in this lethal tripartite interaction *in vivo* with H-NS and the *rpoB364* gene product.

2. Materials and methods

2.1 *Bacterial strains, phages and plasmids*

All bacterial strains used in this study are derivatives of *E. coli* K-12, and are listed in table 1. Phage P1*kc* was from our laboratory stock; the transposon-vehicle phages λNK370 and λ1105, for Tn*10* and Tn*10*dKan respectively, have been described earlier (Kleckner *et al.* 1978; Way *et al.* 1984).

Previously described plasmids that were used in this study are listed in table 2. In addition, the following plasmids were constructed (see figure 4a): Plasmids pHYD105 and pHYD115, which carry, respectively, the 0·63-kb *Xba*I–*Sph*I and 0·58-kb *Eco*RI–*Nhe*I fragments from pACYC184 cloned into the corresponding sites in pUC19 and pBR322; plasmid pHYD111, which carries the 0·41-kb *Pvu*II fragment of pACYC184 in the *Sma*I site of pUC19; plasmids pHYD107, pHYD109 and pHYD110, constructed by cloning the 0·45-kb *Bam*HI fragment of pHYD105 (which

Table 1. List of *E. coli* K-12 strains.

Strain	Genotype[a]	Source
CAG12169	*zch-506*::Tn*10*	Singer *et al.* (1989)
CAG18618	*thi-3178*::Tn*10*Kan	Singer *et al.* (1989)
GC2553	*ftsRI* (*rpoB369*)	Vinella and D'Ari (1994)
GC3698	*ftsRI* (*rpoB369*) *relA1*	Vinella and D'Ari (1994)
JM101	*supE thi* Δ(*lac–proAB*)/ F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	Sambrook *et al.* (1989)
MC4100	Δ(*argF–lac*)*U169 rpsL150 araD139 relA1 flbB5301 deoC1 ptsF25*	Casadaban (1976)
PD#5:NO1736	*lac*(Am) *trp*(Am) [λd*rif18*] [λ*c*I857 *S7 b519 xis6*]	Patrick Dennis[b]
GJ11	MC4100 *proU224*::*lac* (footnote *c*)	Gowrishankar (1985)
GJ1307	MC4100 *rpoB364*	This study
GJ1308	GJ1307 *proU224*::*lac zfi-900*::Tn*10*	This study
GJ1312	GJ11 *zja-900*::Tn*10*	This study
GJ1313	GJ11 *rpoB364 zja-900*::Tn*10*	This study
GJ1313-a	GJ11 *rpoB364 thi-3178*::Tn*10*Kan	This study
GJ1318	MC4100 *rpoB364 proU*::*lac* Δ(*zfi-900*::Tn*10*) *recA srl*::Tn*10*	This study
GJ1319	GJ1318 [λd*rif18*]	This study
GJ1320-a	GJ1313 [λd*rif18*]	This study
GJ1323	GJ11 *rpoB364 hns-200 zja-900*::Tn*10*	This study
GJ1327	GJ11 *rpoB364 hns-200 zch-900*::Tn*10*dKan *zja-900*::Tn*10*	This study
GJ1328	GJ11 *rpoB364 zch-900*::Tn*10*dKan *zja-900*::Tn*10*	This study
GJ1329	GJ11 *hns-200 zch-900*::Tn*10*dKan *zja-900*::Tn*10*	This study
GJ1332	GJ11 *zch-900*::Tn*10*dKan *zja-900*::Tn*10*	This study
GJ1335	JM101 *thi-3178*::Tn*10*Kan	This study
GJ1336	JM101 *rpoB364 thi-3178*::Tn*10*Kan	This study

a, Genetic nomenclature is according to Bachmann (1990) with the exception that the *osmZ* locus has been renamed *hns* (Higgins *et al.* 1990). All strains are F^- unless otherwise indicated. Allele numbers are indicated where they are known. Allele numbers for new mutations obtained in this study were provided by Barbara Bachmann (*E. coli* Genetic Stock Center).
b, Department of Biochemistry, The University of British Columbia, Vancouver, Canada V6T 1W5.
c, The *proU224*::*lac* allele is stabilized by λp1(209) prophage (Gowrishankar 1985).

consists primarily of the *Xba*I–*Bam*HI fragment from pACYC184) into the *Bam*HI sites of the vectors pACYC177, pMU575 and pCL1920 respectively; and plasmids pHYD106 and pHYD108, obtained following *Hind*III digestion and self-recircularization of pHYD105 and pHYD107 respectively—the shortened plasmids thus contain the *Xba*I–*Hind*III fragment of pACYC184 cloned into the vectors pUC19 and pACYC177 respectively.

2.2 *Growth media*

Glucose–minimal A and LB media have been described (Miller 1972). Low-osmolarity media that were used included K medium (Gowrishankar 1985), half-strength minimal

Table 2. List of previously described plasmids.

Plasmid	Features	Reference
pBR322	Tet^r, Amp^r, ColE1-derived cloning vector	Sambrook *et al.* (1989)
pBR329	Tet^r, Amp^r, Cm^r, ColE1-derived cloning vector	Covarrubias and Bolivar (1982)
pUC19	Amp^r, ColE1-derived cloning vector	Sambrook *et al.* (1989)
pACYC177	Amp^r, Kan^r, P15A-derived cloning vector	Chang and Cohen (1978)
pACYC184	Tet^r, Cm^r, P15A-derived cloning vector	Chang and Cohen (1978)
pMU575	Tp^r, IncW-based promoter-cloning vector	Andrews *et al.* (1991)
pCL1920	Sm^r, Sp^r, pSC101-based cloning vector	Lerner and Inouye (1990)
pIJ699	Kan^r, pACYC184-derived *Streptomyces* shuttle vector	Kieser and Melton (1988)
pALS10	Amp^r, pBR322-derived, carrying p*tac-relA*$^+$	Svitil *et al.* (1993)
pDV1	Amp^r, pBR322-derived, carrying *rplL*$^+$ *rif18*	Vinella and D'Ari (1994)
pDV2	Amp^r, pBR322-derived, carrying *rplL*$^+$	Vinella and D'Ari (1994)
pDIA510	Amp^r, pBR322-derived, carrying *hns*$^+$	Bertin *et al.* (1994)
pHYD94	Cm^r, pACYC184-derived, carrying *proWX* and Muc(Ts)	Gowrishankar *et al.* (1986)
pHYD103	Tp^r, pMU575-derived, carrying *proU–lac* fusion	Jayashree and Gowrishankar (1995)

A (supplemented with 0·2% glucose), and LBON (that is, LB with NaCl omitted [Dattananda *et al.* 1991]). MacConkey agar (Difco) plates supplemented with different concentrations (0 to 0·3 M) of NaCl were used in screening for changes in *proU–lac* expression in the mutants. Antibiotics were included at the following concentrations (μg/ml) in LB: tetracycline (Tet), 15; ampicillin (Amp), 50; trimethoprim (Tp), 40; chloramphenicol (Cm), 25; streptomycin (Sm), 200; spectinomycin (Sp), 50; rifampicin (Rif), 50; and kanamycin (Kan), 50; these concentrations were halved in glucose-minimal A and the low-osmolarity media.

2.3 *Genetic and recombinant DNA techniques and enzyme assays*

The methods for nitrosoguanidine mutagenesis, conjugation and transduction have been described (Miller 1972; Gowrishankar 1985). Bgl$^+$ and Bgl$^-$ phenotypes of strains were scored as yellow and blue colonies respectively on K medium agar plates supplemented with 1% salicin and 0·02% bromothymol blue. Spontaneous Tet^s derivatives of Tn*10*-carrying strains were obtained by quinaldic acid selection, as described by Maloy and Nunn (1981). Plasmid transformation and recombinant DNA techniques were carried out by the methods described in Sambrook *et al.* (1989). Spontaneous plasmid-cured derivatives of strains carrying pHYD103 or pACYC184 were obtained by screening single colonies for antibiotic sensitivity following several generations of growth in antibiotic-free medium. Specific activity of β-galactosidase in cultures was determined by the method of Miller (1972), and the values are expressed in units (U) defined therein.

2.4 *Isolation of rpoB364 mutant*

The *rpoB364* mutation was identified in a search for *trans*-acting mutations that derepress the osmotically regulated *proU* operon. The strategy for such a search

has been described by us earlier (Jayashree and Gowrishankar 1995). A population of MC4100 cells mutagenized with nitrosoguanidine was transformed with the *proU–lac* plasmid pHYD103, and individual colonies were scored for their Lac phenotype on MacConkey–lactose–Tp plates at 30°C. The parental MC4100/pHYD103 colonies are Lac^- on this medium. One mutant, GJ1307/pHYD103, identified as Lac^+ on the MacConkey agar plates, was shown to have a mutation (subsequently designated *rpoB364*) unlinked to the plasmid itself that resulted in a three-fold derepression of *proU–lac* expression at low osmolarity (data not shown). A Tp^s segregant of this mutant was obtained and a chromosomal *proU–lac* allele was transduced into it with the aid of the linked *zfi-900*::Tn*10* insertion, to generate the strain GJ1308. The latter strain also exhibited a three-fold derepression of *proU* expression at low osmolarity (see figure 1 and table 4).

2.5 *Transposon insertions linked to rpoB364 and to hns-200*

In strains carrying a chromosomal *proU–lac* fusion, either the *rpoB364* or the *hns-200* mutation confers a Lac^+ phenotype on MacConkey–lactose plates, and this fact was exploited in obtaining transposon insertions cotransducible with each of these loci. Random transpositions in the wild-type strain MC4100 of Tn*10* or of Tn*10*dKan were obtained following infection with λ-NK370 or λ1105 respectively, as described (Kleckner *et al.* 1978; Way *et al.* 1984). A P1 lysate prepared on the pool of Tn*10*-insertion strains was used to infect a Tet^s derivative of the *rpoB364* strain GJ1308 and Tet^r transductants were selected on MacConkey agar plates. One of the Tet^r Lac^- colonies identified in this cross was subsequently shown to have a Tn*10* insertion (*zja-900*::Tn*10*) 54% linked to *rpoB364*, and this insertion in turn was used to construct a pair of isogenic *rpoB*$^+$ and *rpoB364 proU–lac* derivatives, GJ1312 and GJ1313, respectively. In a similar manner, a Tn*10*dKan insertion (*zch-900*::Tn*10*dKan) 90% linked to the *hns* locus was identified following transduction into the *hns-200* strain GJ1323.

2.6 *Lysogenization of GJ1313 and GJ1318 with λdrif18*

Phage lysate of λd*rif18* was prepared from the strain PD#5:NO1736 by temperature induction, as described (Kirschbaum and Konrad 1973). Selection for Rif^r colonies was done following infection of the *rpoB364* strains GJ1313 and GJ1318 with the λd*rif18* lysate. In GJ1313, Rif^r colonies can arise either by integration of λd*rif18* as a prophage (since *rif18* is a dominant Rif^r mutation), or by a double-crossover homologous recombination event that leads to replacement of the chromosomal *rpoB* gene with the *rif18* allele. The *zja-900*::Tn*10* insertion in GJ1313 would be expected to remain linked to *rpoB364* and to be unlinked to *rif18* in the former case, and vice versa in the latter. The two classes of Rif^r colonies could thus be distinguished with appropriate P1 transduction experiments, and the merodiploid status of one representative λd*rif18* lysogenic derivative of GJ1313, GJ1320-a, was verified. A similar strategy was used to establish that GJ1319, a Rif^r derivative obtained after λd*rif18* infection of GJ1318 (which is *recA* and therefore cannot exhibit homologous recombination), is also merodiploid for both *rpoB364* and *rif18*; in this case, it was shown that the *metA*$^+$ locus continues to be 5% linked to

rpoB364 and is unlinked to the *rif18* marker. We noticed that the *rif18/rpoB364* merodiploid strains are less rifampicin-resistant than either the *rif18* haploid or the *rif18/rpoB*$^+$ diploid derivatives (data not shown), an observation consistent with the rifampicin-sensitivity phenotype associated with *rpoB364* (see below).

3. Results

3.1 *Isolation of a conditional-lethal* rpoB *mutant*

As described above, the *rpoB364* mutation was identified in the course of a search for *trans*-acting mutations that affect transcription of the osmotically regulated *proU* operon. This mutation caused a modest increase in *proU–lac* expression at all osmolarities tested (figure 1), to an extent sufficient to confer a clearly distinguishable phenotype on MacConkey–lactose agar plates. In addition, the *rpoB364* mutation conferred two interesting phenotypes:

(i) The *rpoB364* mutant was unable to grow or form colonies at 42°C in low-osmolarity medium such as LBON or half-strength minimal A (see table 3); addition of impermeable solutes such as NaCl (0·3 M) or sucrose (0·44 M), or even of permeable solutes such as glycerol (0·8 M), restored growth at 42°C. Thus, the mutants were not temperature-sensitive for growth on LB (which contains around 0·17 M NaCl). Growth under restrictive conditions led to extensive cell filamentation which was *recA*-independent (data not shown).

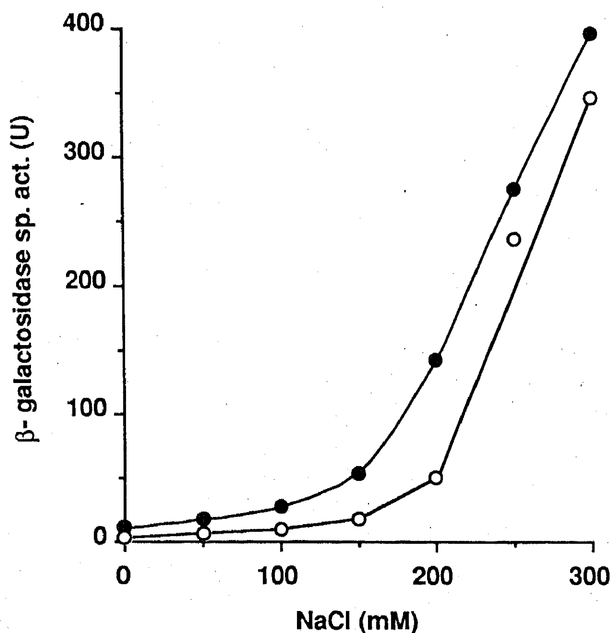


Figure 1. Expression of chromosomal *proU–lac* in GJ1312 (*rpoB*$^+$; ○) and GJ1313 (*rpoB364*; ●) as a function of osmolarity of growth medium. Specific activity of β-galactosidase was measured in cultures that had been grown for at least 10 generations at 30°C in K medium supplemented with the indicated concentration of NaCl.

(ii) When the plasmid vector pACYC184 was introduced into the *rpoB364* strain GJ1313 by transformation, Cm^r transformants were obtained at 30°C on LB plates at roughly the same frequency as that in similarly transformed isogenic *rpoB*$^+$ strain GJ1312. However, colonies of the former were flattened and less opaque, and the cells in them rapidly lost viability after 30 to 40 h of incubation and could no longer be subcultured reliably after this period. Thus, whereas the number of viable cells in each of these colonies was close to normal up to 24 h after plating of the transformants (on LB–Cm at 30°C), it had dropped by a factor of around a hundred after about 42 h (data not shown); after 60 h, the majority of surviving cells in a colony were either revertants or suppressor mutants (see below). In broth-inoculation experiments as well, transformants of the *rpoB364* strain grew to a density of approximately $1–3 \times 10^8$ cells/ml after which the viability (measured on antibiotic-free medium) dropped precipitously in the next 2 to 4 h, although there was no discernible lysis (figure 2). Similar results were obtained when Tet^r was used as the selection marker for introduction and maintenance of the pACYC184 plasmid in the *rpoB364* strain GJ1313-a, or even in a broth subculture without Tet or Cm addition (data not shown), indicating that the observed decrease in plating efficiency was not merely a gratuitous consequence of an inhibition of plasmid replication or partitioning during cell division. On the other hand, the transformants could be maintained almost indefinitely by repeated subculturing in the log phase of growth.

Genetic evidence that all the phenotypes above were the result of a single mutation was obtained in reversion-analysis experiments: following transformation of GJ1318 with pACYC184, one class of mutants that had been selected as survivors

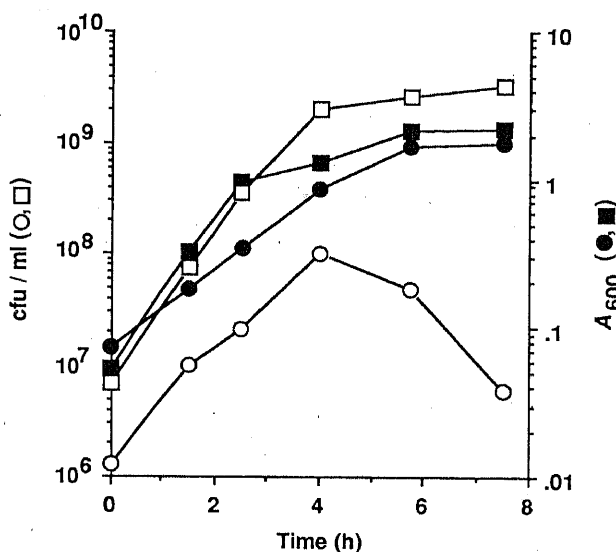


Figure 2. Comparison of viability of pACYC184 derivatives of GJ1312 (*rpoB*$^+$; □, ■) and GJ1313 (*rpoB364*; ○, ●) at different times of growth. At time zero in the figure, exponentially growing cultures of the two strains were diluted suitably in fresh LB–Cm medium to a final density of between 10^6 and 10^7 cfu/ml, and incubated at 30°C. A_{600} and viable counts (on LB at 30°C) were monitored for each culture at various time points as indicated.

into late stationary phase was shown also to have reverted simultaneously for the phenotypes of temperature sensitivity and *proU–lac* derepression (data not shown).

3.2 *Suppression of rpoB364 by intracellular overproduction of ppGpp*

Vinella and D'Ari (1994) have recently described an *rpoB* mutation, which they designated *ftsR1* or *rpoB369*(Fts), that confers an NaCl-suppressible temperature-sensitive-growth phenotype similar to that found by us for *rpoB364*. Temperature sensitivity in the *ftsR1* mutant was also reversed under conditions where the intracellular concentration of guanosine 3′,5′-bispyrophosphate (ppGpp) was increased.

We therefore tested the effect of intracellular ppGpp overproduction on the *rpoB364* mutant phenotype. For this purpose, we used the plasmid pALS10, which carries the full-length *relA* gene fused to the inducible *tac* promoter (Svitil *et al.* 1993). Results of efficiency-of-plating experiments indicated that introduction of pALS10 into the *lacI*^{q} *rpoB364* strain GJ1336 led to alleviation of the temperature-sensitive-growth phenotype on LBON medium (table 3). This effect was observed even when there was no addition of the inducer, isopropylthio-β-D-galactoside, to the plates; conceivably there is sufficient production of stable RelA protein from the multicopy plasmid for build-up of elevated cytoplasmic ppGpp concentrations even in the uninduced cultures (Gentry *et al.* 1993; Svitil *et al.* 1993). Expression of *relA* from the plasmid was associated also with inhibition of lethality mediated by pACYC184 in the *rpoB364* strain GJ1336 (data not shown).

Although these results indicated that the *rpoB364* mutation is very similar to *ftsR1*, strains carrying the latter (such as GC2553 and GC3698) were not killed by pACYC184 as were the *rpoB364* derivatives. This was demonstrated in a transductional cross with a P1 phage lysate prepared on GJ1313-a (which has a

Table 3. Efficiency of plating (eop) on LBON at 42°C of *rpoB364* strains.

Strain derivative	Relevant genotype	Plasmid	eop[a]
GJ1312	*rpoB*$^+$	Nil	0·9
GJ1313	*rpoB364*	Nil	$1·4 \times 10^{-3}$
		/pDV1	0·7
		/pDV2	$1·1 \times 10^{-3}$
GJ1318	*rpoB364 recA*	/pDV1	1·0
		/pDV2	$4·6 \times 10^{-3}$
GJ1335	*lacI*^{q} *rpoB*$^+$	Nil	1·0
		/pALS10	1·0
GJ1336	*lacI*^{q} *rpoB364*	Nil	$2·5 \times 10^{-2}$
		/pALS10	1·1

a, The reported values represent the ratio for cultures of each strain derivative of cfu/ml upon plating on LBON (with antibiotic supplementation as appropriate) at 42°C to that on the same medium at 30°C.

Kan^r-coding insertion linked to *rpoB364*) into GC2553/pACYC184. Approximately 60% of the Kan^r transductants (which is the expected frequency of contransduction of *rpoB364*) exhibited the distinctive colony morphology associated with the lethality described above, whereas the remainder were not killed on the plates; on the other hand, 100% of the Kan^r transductants were unable to grow on LBON medium at 42°C.

Contrary also to the situation with the *ftsR1* mutation (Vinella and D'Ari 1994), the phenotypes associated with *rpoB364* appeared to be independent of the chromosomal *relA* genotype of the strain. The *rpoB364* mutation was originally isolated and characterized in a *relA1* background (whose phenotypic behaviour is much the same as that of null mutants in *relA* [Metzger *et al.* 1989]); transduction of GJ1313-a to *relA*$^+$ (with a linked *recD*::Tn*10* marker) did not cause any alteration in the phenotypes specified by *rpoB364* (data not shown). The strain GJ1336 used in some of the experiments above is also *relA*$^+$.

3.3 *Mapping and complementation studies on rpoB364*

In this section, we describe the findings underlying the conclusion that the conditional-lethal mutation obtained above is an allele of *rpoB*, the gene encoding the β-subunit of RNA polymerase in *E. coli*.

(i) Conjugational and transductional mapping experiments, done with the aid of both a Tn*10* insertion (*zja-900*::Tn*10*) obtained in this study and a Tn*10*Kan insertion (*thi-3178*::Tn*10*Kan) from the collection of Singer *et al.* (1989), which were each linked approximately 50% to *rpoB364*, allowed us to localize the mutation to the vicinity of the *rpoB* locus at 90 min, with a clockwise gene order of *argE–rpoB364–thi-3178*::Tn*10*Kan–*zja-900*::Tn*10*–*metA* (data not shown). *rpoB364* was also linked 96–99% to two different rifampicin-resistance mutations in *rpoB* that were tested (data not shown).

(ii) Lysogens of the *rpoB364* strain GJ1308 or of its *recA* derivative GJ1318 for the specialized transducing phage λd*rif18* were constructed as described. These derivatives, which are merodiploid for genes around the *rpoB* locus, were complemented for all the three *rpoB364*-associated phenotypes (data not shown), indicating that the mutation is recessive to the allele carried on the temperate phage. We also tested different plasmid subclones constructed from λd*rif18* by Vinella and D'Ari (1994) for their ability to complement the temperature-sensitive growth phenotype of *rpoB364* strains. Plasmid pDV1 (carrying *rpoB*[*rif18*] and *rplL* genes) was able to reverse the plating-efficiency defect on LBON medium at 42°C of GJ1313 and GJ1318, whereas plasmid pDV2 (carrying only *rplL*) was unable to do so (table 3). These results permitted the conclusion that the mutation is in the *rpoB* gene.

(iii) Finally, the *rpoB364* mutants exhibited an increased sensitivity to rifampicin whereas their sensitivity to each of two other antibacterial agents, ampicillin and nalidixic acid, was unaltered (figure 3). This observation is consistent with the mutated gene being an *rpoB* allele, since the binding site for rifampicin resides in the β-subunit of RNA polymerase. Interestingly, the *ftsR1* (*rpoB369*) mutation described by Vinella and D'Ari (1994) also confers a rifampicin-sensitive phenotype.

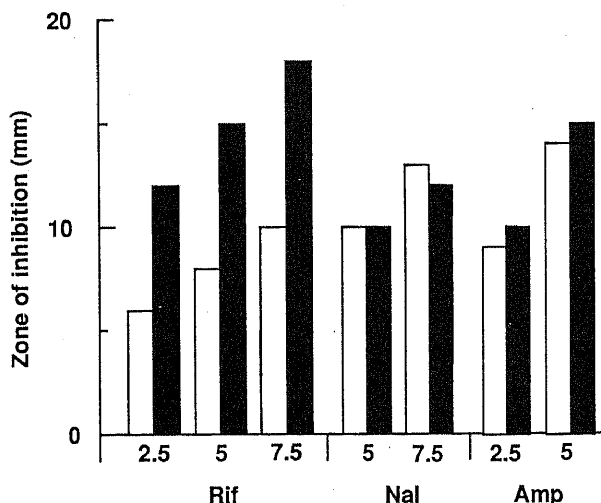
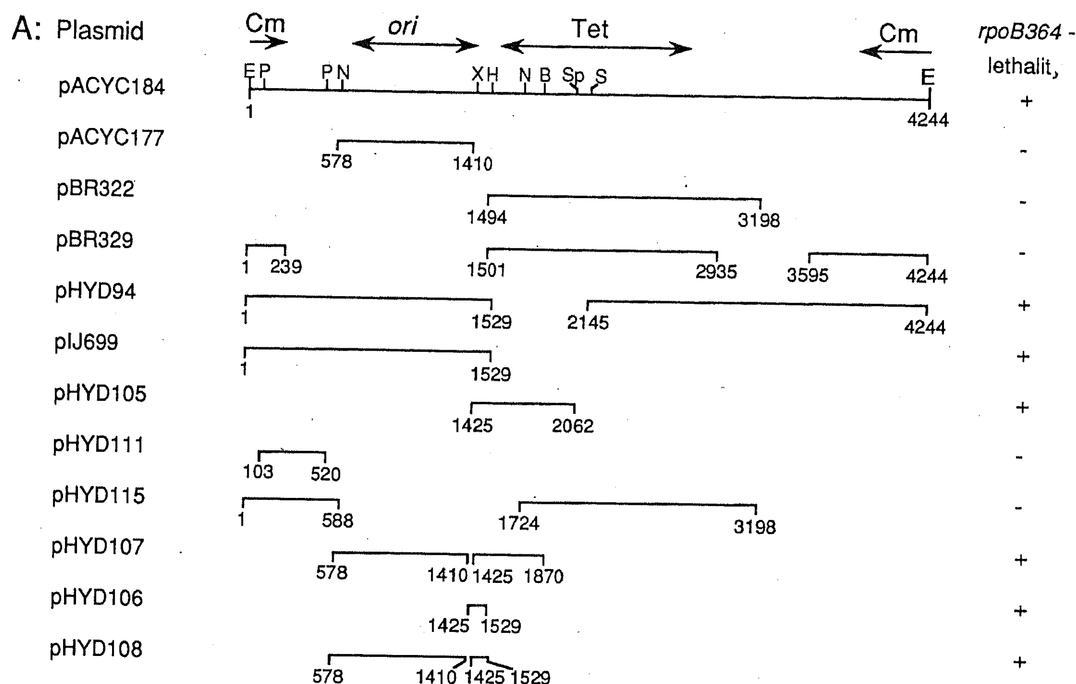


Figure 3. Sensitivity of GJ1312 (*rpoB*$^+$; open bars) and GJ1313 (*rpoB364*; solid bars) to antibacterial agents. The diameters of the zones of inhibition around discs carrying the indicated amounts (in µg) of antibacterial agents are plotted in the histogram. Nal, nalidixic acid.

3.4 *A 105-bp fragment from pACYC184 is sufficient to confer lethality in rpoB364 strains*

To determine whether the *rpoB364*-mediated lethality was associated with any particular segment of the pACYC184 molecule, we examined the effects in the *rpoB364* background of several other plasmids into which we had cloned different regions of pACYC184. The results of these tests are depicted in figure 4A. The plasmid vectors pBR322 and pBR329 did not confer lethality in GJ1313, nor even did pACYC177 which belongs to the same incompatibility group (P15A) as pACYC184. On the other hand, introduction of pIJ699 or pHYD94, derivatives of pACYC184 carrying different extents of vector DNA as shown in figure 4A, resulted in loss of viability of cultures in stationary phase, an observation similar to that described above for pACYC184.

A comparison of the region of pACYC184-equivalent DNA present on pIJ699 (lethality-positive) with that on pACYC177 (lethality-negative) suggested that the region of pACYC184 from base pair (bp) 1 to bp 577 or the region from bp 1411 to bp 1529, or both, was required for lethality (nucleotide numbering according to the scheme of Rose [1988a]). Neither an *Eco*RI–*Nhe*I fragment (1 to 588 bp) nor a *Pvu*II–*Pvu*II fragment (103 to 520 bp) from pACYC184, cloned into pBR322 (resultant plasmid pHYD115) and pUC19 (resultant pHYD111) respectively, conferred lethality upon introduction into GJ1313, whereas an *Xba*I–*Sph*I fragment (1425 to 2062 bp) and an *Xba*I–*Bam*HI fragment (1425 to 1875 bp) cloned into pUC19 (resultant pHYD105) and pACYC177 (resultant pHYD107) respectively did confer lethality. In subsequent subcloning experiments, the 105-bp *Xba*I–*Hin*dIII fragment (1425 to 1529 bp) was inserted into pUC19 and pACYC177 to generate plasmids pHYD106 and pHYD108 respectively, and GJ1313 transformant derivatives carrying



B:

Xba I

```
TCTAGATTTC AGTGCAATTT ATCTCTTCAA ATGTAGCACC TGAAGTCAGC CCCATACGAT
AGATCTAAAG TCACGTTAAA TAGAGAAGTT TACATCGTGG ACTTCAGTCG GGGTATGCTA

ATAAGTTGTA ATTCTCATGT TTGACAGCTT ATCATCGATA AGCTT
TATTCAACAT TAAGAGTACA AACTGTCGAA TAGTAGCTAT TCGAA
```

Hin dIII

Figure 4. (A) Correlation between extent of pACYC184 DNA carried on various plasmids and lethality phenotype conferred by each of them in the *rpoB364* mutant GJ1313. The 4244-bp-long pACYC184 DNA (linearized at the *Eco*RI site) is represented on the top line, and the positions of relevant restriction sites and known functional regions on the molecule are marked. Aligned beneath it are lines each of which represents the extent of pACYC184-equivalent DNA carried by the corresponding plasmid noted in the left column. Numbers below each line refer to the nucleotide coordinates in the pACYC184 sequence (Rose 1988a) that mark the ends of the aligned stretches of identity. Stationary-phase lethality or survival phenotype associated with each plasmid in *rpoB364* strains is denoted, respectively, by + or – in the right column. Restriction sites are: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nhe*I; P, *Pvu*II; S, *Sal*I; Sp, *Sph*I; and X, *Xba*I. References for nucleotide sequence data: pACYC184 (Rose 1988a); pACYC177 (Rose 1988b); pBR322 (Sutcliffe 1978); and pBR329 (Covarrubias and Bolivar 1982). (B) Nucleotide sequence of the 105-bp *Xba*I–*Hin*dIII segment of pACYC184 (Rose 1988a).

either of these plasmids were rapidly killed in stationary phase. This 105-bp contiguous stretch of DNA is present on all plasmids that confer lethality in the *rpoB364* background, whereas the major portion of it is absent from all plasmids that do not (figure 4A).

The 0·45-kb *Xba*I–*Bam*HI fragment of pACYC184 above was also cloned into a low-copy-number IncW-based plasmid vector, pMU575, and into a medium-copy-number pSC101-based plasmid vector, pCL1920, to generate pHYD109 and pHYD110 respectively. Neither of them conferred lethality in GJ1313, in contrast to the behaviour of the pACYC177 derivative (pHYD107) carrying this segment. These results suggest that the pACYC184-derived DNA fragment must be present in a certain minimum copy number (the lower and upper bounds of this copy number may well be represented by the copy numbers of pSC101 and pACYC177 respectively [Sambrook *et al.* 1989]) in the cell in order to be associated with stationary-phase lethality of *rpoB364* mutants.

3.5 *Mutations in hns suppress rpoB364/pACYC184-associated lethality*

Upon prolonged incubation of GJ1313/pACYC184 transformant colonies, mutant cells that had survived the lethality associated with stationary phase grew up to form opaque colonies upon restreaking. On low-osmolarity plates supplemented with Xgal, approximately half of all such colonies were deep blue in colour, suggesting that at least one class of mutations that rescued the cells from the lethal phenotype also led to further derepression of *proU* expression.

A strain (GJ1327) with a Tn*10*dKan insertion 90% cotransducible with the suppressor mutation in a blue papillary clone (GJ1323) that had been cured of pACYC184 was obtained as described above, and was used to show that the suppressor mutation acted independently of, and additively with, the *rpoB364* mutation in derepressing *proU* expression (table 4). Transduction experiments (data not shown) with the Tn*10*dKan insertion also indicated that the suppressor mutation in each of 16 independent blue suppressor mutants that were tested all mapped to the same locus, which we show below is the *hns* gene.

(i) The Tn*10*dKan-insertion strain was used along with the panel of mapping strains of Singer *et al.* (1989) to map the mutations to 27 min, in the *trp–supF* region (data not shown). The *zch-506*::Tn*10* insertion in CAG12169, which is cotransducible 43% with *trpA* and 83% with *supF* (Singer *et al.* 1989), was linked

Table 4. Expression of chromosomal *proU–lac* in *rpoB364* and *hns-200* mutants.

Strain[a]	Relevant genotype	β-Galactosidase sp. act. (U)[b]	
		Low	High
GJ1332	*rpoB*⁺ *hns*⁺	5	260
GJ1328	*rpoB364 hns*⁺	15	410
GJ1329	*rpoB*⁺ *hns-200*	38	305
GJ1327	*rpoB364 hns-200*	61	500

a, This panel of four isogenic strains was constructed by two sequential transductions of the *rpoB* and *hns* regions into GJ11, with the aid of the *zja-900*::Tn*10* and *zch-900*::Tn*10*dKan markers respectively.

b, Specific activity of β-galactosidase was measured in cultures that had been grown for at least 10 generations at 30°C in glucose–half-strength minimal A medium without (Low) or with (High) 0·3 M NaCl supplementation.

88% to the suppressor mutation (*hns-200*) originally isolated in GJ1323, a value similar to one expected for an *hns* mutation.

(ii) The majority of mutations in *hns* that derepress *proU* expression have previously been shown also to activate the cryptic *bgl* operon in *E. coli* (Higgins *et al.* 1988; Lucht and Bremer 1991). It was found that, with the exception of GJ1323, all the independently isolated mutants above had also become Bgl$^+$.

(iii) Finally, in each of three mutants tested (including GJ1323), the plasmid pDIA510 (which carries a 0·9-kb chromosomal DNA fragment encompassing the *hns*$^+$ gene) was able to complement the suppressor mutation, as judged by the *proU–lac* expression phenotype.

The *hns-200* mutation in GJ1323 was also shown to be proficient in suppressing the lethality phenotype associated with the plasmids pHYD106 and pHYD108, which carry the 105-bp *Xba*I–*Hin*dIII fragment of pACYC184 cloned in the vectors pUC19 and pACYC177 respectively.

4. Discussion

In this report we have described the isolation of a mutant that exhibited the following phenotypes: (i) filamentation and inviability in high-water-activity media at 42°C, suppressible either by decreasing the water activity of the growth medium or by increasing the intracellular ppGpp concentration; (ii) partial constitutivity of the osmotically regulated *proU* operon at the permissive temperature; and (iii) lethality in stationary phase at the permissive temperature after transformation with the plasmid vector pACYC184. Analysis of revertants indicated that all the phenotypes were the consequence of a single mutation in the strain. Data from mapping, rifampicin-sensitivity and complementation studies indicate that the mutation is in *rpoB* (*rpoB364*), a conclusion supported by the earlier report by Vinella and D'Ari (1994) of an *rpoB* (*ftsR1*) mutant with a conditional-lethal filamentation phenotype identical to that we have described. As suggested by Vinella and D'Ari (1994) for their *ftsR1* mutant, it is likely that the phenotype of the *rpoB364* strain also is a consequence of reduced affinity of ppGpp binding to the mutant RNA polymerase and the associated alterations in gene expression in the strain.

It is interesting that pACYC184 transformants of the *rpoB364* strain are killed in stationary phase, and that this killing is suppressed either by ppGpp overproduction or by mutations in *hns*. We have shown that the presence of the 105-bp *Xba*I–*Hin*dIII fragment of pACYC184 in multiple copies is sufficient to confer the stationary-phase lethality phenotype. The mechanism by which this novel and unusual tripartite interaction between the 105-bp fragment, the mutant *rpoB* gene product and the H-NS protein result in cell suicide is not clear, but we speculate on some possibilities below.

Is it possible that a *direct* interaction between the DNA fragment, the H-NS protein (perhaps an isoform specific to, or more abundant in, stationary-phase cells), and RNA polymerase enzyme bearing the mutant β-subunit is in some way responsible for cell death? This region of pACYC184 DNA (figure 4B) is immediately upstream of the *tet* gene but does not have any recognizable promoter sequences to which RNA polymerase might bind, nor is it predicted to exhibit structures or motifs that are targets for preferential binding of H-NS (Tanaka *et al.* 1991; Owen-Hughes *et al.* 1992; Zuber *et al.* 1994). We therefore consider it unlikely

that cell death is a consequence of direct interaction between the three players in this macabre drama.

The alternative possibility is that the interaction is *indirect*, and is mediated by one or more additional gene products whose concentration in stationary phase is modulated by RNA polymerase and H-NS. Thus, whereas overproduction of ppGpp in the *rpoB364* strain may restore expression of all genes to levels equivalent to those in the wild type, the absence of H-NS in the *rpoB364* strain might correct the altered expression of only those genes that are involved in stationary-phase lethality. As mentioned above, H-NS has been implicated in several aspects of stationary-phase gene regulation and metabolism, and so too RNA polymerase, in particular the σ^s-bearing holoenzyme (see Matin *et al.* 1989; Kolter *et al.* 1993; Hengge-Aronis 1993 for reviews).

We therefore propose that in stationary-phase *rpoB364 hns*$^+$ cells, the concentration of an as yet unidentified gene product is altered compared to that in *rpoB*$^+$ or in *rpoB364 hns* cells and that this alteration when accompanied by the presence of the 105-bp DNA fragment of pACYC184 results in lethality. Whether this gene is the same as any identified earlier by studies on mutants that are killed in stationary phase, such as *surA* (Tormo *et al.* 1990), *surB* (Siegele and Kolter 1993), *dps* (Almirón *et al.* 1992) or *psp* (Weiner and Model 1994), remains to be determined.

The role of the pACYC184-derived DNA fragment in mediating the cell suicide phenomenon is at present obscure. The fact that the presence of the fragment on low-copy-number (pMU575) or medium-copy-number (pSC101) plasmids is not associated with cell death but its presence on high-copy-number plasmids (pACYC184 or pUC19) is so associated might suggest titration by the fragment of a limiting 'survival' factor in the *rpoB364 hns*$^+$ strain. A similar titration effect might explain the lethality associated with overexpression of either Tn*5* transposase or the core polypeptide of *RhsA* in *E. coli* (Weinreich *et al.* 1994; Vlazny and Hill 1995). We are at present undertaking additional genetic studies, in particular the isolation of extragenic suppressors (chromosomal or multicopy) of the lethality phenotype in genes other than *hns*, and of suppressors that map in pACYC184, in further attempts to understand this unusual phenomenon.

Acknowledgements

We thank Mike Cashel, Richard D'Ari, Patrick Dennis, David Hopwood, Masayori Inouye and Philippe Lejeune for various strains, plasmids and phages used in this study. We are also grateful to Dipankar Chatterji, Durgadas Kasbekar, Ramesh Sonti and colleagues in the laboratory for their advice and comments on the manuscript. The work was supported in part by funds from the Department of Biotechnology and the Department of Science and Technology, Government of India. P.J. was the recipient of a research fellowship from the Council of Scientific and Industrial Research.

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