Spot 42 RNA mediates discoordinate expression of the *E. coli* galactose operon

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The physiological role of *Escherichia coli* Spot 42 RNA has remained obscure, even though the 109-nucleotide RNA was discovered almost three decades ago. Structural features of Spot 42 RNA and previous work suggested to us that the RNA might be a regulator of discoordinate gene expression of the galactose operon, a control that is only understood at the phenomenological level. The effects of controlled expression of Spot 42 RNA or deleting the gene (*spf*) encoding the RNA supported this hypothesis. Down-regulation of *galK* expression, the third gene in the *gal* operon, was only observed in the presence of Spot 42 RNA and required growth conditions that caused derepression of the *spf* gene. Subsequent biochemical studies showed that Spot 42 RNA specifically bound at the *galK* Shine-Dalgarno region of the *galETKM* mRNA, thereby blocking ribosome binding. We conclude that Spot 42 RNA is an antisense RNA that acts to differentially regulate genes that are expressed from the same transcription unit. Our results reveal an interesting mechanism by which the expression of a promoter distal gene in an operon can be modulated and underline the importance of antisense control in bacterial gene regulation.

*Key Words:* Antisense RNA; galactose operon; riboregulation; small RNAs; Spot 42 RNA; translational regulation

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Over the past two decades it has become clear that cells contain a variety of small untranslated RNA molecules (sRNAs). These RNAs play important or essential roles and regulate diverse cellular functions such as RNA processing and splicing, RNA editing and modification, protein stability and secretion, mRNA stability, and translation. Posttranscriptional control by sRNAs has been documented in bacteria and in a variety of eukaryotic cells including nematodes, plants, and mammals [Jorgensen et al. 1998; Panning and Jaenisch 1998; Eddy 1999, Wassarman et al. 1999, Adoutte 2000]. The majority of these riboregulators act as antisense RNAs that pair to complementary regions on target RNAs. Antisense control was first discovered in plasmids, bacteriophages, and transposable elements. In all these cases, the antisense and target RNAs are transcribed from opposite DNA strands of the same region (i.e., cis-encoded; Wagner and Simons 1994; Zeiler and Simon 1996). In a growing number of cases, however, the antisense and target RNAs are expressed from unlinked genes, and complementarity is incomplete and relatively modest. Such trans-encoded antisense RNAs can carry separate determinants for binding/regulation, function by more than one mechanism, and affect more than one target gene [for review, see Altuvia and Wagner 2000].

Until recently only a dozen sRNAs in addition to tRNAs and 5S RNA were known to be encoded by the *E. coli* genome [for review, see Wassarman et al. 1999, Urbanowski et al. 2000, Majdalani et al. 2001]. However, novel approaches including genomic mining have increased the number to more than 40 [Argaman et al. 2001, Rivas et al. 2001, Wassarman et al. 2001]. Thus, sRNAs are much more widespread than previously imagined. At present three of the sRNAs are known to be antisense regulators (i.e., OxyS, DsrA, and MicF) that act at the level of translation initiation by base-pairing to the 5’ end of target mRNAs. They either repress translation by interfering with ribosome binding or activate translation by interfering with an inhibitory intramolecularly base-paired structure of the target RNA [Mizuno et al. 1984, Altuvia et al. 1998, Lease et al. 1998, Majdalani et al. 1998, Delilas and Forst 2001]. Also, unique functions have been discovered for most of the other well-known sRNAs [Romeo 1998, Wassarman and Storz 2000; for review, see Wassarman et al. 1999]; however, the role of the Spot 42 RNA has not yet been established.

Spot 42 RNA was discovered nearly three decades ago by two-dimensional [2D] gel electrophoresis as the most apparent RNA of *E. coli* when cells were labeled briefly with $^{32}$Pphosphate [Ikemura and Dahlberg 1973; Sahuagin and Dahlberg 1979]. The RNA is transcribed from...
the *spf* (spot forty-two) gene, whose expression is negatively regulated by the cAMP–CRP complex (Joyce and Grindley 1982, Rice and Dahlberg 1982, Polayes et al. 1988b). The gene includes a ρ-independent terminator site, and the mature RNA is unmodified and identical to the primary transcript. Spot 42 RNA has a half-life of 12 to 13 min at 37°C and is present in ∼200 copies per cell when cells are growing in media supplemented with glucose (Sahagan and Dahlberg 1979). This number is reduced three- to fivefold when cells are grown on nonglucose carbon sources or when cAMP is added to a glucose-grown culture (Polayes et al. 1988b). The *spf* gene is dispensable, and no major defects are observed in Spot 42 RNA null mutants (Hatfull and Joyce 1986; Polayes et al. 1988a). A 10-fold overproduction of Spot 42 RNA resulted in pronounced growth defects under a variety of conditions (e.g., small colony size and slow growth, inability to grow on succinate as carbon source and to respond normally to nutritional up-shift; Rice and Dahlberg 1982). However, in spite of this knowledge, the function of Spot 42 RNA has remained elusive.

Here we show that the *spf* gene is absolutely required for discordant expression of the *gal* operon cistrons. Moreover, in vitro studies show that Spot 42 RNA specifically binds at the *galK* Shine-Dalgarno region of the *gal* mRNA and that the RNA interferes with the formation of *galK*-30S initiation complexes. The data are discussed in terms of a model wherein Spot 42 RNA acts as an antisense RNA to differentially regulate the *gal* genes by competing with 30S ribosomes for binding to the *galK* translational initiation region.

**Results**

*What is the function of Spot 42 RNA?*

The *E. coli* cAMP receptor protein (CRP) is a global transcriptional regulator that can act as an activator, repressor, coactivator, and corepressor (Kallipolitis et al. 1997; Busby and Ehrbit 1999). Moreover, work on the galactose operon has revealed that the cAMP–CRP complex, directly or indirectly, influences synthesis of the products of this operon differentially (Ullmann et al. 1979; Joseph et al. 1981; Adhya 1987). The *E. coli* *galETKM* operon is unusual in two respects. First, it possesses two overlapping promoters (Fig. 1). The cAMP–CRP complex stimulates transcription from the downstream promoter P1 and inhibits transcription from the upstream promoter P2. Second, although the genes are translated from a polycistronic mRNA, the relative synthesis of the enzymes UDP-galactose-4-epimerase (*GalE*), galactose-1-phosphate uridyl transferase (*GalT*), and galactokinase (*GalK*) has been found to vary under different metabolic conditions, a phenomenon that has been termed discordant expression. When cAMP levels are reduced by mutations (*cya−*) or catabolite repression (glucose as carbon source), the levels of GalK and GalT decline (the GalK level, in particular), but the GalE level remains high, resulting in an approximately fourfold increase in the GalE-to-GalK ratio. We note, however, that there are minor disagreements as to how cAMP levels or carbon source affect *galE* expression (Ullmann et al. 1979; Joseph et al. 1981; Adhya 1987). The discordant expression is more severe in the absence of a functional cAMP–CRP complex and can be suppressed in glucose-grown cells or in *cya−* strains by the addition of cAMP to the growth medium. It has been proposed that transcription termination at intercistronic regions, preferential degradation of the promoter distal portion of the mRNA, or different translational efficiencies of the two *gal* transcripts could account for the regulation (Ullmann et al. 1979; Joseph et al. 1981; Queen and Rosenberg 1981; Adhya 1987).

Several observations suggested to us that Spot 42 RNA is involved in mediating discordant expression of the *gal* operon. First, BLAST searches revealed that Spot 42 RNA has significant complementarity to the region surrounding the translation initiation region of *galK*. Sec-

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**Figure 1.** The *E. coli* galactose operon. (A) Schematic diagram of the *E. coli* galactose operon. Relative positions of the four genes are shown. The genes encode: *galE*, epimerase (*GalE*); *galT*, transferase (*GalT*); *galK*, kinase (*GalK*); and *galM*, mutarotase. The two promoters (P1 and P2) are modulated negatively by Gal repressor, and the cAMP–CRP complex abolishes transcription from P2 and is required for P1 transcription. (B) The pathway for galactose metabolism. Note that the GalE, GalT, and GalK enzymes are part of an amphibolic pathway that produces substrates for biosynthetic glycosylations (UDP-glucose and UDP-galactose). Moreover, the pathway generates galactose from UDP-galactose when cells are growing in other carbon sources. Subscripts: (e) extracellular; (i) intracellular.
ond, Spot 42 RNA was predicted to form three stem–loop structures in which the first hairpin contains a seven-nucleotide single-stranded loop presented by a loosely paired top stem of interrupted helicity. Such features are often observed in antisense RNA regulatory units [Wagner and Brantl 1998]. Third, the opposing regulation of spf and galK by the cAMP–CRP complex was striking.

Effects of Spot 42 RNA on plasmid-encoded GalE, GalT, and GalK proteins

To assess the role of Spot 42 RNA on gal operon regulation, we first investigated the effect of controlled Spot 42 RNA expression on the GalE, GalT, GalK, and GalM polypeptide synthesis pattern by 2D gel electrophoresis. To this end we constructed a low-copy-number plasmid [pSpf33] that expressed spf under the control of the inducible araBAD promoter. Moreover, we constructed a Δspf strain (SØ928∆spf) carrying the entire gal operon on plasmid pBR322 [pGal4]. This strain was transformed with pSpf33 and as a control with pBAD33. Uninduced and induced cultures of the transformed strains were pulse-labeled with [35S]methionine, and their proteins were analyzed by standard 2D gel electrophoresis and autoradiography [Fig. 2]. Induction of spf expression had little effect on the net synthesis rate of the GalE, T, and M polypeptides (~10% increase of GalE, ~10% decrease of GalT, and ~15% decrease of GalM synthesis), whereas a significant reduction of the net rate of synthesis (~two-fold) was observed for GalK [Fig. 2, cf. panel A, uninduced culture, with panel B, induced culture; see legend to Fig. 2]. Addition of arabinose to the control strain [SØ928Δspf/pGal4, pBAD33] had no effect on the net synthesis rates of the four Gal polypeptides (data not shown). These results showed that expression of spf specifically affected GalK synthesis, supporting the idea that Spot 42 RNA might be responsible for discoordinate expression of the gal operon.

Effect of deleting the spf gene on gal operon expression

We next examined the consequences of deleting the spf gene. For these studies, we constructed isogenic spf+ and Δspf strains in gal constitutive [i.e., galR− crp+] and Δcrp backgrounds. The various strains were grown in minimal medium with glucose or glycerol as carbon and energy source to an OD600 of 0.4. Following this, Spot 42 RNA levels and GalE, GalT, and GalK levels were determined by Northern blot and Western blot analysis, respectively. The results of the experiments are presented in Figure 3. We emphasize the following observations. Expression of galE and galT was not significantly altered by deleting spf [Fig. 3, cf. lanes 1–2 with lanes 3–4]. In accordance with previous work, the expression of spf was enhanced [Sahagan and Dahlberg 1979; Polayes et al. 1988b] and the expression of galK was reduced in cells lacking the cAMP–CRP complex [Fig. 3, lanes 5,6] or deficient in cAMP and CRP [Fig. 3, lane 2, glucose grown, Ullmann et al. 1979; Joseph et al. 1981; Queen and Rosenberg 1981; Adhya 1987]. Strikingly, this down-regulation of galK was eliminated in the Δspf strains [Fig. 3, cf. lane 2 with lane 4, and lanes 5–6 with lanes 7–8]. For the spf mutant, expression of the three gal genes was slightly higher in glycerol-grown cells [Fig. 3, lane 3] than in glucose-grown cells [Fig. 3, lane 4], and a similar expression was observed for galE and galT in the wild-type strain [Fig. 3, cf. lanes 1–2 with lanes 3–4]. Accordingly, previous work has shown that the addition of cAMP to a Δcya, galO81 mutant stimulated galE expression 40%–70% [Joseph et al. 1981], and that galT expression in gal constitutive strains [galR+ or galO81] was 40%–70% higher in glycerol-grown cells than in glucose-grown cells (Adhya and Echols 1966). On this basis we conclude that cAMP–CRP also stimulates galE expression. Furthermore, the levels of the three Gal enzymes increased coordinately in the spf mutant when glycerol was used as carbon source [Fig. 3, lanes 3,4; see legend], indicating that transcription initiation is more
Riboregulation by Spot 42 RNA

Spot 42 RNA binds to the translational initiation region of galK

Spot 42 RNA has significant sequence complementarity to the gal mRNA in the region surrounding the translational initiation region of galK [Fig. 5A], and, strikingly, the galK complementary regions reside within exposed regions of Spot 42 RNA [Fig. 6B]. To test whether Spot 42 RNA might bind the complementary regions, we performed binding experiments with 5'-end-labeled Spot 42 RNA and an unlabeled galK' RNA substrate that carried the galK Shine–Dalgarno sequence in the central portion of the molecule. Samples containing a fixed amount of Spot 42 RNA, a 500-fold excess of RNA, and increasing amounts of galK' RNA were incubated on ice, formation of complexes was assayed by gel-retardation assays. The results, presented in Figure 5B, show that Spot 42 RNA and the galK' RNA formed a single retarded complex. A similar complex was observed by adding unlabeled Spot 42 RNA to incubations containing a fixed amount of 5'-end-labeled galK' RNA. Addition of excess unlabeled galT' RNA substrate, which carried the distal part of galE and the proximal part of galT [i.e., the galT translational initiation region], did not affect formation of the retarded complex (data not shown). We infer that Spot 42 RNA specifically binds the galK' RNA, supporting the idea that regulation of galK expression occurs via RNA–RNA interaction.

We used structural probing for further characterization of galK–Spot 42 RNA interaction. Here, 5'-end-labeled Spot 42 RNA was mixed with increasing concentrations of unlabeled galK' RNA, and the incubations were treated with RNase T1. The structural probing is presented in Figure 6 and shows that additions of galK' RNA reduced or abolished cleavage by RNase T1 in the 5' tail, loop I, and at positions 47–48 and 53–58. Thus, three short antisense sequences exposed in single-stranded regions of Spot 42 RNA appear to interact with
separate target sites located at or near the translational initiation region of galK (Fig. 5A).

Spot 42 RNA prevents ribosome binding

To test whether Spot 42 RNA binding to galK mRNA might interfere with ribosome binding, we used toeprinting assays [Hartz et al. 1989]. Briefly, in the presence of initiator tRNA (fMet-tRNA), the ribosomal 30S subunits bind to the Shine–Dalgarno sequence of mRNAs and block for reverse transcription primed downstream. The inhibition of primer extension generates a stop signal at the 3' end of the bound 30S subunit [usually 15–17 nt downstream of the start codon]. The strength of this stop signal provides a measure for the formation of the ternary complexes.

For the toeprint analysis we used the galT* and galK* RNA substrates. The probes were annealed with a 5'-
end-labeled primer complementary to a region downstream of the translation start site and incubated with 30S subunits in the presence or absence of fMet-tRNA. As shown in Figure 7A, the addition of Spot 42 RNA at different molar ratios to galT RNA before the addition of 30S subunits and fMet-tRNA did not interfere with ternary complex formation. In contrast, when the experiments were performed with the galK RNA (Fig. 7B), the presence of Spot 42 RNA resulted in a diminished toeprint signal, and an almost complete inhibition of ternary complex formation was observed at a molar excess of 5:1 of Spot 42 RNA to galK RNA (Fig. 7B, lane 8). A diminished toeprint signal, however, was not detected if 30S subunits and fMet-tRNA were added prior to Spot 42 RNA to incubations containing galK RNA (data not shown). These assays show that Spot 42 RNA binding interferes directly with the formation of galK–30S initiation complexes.

Discussion

In this work we have provided insight into two longstanding questions concerning a physiological function of Spot 42 RNA and the regulation of discoordinate gene expression of the E. coli galactose operon. Our results establish that Spot 42 RNA is a riboregulator that acts to regulate the gal operon genes differentially under certain physiological conditions. Our results further show that Spot 42 RNA inhibits expression of galK, the third gene in the operon, by an antisense mechanism. Three short antisense sequences exposed in single-stranded regions of Spot 42 RNA appear to interact with three separate target sites located at or near the translational initiation region. Finally, toeprinting experiments with 30S ribosomal subunits revealed that galK–Spot 42 RNA interactions interfere with the formation of translational initiation complexes. Taken together, our results suggest that Spot 42 RNA inhibits galK translation by sterically

![Figure 5](image-url) Spot 42 RNA–galK mRNA interaction. (A) The complementarity between Spot 42 RNA (top sequence) and the galK translation initiation region (bottom sequence). The stop codon of galT and the start codon of galK are underlined. (B) Gel mobility shift assays of Spot 42 RNA binding to galK RNA. (Lanes 1–5) 5'-End-labeled transcript of Spot 42 RNA (0.05 pmole) and 500-fold molar excess tRNA were incubated with increasing amounts of unlabeled galK/H11032 RNA (0, 1, 5, 10, and 20 pmole) to allow complex formation and then resolved on a native polyacrylamide gel. (Lanes 6–10) 5'-End-labeled galK/H11032 RNA (0.05 pmole) and 500-fold molar excess of tRNA were incubated with increasing amounts of unlabeled Spot 42 RNA (0, 1, 5, 10, and 20 pmole) to allow complex formation and then resolved on a native polyacrylamide gel.

![Figure 6](image-url) Nuclease probing of Spot 42 RNA–galK' RNA complexes. (A) In vitro synthesized 5'-end-labeled Spot 42 RNA (0.05 pmole) was mixed with increasing concentrations of unlabeled galK' mRNA (1, 5, 10, and 20 pmole), and the incubations were treated with RNase T2. (Lane 1) RNase T1 cleavage of Spot 42 RNA under denaturing condition (G-specific cleavage); (lane 2) alkaline hydrolysis ladder; (lane 3) control (untreated Spot 42 RNA); (lanes 4–8) RNase T1 footprinting reactions in absence (lane 4) and in presence (lanes 5–8) of galK' RNA. (B) Summary of changes in the cleavage pattern induced by galK' RNA. The galK complementary regions are shown on a gray background. Arrows indicate reduced cleavage.
The DNA sequence reactions (GATC) were carried out with assays. The same end-labeled oligonucleotide used in the toeprinting molar ratios to mRNA, 1:2 (lane 30S subunits and fMet-tRNA. Spot 42RNA was added at the presence, Spot 42RNA was added prior to the addition of 0.04 µM. fMet-tRNA was in molar excess over 30S subunits. reactions the concentration of mRNA and 30S subunits was and +17 relative to A of the start codon (marked by stars). In all galT

Figure 7. Toeprinting analysis of 30S ribosomal subunit binding to galIT and galK’ RNA. (A) Ternary complex formation on galIT RNA. The toeprint signal is at position +15 relative to A of the start codon (marked by a star). (B) Ternary complex formation on galK’ RNA. The toeprint signal is at positions +16 and +17 relative to A of the start codon (marked by stars). In all reactions the concentration of mRNA and 30S subunits was 0.04 µM. fMet-tRNA was in molar excess over 30S subunits. When present, Spot 42 RNA was added prior to the addition of 30S subunits and fMet-tRNA. Spot 42 RNA was added at the molar ratios to mRNA, 1:2 (lane 7) and 5:1 (lane 8). [Lanes 1–4] The DNA sequence reactions [G A’ T C] were carried out with the same end-labeled oligonucleotide used in the toeprinting assays.

hindering ribosome binding. As Spot 42 RNA regulation only interfered weakly with the expression of the two proximal genes and the distal gene of the gal operon (Figs. 2, 3), it seems reasonable to suppose that the stability of the gal message is not grossly altered by Spot 42 RNA interaction. This view is further supported by gene array analysis of the expression of the individual genes of the gal operon in spf+ and Δspf cells [T. Møller, unpubl.]. Therefore, we favor the idea that Spot 42 RNA acts primarily at the level of translation initiation.

Previously, the stimulatory effect of cAMP on the GalT levels (−1.5) and on the GalK levels (−fourfold) led to the idea that cAMP–CRP acts, directly or indirectly, at the transcriptional level by modulating polarity (e.g., by interfering with the transcription terminator ρ). (Ullmann et al. 1979). However, the hypothesis was based on the assumptions that the gal operon carried a single cAMP–CRP-independent promoter and that expression of the promoter proximal gene (galE) was unaffected by carbon source and/or cAMP. Moreover, the authors did not take into account the fact that galE expression was stimulated (40%–70%) by cAMP in an acya, galO−81 strain [Joseph et al. 1981]. Also, when the amount of gal mRNA corresponding to promoter proximal and distal genes was measured in an acya strain, grown in the absence or in presence of cAMP, it was found that cAMP could only partly restore coordinate expression (Guidi-Rontani et al. 1984). Therefore, it was concluded that a translational control was probably involved in the discoordinate regulation of the gal operon (Guidi-Rontani et al. 1984). The present results on gal operon expression, consistent with data for gal operator constitutive mutants (Adhya and Echols 1966, Joseph et al. 1981), suggest that the cAMP effect on galE and galT expression is transcriptional. Thus, we showed that the expression of galE and galT in a ΔgalR strain and the expression of galE, galT, and galK in a ΔgalR Δspf strain was affected to a very similar extent by carbon source (i.e., 40%–50% higher in glycerol-grown cells than in glucose-grown cells; see Fig. 3), indicating that transcription initiation is more efficient from the cAMP–CRP-dependent P1 promoter than from the cAMP–CRP-repressed P2 promoter (Fig. 1).

A second interesting aspect of Spot 42 RNA regulation concerns ongoing translation. Because sequences encoding the end of the galT gene overlap with sequences involved in ribosome binding and initiation of galK translation as well as with sequences involved in Spot 42 RNA binding (Fig. 5), a terminating ribosome would be expected to interfere with Spot 42 RNA binding. We speculate that the proposed interaction between Spot 42 RNA and separated target regions could be crucial for an efficient control. Thus, the three binding regions of Spot 42 RNA are all exposed, and each of these may possibly facilitate bimolecular RNA–RNA interaction. Moreover, the separated target regions within the coding region of galK may allow formation of initial duplex formation in the presence of a terminating ribosome. Such binding features could lead to an increase in the local concentration of Spot 42 RNA, and facilitate rapid formation of repression complexes.

Finally, we note that recent work has shown that Spot 42 RNA regulation of gal operon expression relies on the global posttranscriptional regulator Hfq, which shares functional and structural features with the eukaryotic Sm and Sm-like proteins (Møller et al. 2002; Zhang et al. 2002). In hfq− strains, the half-life of Spot 42 RNA is strongly reduced and Hfq binds specifically to the RNA. Moreover, in vitro Hfq strongly cooperates in complex formation between Spot 42 RNA and galK mRNA (Møller et al. 2002). Because Hfq also increases Oxys RNA interaction with target mRNAs (Zhang et al. 2002), is required for riboregulation of rpoS by DsrA and RprA RNA (Majdalani et al. 2001; Sledjeski et al. 2001), and binds to several other sRNAs (Wassarman et al. 2001), it has been proposed that Hfq acts as a general cofactor for antisense RNAs that rely on short stretches of base pairing (Wassarman et al. 2001; Møller et al. 2002; Zhang et al. 2002).

Physiological significance of Spot 42 RNA regulation

Despite the great understanding of D-galactose metabolism in E. coli, the physiological significance of the uncoordinated expression of the gal operon genes is still unclear. It is intriguing, however, that the GalE, T, and K enzymes form part of an amphibolic pathway that pro-
duces substrates for biosynthesis of lipopolysaccharides [i.e., UDP-glucose and UDP-galactose; see Fig. 1, for review, see Adhya 1987]. When the cell derives its energy from galactose, all three enzymes are required. The net result is the conversion of galactose to the glycolytic intermediate glucose-1-phosphate. When the cell derives its energy from other carbon sources, a relatively high basal level of epimerase (GalE) is required to produce UDP-galactose for biosynthetic glycosylations. Under such metabolic conditions, the amphibiotic pathway, together with a galactose-1-phosphatase activity, generates galactose from UDP-galactose [i.e., GalT is also required]. However, the galactose generated intracellularly does not accumulate to a level that is high enough to induce the gal operon because it is either excreted or recycled. Excretion takes place in the presence of glucose, the preferred carbon source, whereas the intracellular galactose is recycled when the cell uses less favorable carbon sources. These explanations assign a physiological significance to Spot 42 RNA regulation and reveal a form of cellular regulation that may play a decisive role in the fine-tuning of energy utilization for carbon sources.

**Materials and methods**

**Bacterial strains**

All strains used were *E. coli* K-12 derivatives: S0928 (Δdeo, Δlac, Valentim-Hansen et al. 1978), S02928 (Δdeo, Δlac, Δtrp, 9626dh-732:Trn10, Søgaard-Andersen et al. 1991); MW130 (ΔgalR::Cm); Weickert and Adhya 1993); KM22 [ΔrecC, ptr, recD]; P222-bet, exo3, kari (Murphy 1998), and MC1000Δssf (Murphy 1998). PCR and Northern blot analysis verified gene replacements and the absence of Spot 42 RNA.

**Plasmid construction**

To construct pSpf34, the DNA regions flanking the *spf* gene were amplified from S0928 chromosomal DNA using primers ospfdel1 [5′-CCCGGATCCCGCGGCGTGGCCGCGTCGCT-3′] plus ospfdel2 [5′-GCGGGAATTCGCGGACGTGGTTTCAGTGGACGCA-3′], and Ospde15 [5′-CCCGTCTGAGGTTACCTACGACCTACTCCTCGGATCGTATAGG-3′] plus ospfdel4 [5′-CCCGGAAATTCGGTTACGCTGTTGCTCTGG-3′], respectively. The upstream DNA fragment was digested with EcoRI and BamHI and cloned into EcoRI/BamHI-restricted pUC19, generating pUSpf. Subsequently, pUDspf was constructed by replacing the Xhol–EcoRI segment of pUSpf with the downstream DNA fragment (digested with Xhol and EcoRI). The *aphA* gene was amplified from chromosomal DNA of strain MC1000Δssf using osr1 [5′-GCCGGGATCCCGGAGTGGTTATGTTGTTTTTCA-3′] and osr2 [5′-CGCGGAGTCTCACGGTACCGGATTTACGATCGGAGGCGGACGCA-3′] as primers. The *aphA* fragment was digested with *KpnI* and cloned into the unique KpnI site of pUSpf, generating pSpf11 and pSpfΔ4 (with *aphA* in the opposite or the same orientation as the original *spf* gene, respectively). Concerning construction of *spf* knockout strains, we did not observe any importance of the orientation of the kanamycin cassette.

To create pSpf33, two PCR products were generated using [1] pBAD33 as template and osp13 [5′-CATCTTACCTCTGTTACCTCATGAGAAGCAGAAACCATAGGATTGTTGTTGTTTTTCA-3′] plus osp15 (5′-CCCGGATCCGCGGACGTGGTTTCAGTGGACGCA-3′) as primers, and [2] S0928 chromosomal DNA as template and oarsp3 [5′-CAACCCTCTTACGATCGGAGCAGGAAACCATAGGATTGTTGTTGTTTTTCA-3′] plus oarsp1 (5′-GCGGGAATTCGCGGACGTGGTTTCAGTGGACGCA-3′) as primers. These two products were amplified using *EcoRI* and *XhoI* as primers and S0928 chromosomal DNA as template, followed by digestion with *EcoRI* and BamHI.

**Plasmid construction**

Plasmid pSpf3 was constructed by replacing the *EcoRI*–BamHI fragment of pUC18 with a *PCR*-generated fragment, prepared using oT7spf [5′-CCGGATCCGCGGACGTGGTTTCAGTGGACGCA-3′] plus oT71 (5′-CGCGGAGTCTCACGGTACCGGATTTACGATCGGAGGCGGACGCA-3′) as primers and o3spf (5′-CCCGGATCCGCGGACGTGGTTTCAGTGGACGCA-3′) as primers. A third round of amplification with these two products as template and o33mluI and ospfpstI as primers generated a DNA fragment carrying the *spf* gene fused to the arabinose promoter. Finally, pSpf33 was constructed by replacing the *MluI*–*PstI* fragment of pBAD33 with the restricted *araP*–*spf* fragment.

Plasmid pSpfΔ3 was constructed by replacing the *EcoRI*–BamHI fragment of pUC18 with a *PCR*-generated fragment, prepared using oT7spf [5′-CCGGATCCGCGGACGTGGTTTCAGTGGACGCA-3′] plus oT71 (5′-CGCGGAGTCTCACGGTACCGGATTTACGATCGGAGGCGGACGCA-3′) as primers and o3spf (5′-CCCGGATCCGCGGACGTGGTTTCAGTGGACGCA-3′) as primers. A third round of amplification with these two products as template and o33mluI and ospfpstI as primers generated a DNA fragment carrying the *spf* gene fused to the arabinose promoter. Finally, pSpf33 was constructed by replacing the *MluI*–*PstI* fragment of pBAD33 with the restricted *araP*–*spf* fragment.

Plasmid pSpf3 was constructed by replacing the *EcoRI*–BamHI fragment of pUC18 with a *PCR*-generated fragment, prepared using oT7spf [5′-CCGGATCCGCGGACGTGGTTTCAGTGGACGCA-3′] plus oT71 (5′-CGCGGAGTCTCACGGTACCGGATTTACGATCGGAGGCGGACGCA-3′) as primers and o3spf (5′-CCCGGATCCGCGGACGTGGTTTCAGTGGACGCA-3′) as primers. A third round of amplification with these two products as template and o33mluI and ospfpstI as primers generated a DNA fragment carrying the *spf* gene fused to the arabinose promoter. Finally, pSpf33 was constructed by replacing the *MluI*–*PstI* fragment of pBAD33 with the restricted *araP*–*spf* fragment.
TTTATAATGCACTACCATACATCACCATCACCC AATTATACCCGGTATGATAC-3' and oT [5'-GCCCG GATCCCCCGCCAGCCCGCTATGTTG-3'], galK, ohsK [5'-CGCCCGAATCTAAGGACCTTATTAAATGCTACCC ATACCACTATCACTACAGTGAAGAAAACACAA TCTC-3'] and oK [5'-GCCCGGATCCACCCAGGACATCTTG TCATGACGCC-3'].

**Growth media**

Strains were grown at 37°C in AB minimal medium [Clark and Maaløe 1967] containing 1 µg/mL thiamine, 0.2% carbon source (glucose or glycerol), and 0.05% casamino acids. For the growth media used for [35S]methionine labeling, casamino acids were replaced by a 1/20 volume defined amino acid mixture (concentrations: L-alanine, 1.75 g/L; L-arginine, 1.75 g/L; L-histidine, 0.78 g/L; L-lysine, 1.5 g/L; L-proline, 1.15 g/L; L-threonine, 1.20 g/L; glycine, 1.5 g/L; L-asparagine, 1.33 g/L; L-glutamine, 2.18 g/L; L-isoleucine, 1.30 g/L; L-phenylalanine, 0.60 g/L; L-tryosine, 0.90 g/L; L-valine, 1.75 g/L; L-aspartic acid, 1.33 g/L; L-glutamic acid, 2.2 g/L; L-cysteine, 0.30 g/L; L-serine, 25.0 g/L; L-tryptophan, 0.50 g/L. Ampicillin (100 µg/mL), spectinomycin (100 µg/mL), and chloramphenicol (50 µg/mL) were added where appropriate.

**2D gel electrophoresis**

Samples [1 mL] of exponentially grown cells were labeled with 10 µCi of carrier-free [35S]methionine for 1 min, followed by a chase with unlabeled methionine (final concentration 200 µg/mL) for 1 min. The chase was terminated by the addition of 5 µL of chloramphenicol [50 mg/mL]. Cells were collected by centrifugation at 0°C, resuspended in 50 µL of loading buffer [20% sucrose, 3% SDS, 0.16 M Tris-HCl at pH 6.8, with freshly added β-mercaptoethanol], boiled for 3 min, and mixed with 25 µL of 10% NP-40. A 30-µL sample was loaded onto the first dimension gel. Gel electrophoresis was carried out as described by O'Farrell [1975]. Quantification of protein spots was done by PhosphorImager and the Imagequant software package (Molecular Dynamics).

**Protein purification, antibody production, and Western blotting**

The N-terminally histidine-tagged Gal enzymes were expressed from pH8-E, pH8-T, or pH8-K, respectively, in strain SØ928. TheN-terminally histidine-tagged Gal enzymes were expressed and Western blotting. Protein purification, antibody production, O'Farrell (1975). Quantification of protein spots was done by 2D gel electrophoresis

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**Gel mobility shift assay**

The binding assays were performed as follows: 5’-end-labeled RNA (0.05 pmole of Spot 42 RNA or galk RNA), 1 µg of tRNA (Boehringer Mannheim), and unlabeled RNA [1, 5, 10, and 20 pmole of Spot 42 RNA or galk RNA] were mixed in binding buffer (20 mM Na-HEPES, 100 mM KCl, 1 mM DTT, 1 mM MgCl₂) and incubated on ice for 20 min. The binding reactions (10 µL) were mixed with 5 µL of loading dye (10% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol) and analyzed on a 5% nondenaturing polyacrylamide gel in TBE buffer at 200 V at 4°C for 1.5 h. Subsequently, the gel was dried and subjected to autoradiography.

**Toeprinting analysis**

Purification of ribosomal 30S subunits and toeprinting assays were carried out as described previously [Franc et al. 1997]. The 5’-end-labeled oligonucleotides otpk [5’-GCCCTGAATG TGTGTAGTGG-3’], complementary to the galk gene, and otp [5’-GAACAGAATCCATTGCC-3’], complementary to the galt gene, were used as primers for cDNA synthesis in the toeprinting reactions.

**Enzymatic and chemical probing of Spot 42 RNA**

RNase T₁ [Sigma] and RNase V₁ [Amersham Pharmacia Biotech] digestion was performed as described previously [Franc et al. 1997]. 5’-end-labeled Spot 42 RNA (0.05 pmole) was incubated in RNase buffer (20 mM Tris-acetate at pH 7.8, 100 mM NH₄Cl, 10 mM Mg-acetate, 200 mM K-glutamate, and 5 µg of tRNA) at 37°C. Partial digestion was carried out with 0.001 units of RNase T₁ or 0.005 units of RNase V₁ for 3 min and 15 min. For lead (II) acetate probing, 5’-end-labeled Spot 42 RNA (0.05 pmole) was incubated in 50 mM HEPES-KOH (pH 7.5), 10 mM MgCl₂, and 50 mM KCl, at 37°C. Samples were treated with PbAc₂ (final concentration, 5 mM) for 3 min and 15 min. Alkaline hydrolysis and RNase T₁ cleavage of Spot 42 RNA were carried out as described in Franck et al. [1999].

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