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Common sequence determinants of the response of a prokaryotic promoter to DNA bending and supercoiling

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Inhibiting the activity of DNA gyrase by mutation or by drugs in *S.typhimurium* causes the loss of transcription attenuation in the histidine operon. We show that gyrase activity is needed to maintain high-level expression of the $tRNA^{His}$ gene (*hisR*), a prerequisite for proper functioning of the attenuation mechanism. A point mutation in the promoter of the tRNA gene cluster which includes the *hisR* gene, specifically relieves the promoter response to negative supercoiling thereby restoring full *hisR* transcription (and, in turn, *his* attenuation) in the presence of a defective gyrase. The very same mutation, a single base-pair substitution between the −10 box and the transcription start site (−7), was found independently among suppressors of the transcriptional deficiency caused by disruption of DNA curvature upstream from the *hisR* promoter. We show that the −7 change does not lead to a generalized increase of promoter strength; the effects of the mutation are seen only when gyrase is inhibited or the upstream curvature is altered. This suggests that the upstream curvature intervenes in the same initiation step which is sensitive to superhelical tension. Three additional promoter mutations correcting (or alleviating) the effects of the upstream alteration (including a change at −8) are described and discussed.
Key words: bent DNA/gyrase/stable RNA/supercoiling/upstream activation

Introduction

The topological state of DNA is an important determinant of its biological activity. In prokaryotes, DNA supercoiling has long been known to influence transcription of many genes (reviewed by Pruss and Drlica, 1989; Drlica, 1984). In spite of extensive scrutiny, this phenomenon eludes full understanding; different mechanisms appear to operate in different systems as some genes are activated, others are inhibited and others still are unaffected by the same supercoiling change (Menzel and Gellert, 1983, 1987; Jovanovich and Lebowitz, 1987; Tse-Dinh and Beran, 1988; Higgins *et al.*, 1988; Richardson *et al.*, 1988). Underlying such complexity is the fact that supercoiling can affect the DNA helix in various ways by modifying its energy (torsional strain) and structure (helical pitch and axial writhing; reviewed by Wang, 1985; Maxwell and Gellert, 1986). Any of these factors can influence promoter reactivity either directly or indirectly through effects on bending and wrapping of the DNA around proteins in chromatin-like structures (Dorman *et al.*, 1990; Hulton *et al.*, 1990; Schmid, 1990). One may expect that in some cases, particularly those where unconstrained superhelicity plays the predominant role, the information specifying a given response is encoded in the promoter primary sequence. To date, little is known concerning these sequence determinants. In a systematic dissection of the *gyrA* promoter of *E.coli* (a promoter whose activity is stimulated by gyrase inhibition, *i.e.*, by DNA relaxation), Menzel and Gellert (1987) found the minimal sequence required for the response to lie within a 20 bp segment spanning positions −19 to +1 of the promoter region. More elusive is the analysis of genes which, contrary to *gyrA*, require high levels of negative supercoiling for optimal transcription. Most current information stems from *in vitro* work with deproteinized templates, in which all main promoter elements have been implicated in the supercoiling response (Borowiec and Gralla, 1987; Meiklejohn and Gralla, 1989). However, it is also apparent that *in vitro* results may not always be representative of the *in vivo* situation. Transcription of a tyrosine tRNA gene and of ribosomal RNA genes were reported to be critically dependent on template supercoiling *in vitro* (Lamond, 1985; Glaser *et al.*, 1983) but unaffected by supercoiling changes *in vivo* (Lamond, 1985; Wahle *et al.*, 1985). Furthermore, topological features that exist on the chromosome may be lost when genes are carried on multi-copy plasmids. For example, suppression of promoter mutant *leu-500* by topoisomerase I mutations in *Salmonella typhimurium* is only observed in the chromosome and not on plasmid clones (Richardson *et al.*, 1988).

In *S.typhimurium*, regulation of histidine operon transcription attenuation relies on efficient expression of the locus (a tRNA gene cluster) which includes the *hisR* gene, the sole $tRNA^{His}$ gene in the cell (Johnston *et al.*, 1980; Bossi, 1983). As little as a 50% reduction in the rate of synthesis of $tRNA^{His}$ offsets the attenuation mechanism resulting in a several-fold increase in *his* expression levels (Lewis and Ames, 1972). This phenotype has been useful in the identification of *cis*- and *trans*-acting elements needed for full performance of the *hisR* promoter *in vivo* at its natural chromosomal location. One such study revealed that a significant fraction of *hisR* transcriptional activity derives from the activating effect of an upstream sequence responsible for introducing curvature in the DNA. In a particular His-constitutive strain, *hisR* transcription is diminished as a result of a three base-pair deletion centered around −70 (*hisR1223*) which alters the DNA bending pattern (Bossi and Smith, 1984). Two additional classes of mutations causing *his* deattenuation (*hisW* and *hisU*) were more recently shown to affect the genes encoding the two subunits of DNA gyrase: *gyrA* and *gyrB*, respectively (Rudd

and Menzel, 1987). The possibility that the regulatory defect reflects a drop in $tRNA^{His}$ gene expression (as opposed to an alteration of histidine operon promoter/attenuator functions) was suggested by some lines of evidence such as the reduced $tRNA^{His}$ charging activity of *hisW* and *hisU* extracts (Lewis and Ames, 1972) and the dramatic dependence on negative supercoiling exhibited by the *hisR* promoter in an *in vitro* transcription system where the promoter is at least one order of magnitude stronger if the template is fully supercoiled than if it is relaxed (our unpublished results). Conclusive demonstration of gyrase involvement in the transcription of the $tRNA^{His}$ gene is provided in this paper. We show that the His-constitutive phenotype of a *hisU (gyrB)* mutant is suppressed by a point mutation in the *hisR* promoter. Thus, the mutation identifies a specific determinant of the transcriptional response to negative supercoiling. Intriguingly, the same sequence change appeared independently in a hunt for phenotypic suppressors of the upstream DNA bending defect. This change is outside the consensus regions (position −7) and all by itself (proficient gyrase and intact upstream region) has no significant effect on promoter performance under standard growth conditions.

Results

Altered DNA supercoiling in a histidine regulatory mutant

Based on genetic analysis, Rudd and Menzel (1987) suggested that *hisU1820*, initially isolated as a *his* regulatory mutation in *S.typhimurium* (Roth *et al.*, 1966; Anton, 1968), is an allele of *gyrB*. This lesion is distinct from other *hisU* mutations which affect *rnpA*, a nearby gene coding for the protein component of RNase P (Bossi and Cortese, 1977). A direct test for the presence of a DNA supercoiling defect in a strain carrying *hisU1820* is provided by the experiment in Figure 1. Plasmid DNA extracted from transformed cells is subjected to electrophoresis on an agarose gel containing the intercalating agent chloroquine. One can see that at a drug concentration at which plasmid purified from a wild-type strain is still nearly fully supercoiled, DNA from an isogenic derivative carrying *hisU1820* exhibits extensive relaxation. That the supercoiling defect is directly responsible for the altered regulation is confirmed by the finding that the *his* deattenuation phenotype of *hisU1820* is suppressed by mutations in the topoisomerase I gene (*topA*; results not shown).

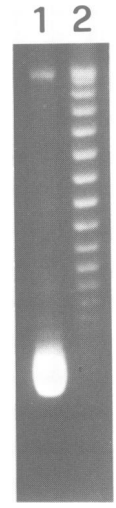

Fig. 1. Effect of the *hisU1820* mutation on plasmid DNA supercoiling. Isogenic strains were transformed with plasmid pKK232-8. Overnight NB cultures of transformed cells, grown at 30°C, were diluted two-fold in NB and shaken for 2 h at 37°C. Plasmid DNA was extracted and analyzed on a 10 μg/ml chloroquine/1.2% agarose gel as previously described (Figueroa and Bossi, 1988). At this chloroquine concentration molecules which are more negatively supercoiled migrate faster in the gel. **1**. plasmid DNA extracted from strain MA463 ($hisU^{wt}$); **2**. plasmid DNA extracted from strain MA578 (*hisU1820*).

Promoter mutation relieves supercoiling response

As indicated above, some lines of evidence suggested that the role of DNA gyrase in *his* regulation is at the level of $tRNA^{His}$ gene transcription. Accordingly, high levels of negative supercoiling might be needed for optimal activity of the promoter of the tRNA gene cluster which includes *hisR*, the single-copy $tRNA^{His}$ gene (Bossi, 1983). If correct, the *his* regulation circuitry would constitute a suitable system for analysis of the sequence determinants of a promoter response to DNA supercoiling. Mutations relieving the supercoiling sensitivity of the *hisR* promoter should restore optimal $tRNA^{His}$ gene expression and, in turn, *his* attenuation in a mutant gyrase background. We undertook a search for such phenotypic suppressors (starting with the *hisU1820* mutant) by setting up selective conditions against the growth of cells which express the *his* operon at high level (see Materials and methods). Following isolation, mutants were screened for the presence of *hisR*-linked mutations. One strain was found which passed all genetic tests, including the verification of the suppressor phenotype in a background other than the one where the mutation was initially isolated. The *hisR* region from the mutant (*hisR10107*) was cloned and sequenced. This analysis revealed that *hisR10107* results from a single base-pair change, a C:G to T:A transition 7 base-pairs upstream from the main transcription start site (Figure 2).

The effects of the −7 mutation on *his* attenuation were quantified using a chromosomal *his−lac* transcriptional fusion. Measurements of β-galactosidase activity show that *hisR10107* does not significantly change the levels of *his−lac* expression in a strain carrying a wild-type gyrase (Table I). In contrast, the phenotype of *hisR10107* is clearly detected in the gyrase mutant background. Whereas β-galactosidase activity increases sharply upon introduction of *hisU1820* into a $hisR^{wt}$ strain, effects are marginal when the gyrase mutation is combined with *hisR10107* (Table I). This strongly suggests that *his* deattenuation in the gyrase mutant results from a drop of *hisR* promoter activity and that the −7 mutation suppresses the defect because it renders the *hisR* promoter insensitive to the gyrase mutation. It should be noticed that suppression is not specific to the particular *hisU1820* allele but is also observed with other *gyrB* mutations (not shown) and, most significantly, even in a gyr^{wt} strain exposed to a gyrase inhibitor (see below).

Further work was done in order to verify that the suppressive properties of *hisR10107* stem from a specific effect on supercoiling response and not from a generalized increase of *hisR* promoter strength or from creation of a new, supercoiling-insensitive promoter site (an alternative −10 box is produced as a result of the sequence change). Wild-

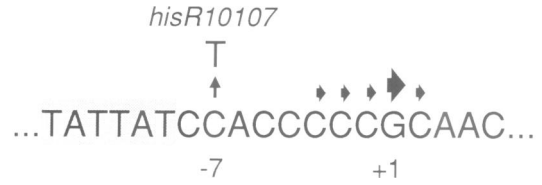

Fig. 2. DNA sequence change in suppressor of the *his* deattenuation defect of a gyrase mutant. Isolation, cloning and sequencing of suppressor mutation *hisR10107* is described in the text. The pattern of transcription initiation sites was determined in the experiment in Figure 3.

Table I. Effect of *hisR* promoter mutations on expression of a *his−lac* operon fusion in the presence and absence of the *hisU1820* allele[a].

	β-galactosidase activity[c]		
relevant allele[b]	wild-type	*hisU1820*	deattenuation ratio
hisR$^{wild-type}$	467	7860	16.8
hisR10107	348	490	1.4

[a] The *lacZ* gene is under the control of the *his* operon promoter/attenuator. Due to the inverse relationship between *his* deattenuation and tRNA^{His} gene (*hisR*) expression, higher β-galactosidase values are indicative of reduced *hisR* promoter activity.
[b] All strains used (MA463, MA 465, MA578 and MA580) are isogenic except for the indicated alleles. Their construction is described in the Methods section; their complete genotypes are given in Table III.
[c] Cells were grown in E medium supplemented with all aminoacids at 37°C (including the *hisU1820* strains which, because of their ts phenotype, were otherwise routinely cultured at 30°C). Values of β-galactosidase activity represent the averages of 3 independent, duplicated determinations. Standard errors were <10% in all cases.

type and mutant promoters were fused to the coding sequence of the chloramphenicol acetyl transferase gene (*cat*) on plasmid pKK232-8 (Brosius, 1984) as described in Materials and methods. Transcripts from transformed *E.coli* cells were purified and analyzed. Results in Figure 3 show that the −7 mutation does not alter the selection of initiation sites nor their relative utilization frequencies. Furthermore, these data indicate that there is no detectable difference in steady-state levels of RNA transcribed from mutant and wild-type promoters. In agreement with the latter findings, the levels of CAT activity in cells transformed with plasmid pKP6-1 (carrying the mutant promoter) are only slightly higher than those measured in cells that harbor the wild-type promoter clone (pKP1-13; Table II). This confirms that the *hisR10107* mutation does not produce a significant increase of *hisR* promoter strength on plasmid.

The responses of cloned promoters to supercoiling modulation were initially tested by assaying CAT activity in extracts of a *hisU1820* strain transformed with plasmids pKP1-13 or pKP6-1. These experiments showed small effects suggesting a reduced sensitivity of plasmid-borne promoters to DNA supercoiling changes. Responses were therefore tested under more stringent conditions involving a treatment expected to inactivate gyrase completely. The levels of newly made *cat* transcripts (^{32}P-labelled) were determined immediately before or 10 min after exposing growing cells to a lethal dose of gyrase inhibitor

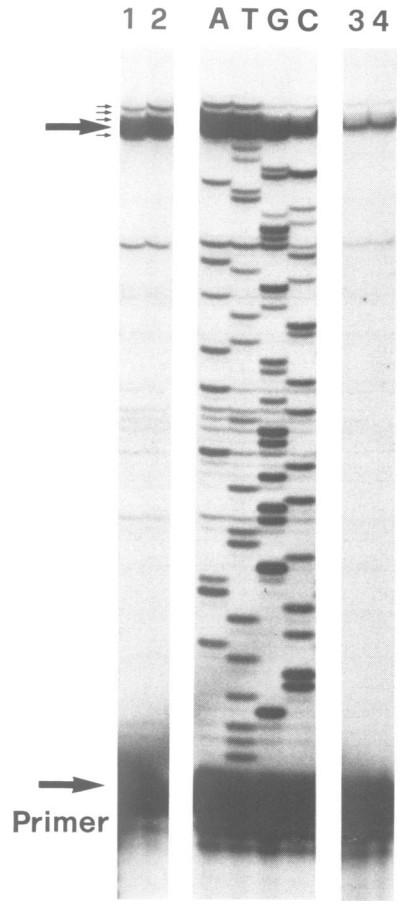

Fig. 3. 5′-end mapping and sequence analysis of transcription products from wild-type and mutant *hisR* promoters. RNA was extracted from strain SU1675 transformed either with plasmid pKP1-13 (*hisR*^{wt}) or with plasmid pKP6-1 (*hisR10107*) and analysed as described in Materials and methods; odd-numbered lanes: primer-extension products of RNA from *hisR*^{wt}; even-numbered lanes: primer-extension products of RNA from *hisR10107*. In lanes 3 and 4, primer was in a 20-fold excess relative to RNA in order to detect possible differences in the level of transcripts. The sequence reference was obtained using RNA from *hisR*^{wt} as template.

Table II. Comparison of the activities of wild-type and mutant *hisR* promoters on plasmid[a].

plasmid	*hisR* allele	CAT/BLA ratio×1000[b]
pKP1-13	wild-type	758
pKP6-1	*10107*	801

[a]Promoter activities are expressed as ratios of CAT to BLA specific activities (in nmol/min per mg of protein) measured as previously described (Figueroa and Bossi, 1988). Normalization to BLA activity automatically corrects for possible copy number differences.
[b]Values are the averages of three independent determinations. Standard error was <5% in all cases.

coumermycin. Results in Figure 4 show that the amount of transcript from the wild-type promoter is significantly reduced in drug-treated cells whereas transcription from the *hisR10107* promoter remains nearly unaffected. These data are consistent with what observed in the chromosome (above) and confirm that the −7 change identifies a specific

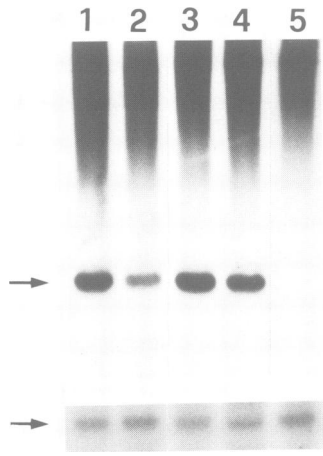

Fig. 4. Effect of gyrase inhibition on the activities of wild-type and mutant promoters on plasmid. ^{32}P-pulse-labelled *cat* RNA was hybrid-selected using the complementary sequence present on an M13 recombinant phage. Hybridized RNA was detected by autoradiography in an agarose gel where it co-migrates with the phage DNA band (see Materials and methods). Similar detection of the *bla* transcript in a separate aliquot of the RNA preparation (using a suitable M13 clone) provided an internal standard. Coumermycin A_1 (Sigma) was added to a final concentration of 100 μg/ml from 10 mg/ml stock solution in dimethyl sulfoxide (DMSO). Cells which were not treated with the drug were exposed to equivalent amounts of the drug solvent. Labelling of plasmid-carrying cells (SU1675) was started 10 min after addition of the drug (or of the DMSO in the untreated samples) and lasted for 5 min. **1**. RNA from plasmid pKP1-13 (wild-type promoter) in the absence of coumermycin; **2**. RNA from pKP1-13 following coumermycin addition; **3**. RNA from plasmid pKP6-1 (*hisR10107* promoter) in the absence of coumermycin; **4**. RNA from pKP6-1 following coumermycin addition; **5**. RNA from cloning vector pKK232-8 which carries the intact *cat* coding sequence but no promoter to express it. The top arrow indicates the RNA selected with the *cat* sequence; the bottom arrow indicates the RNA selected with the *bla* sequence.

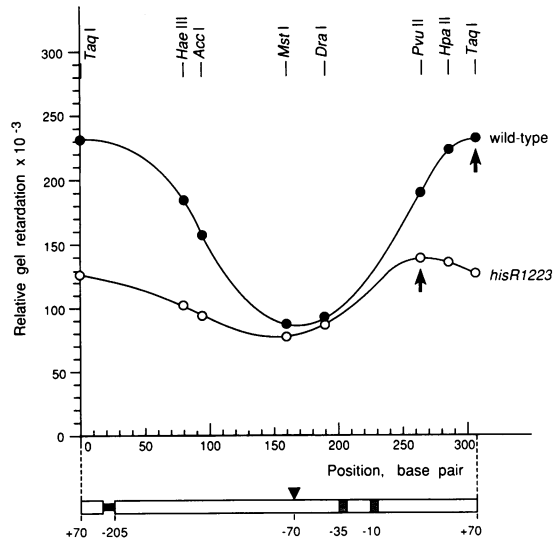

Fig. 5. Mobilities of circularly permutated fragments in polyacrylamide gels. Tandem direct duplications of promoter-containing fragments from *hisR*^{wt} and from *hisR1223* were constructed as described in Materials and methods. Duplicated material spans positions −205 to +86 relative to the main transcription start site (Bossi, 1983). A 10 bp linker region separates each duplicated unit from the other. Gel electrophoresis was as previously described (Bossi and Smith, 1984). Migration distances were normalized to that of a 267 bp fragment of *Hae*III-cleaved pBR322 DNA used as reference in all of the electrophoretic separations. The peaks of maximal gel retardation are indicated by arrows.

determinant of the *hisR* promoter response to negative supercoiling.

A promoter mutation affecting bending of the DNA

Existence of an intrinsic curvature in the DNA upstream from the *hisR* promoter was originally inferred from the abnormally retarded migration of restriction fragments in polyacrylamide gels (Bossi and Smith, 1984). The upstream sequence contains the typical, periodically spaced A:T runs known to induce bending of the helix axis (Koo *et al.*, 1986). A role of the curvature in transcriptional stimulation was suggested by the lower activity of a promoter variant in which a small upstream deletion restores 'closer to normal' fragment mobility (Bossi and Smith, 1984). The deletion mutation, *hisR1223*, spans positions −70 to −72 relative to the main transcription start site. To better define the effects of this lesion on DNA conformation and locate its position relative to the overall bending pattern, we applied the technique of Wu and Crothers (1984) based on comparing the mobilities of circularly permutated fragments where the presumptive curvature is at varying distances from the fragment ends. This was done for wild-type as well as for the *hisR1223* mutant. Results in Figure 5 clearly show that the centre of bending falls very near the site of the *hisR1223* mutation (−70). Presence of the three base-pair deletion causes a drastic decrease of planar curvature but not its complete loss. Furthermore, the mutation appears to modify the overall phase of the curvature as shown by the shift in the point of maximal electrophoretic retardation (see arrows). The residual curvature may be responsible for the relatively high activity of the *hisR1223* promoter (only ~50% lower than wild-type; Bossi and Smith, 1984) compared to promoters with *in vitro* deletions which remove the bent segment entirely (our unpublished results).

Suppression of the bent DNA mutant

As in the case of the gyrase mutant above, lower tRNA^{His} gene expression in strains carrying *hisR1223* causes constitutive *his* operon deattenuation. By analogy to the former, finding second-site suppressors of *hisR1223* could provide some new clues as to the mechanism of upstream activation. We applied the genetic selection to strain MA62 as described in Materials and methods. Suppressors of the *his* regulation defect occurred at high frequency. When tested, the vast majority of these mutants appeared to be genetically unstable and to result from the genetic amplification of the *hisR* region. Stable suppressors were rare and, when characterized, they all turned out to be genetically linked to the initial *1223* mutation. Cloning and sequence analysis of 7 such mutants revealed that they represent 4 classes of changes in different promoter domains: the region upstream from −35, the −35 box and the segment between −10 and the transcription start site (Figure 6). Intriguingly, one of the four changes is the same *hisR10107* allele independently found as a suppressor of the gyrase effects.

The relative strengths of the four suppressors were measured both on the chromosome (*via* the inverse relationship with *his* attenuation levels) as well as on plasmid-borne fusions to the *cat* gene. The two sets of data, graphically combined in Figure 7, are consistent with each

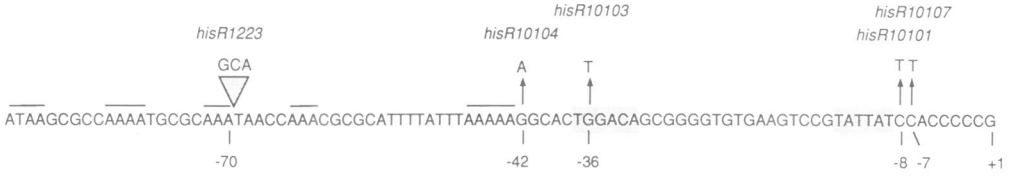

Fig. 6. DNA sequence changes in suppressors of bent DNA mutant. The isolation of *hisR1223* phenotypic suppressors and their molecular characterization are described in Materials and methods. The sequence is that of the anti-sense strand, 5′ to 3′.

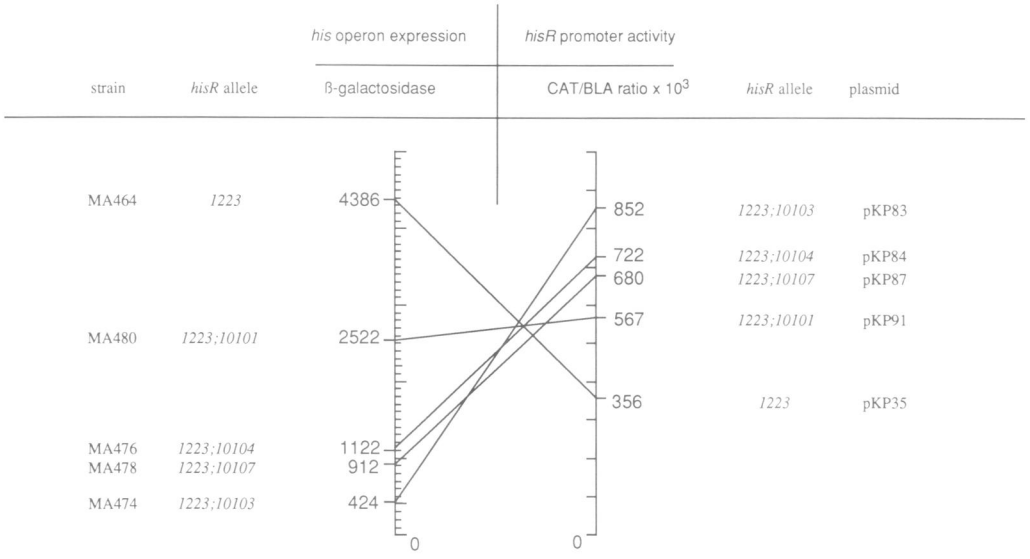

Fig. 7. Quantification of activities of *hisR1223* and second-site suppressors. The relative strengths of the four phenotypic suppressors of *hisR1223* can be inferred from the extents to which they restore attenuation of a *his-lac* operon fusion. These data inversely correlate with the activities of the various promoter variants measured directly on plasmid-borne fusions to the *cat* gene (see text). Numbers of measurements and standard errors are the same as in Tables I and II. An independent calibration (on other mutants) of the negative correlation between *his* deattenuation and $tRNA^{His}$ gene expression is obtained combining the data in Lewis and Ames (1972) and Bossi and Smith (1984).

other. The strongest promoter is produced by the G:C to T:A transversion which brings the sequence of the −35 box (TGGACA) to a perfect match with the consensus hexamer (TTGACA; Figure 6). This change could be regarded as a classical 'promoter up' mutation although conclusive demonstration must await measuring its effects separately from the upstream deletion. In the case of the −7 mutant, where such separation has been possible (above), we have shown that the change does not lead to a generalized increase of promoter strength provided that gyrase is fully functional and the upstream region is intact. It appears, therefore, that the step affected by the −7 mutation is rate-limiting only under certain promoter weakening conditions. Possibly, this is a general property of mutants in this region (Delamarche *et al.*, 1987). Suppression of the −70 lesion suggests that the need for upstream activation is bypassed upon removing the supercoiling-sensitive step (see Discussion). It seems likely that the change at −8, although less efficiently, acts by a similar mechanism. The double mutant −8/−70 appears to be less sensitive to gyrase inhibition than either the −36/−70 or the −42/−70 combinations (Figure 8*b*). The data in Figure 8 also confirm the notion that the supercoiling response is unrelated to promoter strength. Although weaker than the wild-type promoter, the −7/−70 and −8/−70 mutants are less responsive to supercoiling changes (Figures 7 and 8). Finding that none of the *hisR1223* suppressors exhibits a supercoiling sensitivity as dramatic as that observed with the wild-type promoter (compare panels *a* and *b* in Figure 8), suggests that integrity of the upstream region is needed for a full response. Unfortunately, this cannot be adequately tested using the *his* regulation circuitry as the strain carrying the −70 mutation alone shows high *his* deattenuation values which reach a plateau at the lowest novobiocin concentration (not shown).

The change at −42 is the only suppressor likely to act by restoring upstream activation. Such restoration does not seem to involve the reintroduction of the initial curvature as the suppressor mutation has only a slight retarding effect on fragment mobility in gels (data not shown). Yet, it seems likely that DNA trajectory is affected in the mutant. The −42 substitution is adjacent to one of the A:T tracts which determines overall curvature (Figure 6). As a result of the G:C to A:T transition, the beginning of the A:T tract, thus presumably the beginning of the curved region on the promoter−proximal side, is shifted one base-pair closer to the −35 region (see Figure 6). This is expected to modify the phase of the entire upstream portion relative to the main promoter elements. Conceivably, the change could bring back some geometric features which were lost as a result of the *1223* mutation. Alternatively, the action of the −42 suppressor could derive from the positive effect of having the residual curvature of *hisR1223* closer to the promoter (Bracco *et al.*, 1989; McAllister and Achberger, 1989).

Discussion

Promoter DNA undergoes drastic conformational changes during initiation of transcription. Following binding of RNA

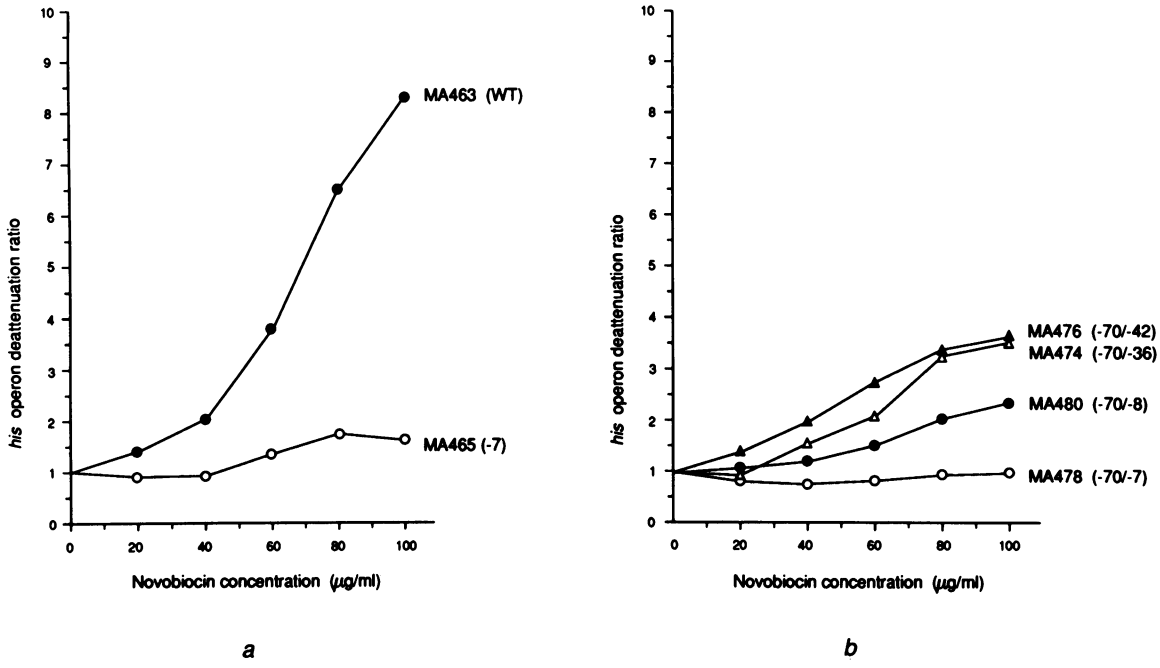

Fig. 8. Effect of inhibiting DNA gyrase on *his* operon deattenuation in *hisR* mutants. All strains harbour the same *his*−*lac* transcriptional fusion. Cells were grown in the presence of the indicated, sublethal doses of novobiocin (Gellert *et al.*, 1986). Plotted values represent the ratios of β-galactosidase activity measured in the presence of novobiocin over the corresponding activity in the absence of the drug. Each point is the average of at least three independent measurements in duplicate. Standard error was $<10\%$ in all cases.

polymerase, which may induce a substantial curving of the helix axis (Buc, 1986), the DNA is melted in a short region encompassing the site of initiation (Siebenlist, 1979). While it is generally accepted that the strength of a promoter signal largely reflects the quality of its contacts with the enzyme, less clear is the extent to which sequence information also specifies the 'predisposition' of the region to the above structural transitions. Although it may not be essential for promoter function (and thus not revealed in most hunts for promoter mutants) this intrinsic reactivity could influence initiation kinetics and be particularly important in highly expressed genes (Deuschle *et al.*, 1986). Furthermore, the availability of a promoter sequence to bending and/or melting during initiation is expected to determine its sensitivity to topological determinants. In the work described here, we isolated and studied mutations affecting a bacterial promoter response to negative supercoiling and to the effects of upstream DNA curvature. Three out of four such mutations lie outside the canonical promoter elements and may act by directly affecting the physical properties of DNA. The C:G to T:A transitions at positions −8 and −7 are likely to influence melting of promoter DNA. Both changes are within the promoter portion which is unwound by polymerase in the formation of the open complex (Siebenlist, 1979). The high C/G content of this region, perhaps in conjunction with other sequence features leading to incomplete polymerase action, could make unwinding the rate-limiting step under conditions of reduced superhelical tension. Accordingly, the −7 mutation specifically reverses the supercoiling effects but it is totally silent when DNA gyrase is fully functional. The effectiveness of this C:G to T:A change in relieving the supercoiling sensitivity may stem from the fact that, in falling next to the sole A:T bp present in the C/G-rich region, it generates a TpA doublet which could act as a critical nucleation point for unwinding (Drew *et al.*, 1985). This could explain the weaker effects of the same sequence change at the −8 position (see Results).

In addition to relieving the transcriptional sensitivity to supercoiling, the −7 mutation alleviates the effects of an alteration in the upstream DNA curvature. This suggests that part of the action of the upstream curvature is directed toward the promoter unwinding step. Intriguingly, negative superhelical tension is expected to favour both, DNA unwinding as well as writhing, *i.e.* bending of the helix axis. Thus, supercoiling could either have a direct action on melting or, indirectly, assist in the formation of a bent structure which helps polymerase to unwind the helix. The possibility that bending the DNA can influence unwinding reactions was previously recognized. Intrinsic curvature could favour wrapping of the DNA around the polymerase and provide important additional contacts for isomerization (Buc, 1986). Alternatively, as recently proposed (Travers, 1990), the trajectory determined by the bending pattern may actually be at odds with the conformation that the DNA must assume upon polymerase binding. Under these conditions, the initial complex would be a metastable structure and the resulting torsional strain would be the force driving the promoter DNA into an unwound state (Travers, 1990). We envision a third possibility based on the idea that DNA must rotate around its axis during transcription elongation (Liu and Wang, 1987). Any impediment to axial rotation behind a moving polymerase should result in the accumulation of negative superhelical tension (Wu *et al.*, 1988; Tsao *et al.*, 1989; Rahmouni and Wells, 1989). A static bend immediately upstream from a promoter could drag the transmission of rotational motion thereby causing a build-up of torsional strain near the initiation site. In this scenario, a substantial fraction of negative superhelicity at the promoter site would originate from transcription elongation. The involvement of DNA gyrase would be indirect and derive

from allowing efficient elongation (*i.e.* rapid rotation) by a swivelling action ahead of the transcription ensemble (Liu and Wang, 1987; Wu *et al.*, 1988). A promoter response to supercoiling could then serve as a mechanism that renders initation frequency dependent on variables affecting elongation rates (see below).

All of the above models assign a major role to the physical properties of naked DNA. This view largely stems from our own data which show that the effects of both upstream bending and negative supercoiling can be detected in a minimal *in vitro* transcription system (Bossi and Smith, 1984; unpublished results). This does not exclude the possibility that the situation *in vivo* is more complex involving the action of auxiliary protein components. Possible candidates are the FIS protein which could potentiate the action of the upstream curvature (Nilsson *et al.*, 1990; Ross *et al.*, 1990) and the *dnaA* gene product whose binding sequence (5′-TTATCCACA-3′; Polaczek and Wright, 1990) differs in only one position (the last one) with the *hisR* promoter portion which includes the site of supercoiling response mutations (Figures 2 and 6). The possible role of these proteins in *hisR* promoter function and in *his* operon regulation is being examined.

Some of the promoter features highlighted in this discussion overlap with sequences known to be important for the coordinate control of tRNA and rRNA genes (Duester *et al.*, 1982; Lamond and Travers, 1983, 1985; Gourse *et al.*, 1986; Dickson *et al.*, 1989). It is tempting to speculate that transcriptional sensitivity to supercoiling is somehow linked to regulation. For example, factors affecting the rotational freedom of the DNA or the rate of transcription elongation could affect transcription-induced superhelicity (Liu and Wang, 1987) and therefore specifically modulate the activity of supercoiling sensitive promoters.

Materials and methods

Materials

Restriction enzymes were purchased from New England Biolabs, Bethesda Research Laboratories and Boehringer Mannheim. T4 DNA ligase was from Collaborative Research (Waltham, MA, USA). T4 polynucleotide kinase, the Klenow fragment of *E.coli* DNA polymerase I and RNase T_1 were from Boehringer Mannheim. AMV reverse transcriptase was obtained from Life Sciences Inc. (St Petersburg, FL, USA). All enzymes were used according to the manufacturer's specifications. Deoxy- and dideoxynucleotides were from P-L Biochemicals. Radiochemicals were obtained from Amersham or from New England Nuclear. Oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems DNA synthesizer.

Bacterial strains, media and growth conditions

The genotypes and the sources of all strains used in this study are listed in Table III. All *Salmonella typhimurium* strains are derivatives of strain LT2. Bacteria were cultured either at 30°C or at 37°C in liquid media or in media solidified by addition of 1.5% agar (Difco). Nutrient broth (NB; 0.8%, Difco) containing 0.5% NaCl was used as a complex medium for the routine growth of *S.typhimurium* strains. *E.coli* strains were normally grown in Luria broth (LB). When required, the above media were supplemented with any of the following antibiotics (from Sigma): tetracycline (25 μg/ml), kanamycin sulfate (50 μg/ml) ampicillin (50 μg/ml) chloramphenicol (25–400 μg/ml) and novobiocin (20–80 μg/ml). The E medium of Vogel and Bonner (1956) supplemented with 0.2% glucose and, if needed, 0.3 mM amino acids, was used as a minimal medium. Glucose concentration was raised to 2% in the high-salt selection medium and when scoring colony morphology (below). Glycerol (0.2%) substituted glucose as the sole carbon source in the selection of Cya^+ transductants. MOPS medium (Neidhardt *et al.*, 1974) supplemented with glucose (0.2 %), all amino acids (0.3 mM) and vitamin B_1 was used in all experiments involving RNA analysis. *lacZ* gene expression was monitored on MacConkey–lactose indicator plates (Difco) which were occasionally supplemented with the above antibiotics.

Table III. List of bacterial strains.

Strain	Genotype	Source
S.typhimurium		
TA790	*hisC202 hisC2493 hisR1223*	B.N.Ames *via* J.R.Roth
TR35	*his-712 ser-821 arg-501* /F′[*his0-E* (*E.coli*)]	J.R.Roth
TR6673	*hisU1820* (Anton, 1968)	J.R.Roth
PP1002	*trpB223 cya-1091*::Tn*10*	P.W.Postma *via* J.R.Roth
TT2043	*argI539 proAB47 amtA1 trp-130 zid-62*::Tn*10*	J.R.Roth
TT7759	*hisC9968*::Mu d1-8(Ap) *supD501 zeb-609*::Tn*10*	J.R.Roth
TT12116	*nadA213*::Tn*10*/F′152 *zzf-1878*::Mu dF(Km)	J.R.Roth
KR2240	*zib-748*::Tn*10 hisU1820*	K.E.Rudd
MA6	*hisU1820 hisR10107*	This work
MA62	*hisC9968*::Mu d1-8(Ap) *hisR1223* /F′[*his0-E* (*E.coli*)]	This work
MA83	*hisC9968*::Mu d1-8(Ap) *hisR1223 hisR10103* /F′[*his0-E* (*E.coli*)]	This work
MA84	*hisC9968*::Mu d1-8(Ap) *hisR1223 hisR10104* /F′[*his0-E* (*E.coli*)]	This work
MA87	*hisC9968*::Mu d1-8(Ap) *hisR1223 hisR10107* /F′[*his0-E* (*E.coli*)]	This work
MA91	*hisC9968*::Mu d1-8(Ap) *hisR1223 hisR10101* /F′[*his0-E* (*E.coli*)]	This work
MA411	*hisC9968*::Mu dJ(Km) *hisR1223 cya-1091*::Tn*10*	This work
MA463	*hisC9968*::Mu dJ(Km)	This work
MA464	*hisC9968*::Mu dJ(Km) *hisR1223*	This work
MA465	*hisC9968*::Mu dJ(Km) *hisR10107*	This work
MA474	*hisC9968*::Mu dJ(Km) *hisR1223 hisR10103*	This work
MA476	*hisC9968*::Mu dJ(Km) *hisR1223 hisR10104*	This work
MA478	*hisC9968*::Mu dJ(Km) *hisR1223 hisR10107*	This work
MA480	*hisC9968*::Mu dJ(Km) *hisR1223 hisR10101*	This work
MA578	*hisC9968*::Mu dJ(Km) *zib-748*::Tn*10 hisU1820*	This work
MA580	*hisC9968*::Mu dJ(Km) *hisR10107 zib-748*::Tn*10 hisU1820*	This work
E.coli K12		
SU1675	*pro lac thi recA56*	R.Weiss
71/18	*supE thi* Δ(*lac-proAB*)/F′[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	R.Cortese

All *S.typhimurium* strains are derivative of strain LT2. The *hisC9968*::Mu d-*lac* insertion (Casadaban and Cohen, 1979) puts the *lacZ* gene under the control of the *his* operon promoter/attenuator. Replacement of Mu d1-8 (Hughes and Roth, 1984) with the smaller Mu dJ derivative (Castilho *et al.*, 1984) was as previously described (Hughes and Roth, 1988).

Genetic techniques

Transductional crosses using the high-frequency generalized transducing mutant of phage P22 (HT 105/1 *int-201*) and conjugational matings were as previously described (Maloy and Roth, 1983; Hughes and Roth, 1984).

Isolation of suppressors of His-constitutive mutants

High-level expression of the *his* operon produces a complex array of phenotypes including changes of colony morphology (Murray and Hartman, 1972) and growth inhibition by temperature, by adenine and by high salt (Kohno *et al.*, 1980). While these defects remain poorly understood, they have been used widely for the selection and screening of mutations decreasing *his* expression. One such selection involves spreading His-constitutive bacteria on plates containing three times the normal concentration of E medium salts (3×E; Casadesús and Roth, 1989). We looked for spontaneous, salt-resistant

derivatives of strains TR6673 and MA62. To facilitate identification of *trans*-acting mutations, strain MA62 carries two copies of the *his* operon, an intact one on an F′ factor (from *E.coli*), the other fused to *lacZ* on the chromosome (by a Mu d-*lac* insertion; see Table III). This allowed screening simultaneously colony morphology on 1×E plates, 2% glucose (Murray and Hartman, 1972) as well as color on MacConkey−lactose indicator plates (MA62 forms deep red colonies; suppressors of the deattenuated phenotype form white, pink or light red colonies). The same scheme could not be applied to TR6673 where the *gyrB* mutation causes frequent F′ loss under the selective conditions. In the latter case, spontaneous survivors of the high-salt selection were directly scored for the presence of *hisR*-linked mutations by seeing whether the initial salt-sensitivity could by rescued in a transductional cross with a strain carrying a Tn*10* insertion near a wild-type *hisR* locus (TT2043). Verification of the mutant phenotypes in a background other than that of the initial isolation was carried out by first introducing *hisR1223* into strain MA463 with a cotransducible Tn*10* insertion in *cya*, then crossing the insertion out (selecting Cya^+) with donor lysates from the various *hisR* mutants. Scoring *hisR1223* as well as the suppressor derivatives was done in lactose indicator plates. Derivatives carrying *hisU1820* were constructed using cotransducible insertion *zib-748*::Tn*10*.

Cloning of hisR mutations and sequence analysis

Chromosomal DNA was prepared as previously described (Bossi and Smith, 1984) and subjected to *Sau*3A digestion. Digestion products were separated on a 1% agarose gel and DNA eluted from a region of the gel encompassing the migrating position of the *hisR* containing fragment (~1.4 kb long). Following purification, DNA was ligated into M13 derivative mWJ43 previously cleaved with *Bam*HI. Ligation, transformation and identification of *hisR*-containing recombinant phages were as previously described (Bossi and Smith, 1984). The sequences of mutant promoter regions were determined by the method of Sanger *et al.* (1977).

Plasmid constructions

M13 clones were subjected to a double *Eco*RI−*Pvu*II digestion. Digestion products were separated on 5% polyacrylamide gel and the *hisR* promoter-containing fragments (spanning positions −293 to +28; Bossi, 1983) were eluted and purified. These fragments were ligated to *Bam*HI-cleaved DNA of plasmid pKK232-8 (Brosius, 1984) following staggered end fill-in. Mixtures were used to transform *E.coli* strain SU1675 selecting ampicillin-resistance. Transformants were screened for the acquisition of chloramphenicol-resistance. Correct plasmid constructions were verified by restriction analysis and direct DNA sequencing.

RNA extraction and 5′-end analysis

Aliquots of exponentially growing cultures (A_{600} = 0.3−0.4) were quickly mixed with one-volume of REB kept in a boiling water bath (REB: **R**NA **E**xtraction **B**uffer: 20 mM Na acetate pH5.2, 2% SDS, 0.3 M sucrose). The mixture was left in boiling water for 30 s, then shaken with one vol of phenol (saturated with 0.1 M Na acetate pH 5.2) at room temperature for 30 s. Samples were put on ice for 2−5 min, then centrifuged. The aqueous phase was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1). Nucleic acids were precipitated with 2.5 vol of 95% ethanol. A 40 bp long synthetic oligonucleotide complementary to a portion of the *cat* transcript (as modified in pKK232-8; Brosius, 1984) was used as primer for RNA sequencing and 5′-end mapping. Typically, 50 ng (~4 pmoles) of 40-mer were phosphorylated with (γ-^{32}P)ATP (50 μCi) and kinase in 70 mM Tris−Cl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT. Labelled oligonucleotide (most commonly 5 ng) was hybridized to RNA obtained from 0.2 ml of original culture in a buffer containing 50 mM Tris−Cl pH 8, 20 mM KCl, 10 mM DTT. Extension reactions were started by adding the 4 deoxynucleotides (0.2 mM each) together with 5 units of reverse transcriptase. Sequencing ladders were produced by including dideoxynucleotides at approximately twice the deoxy concentration.

In vivo pulse-labelling of RNA and hybrid-selection

Cells were grown in MOPS−glucose medium (Neidhardt *et al.*, 1974) supplemented with all amino acids and 0.2 mM phosphate (K_2HPO_4). [^{32}P]phosphate (100 μCi/ml) was added at an A_{600} = 0.25−0.30. After 5 min, RNA was extracted as above. The amount of RNA obtained from 0.1 ml of bacterial culture was mixed with 2 μg of single-stranded DNA from either of two M13 recombinant phages: one carrying 330 bases of the coding strand of the promoter−proximal portion of the *cat* gene (from pKP1-13); the other carrying 340 bases of the coding strand of the promoter-proximal portion of the *bla* gene (from pKK232-8). Following incubation under standard hybridization conditions, the mixture was treated with RNase T_1 and loaded immediately after on a 1% agarose gel. It was found that under non-denaturing elecrophoresis conditions, hybridized RNA co-migrates with the phage DNA band in a region of the gel where there are no other labelled species. Blotting the gel with Whatman 3MM paper, prior to autoradiography, helped to considerably reduce background.

Construction of tandem duplications

A DNA fragment containing the *hisR* promoter and neighboring sequence (−205 to +86; Bossi, 1983) was produced by *Rsa*I digestion. This fragment was cloned into both M13 mp8 and M13 mp9 at their unique *Sma*I site. Recombinant phages were selected in which, due to the opposite orientations of the polylinker region in the two vectors, cloned DNA was flanked by *Eco*RI and *Bam*HI sites in reversed order. Two such constructs were cleaved with *Eco*RI , mixed in a one to one ratio, ligated and recut with *Bam*HI. The products were ligated to *Bam*HI-cleaved M13 mp8 and the ligation mixture used to transform *E.coli* strain 71-18. Subsequent analysis confirmed the presence of recombinant phage carrying two copies of the initial *Rsa*I insert in a head-to-tail arrangement. The two copies are separated by an *Eco*RI site and the duplicated material is flanked by *Bam*HI sites.

Enzyme assays

Activity of β-galactosidase was measured in toluene-permeabilized cells as described by Miller (1972) and it is expressed in Miller units throughout the paper. Chloramphenicol acetyltransferase (CAT) and β-lactamase (BLA) were assayed in cell-free extracts prepared from exponential LB cultures of plasmid-harbouring SU1675. Activities were measured spectrophotometrically at 37°C as previously described (Figueroa and Bossi, 1988).

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