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Characterization of Mutations in the *metY-nusA-infB* Operon That Suppress the Slow Growth of a Δ*rimM* Mutant

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The RimM protein in *Escherichia coli* is associated with free 30S ribosomal subunits but not with 70S ribosomes. A Δ*rimM* mutant shows a sevenfold-reduced growth rate and a reduced translational efficiency, probably as a result of aberrant assembly of the ribosomal 30S subunits. The slow growth and translational deficiency can be partially suppressed by increased synthesis of the ribosome binding factor RbfA. Here, we have identified 14 chromosomal suppressor mutations that increase the growth rate of a Δ*rimM* mutant by increasing the expression of *rbfA*. Nine of these mutations were in the *nusA* gene, which is located upstream from *rbfA* in the *metY-nusA-infB* operon; three mutations deleted the transcriptional terminator between *infB* and *rbfA*; one was an insertion of IS*2* in *infB*, creating a new promoter for *rbfA*; and one was a duplication, placing a second copy of *rbfA* downstream from a promoter for the *yhbM* gene. Two of the *nusA* mutations were identical, while another mutation (*nusA98*) was identical to a previously isolated mutation, *nusA11*, shown to decrease termination of transcription. The different *nusA* mutations were found to increase the expression of *rbfA* by increasing the read-through of two internal transcriptional terminators located just downstream from the *metY* gene and that of the internal terminator preceding *rbfA*. Induced expression of the *nusA*$^+$ gene from a plasmid in a *nusA*$^+$ strain decreased the read-through of the two terminators just downstream from *metY*, demonstrating that one target for a previously proposed NusA-mediated feedback regulation of the *metY-nusA-infB* operon expression is these terminators. All of the *nusA* mutations produced temperature-sensitive phenotypes of *rimM*$^+$ strains. The *nusA* gene has previously been shown to be essential at 42°C and below 32°C. Here, we show that *nusA* is also essential at 37°C.

The *Escherichia coli* RimM protein shows affinity for free 30S ribosomal subunits but not for 70S ribosomes (3) and is important for the maturation of the 30S subunits (4). Mutants lacking RimM show a severalfold-decreased growth rate and a reduced translational efficiency (3). These defects can be partially suppressed by increased expression of the ribosome binding factor RbfA (4) encoded by the *metY-nusA-infB* operon. RbfA is a cold shock protein that is essential for the resumption of growth after a downshift in temperature (19); however, it also has an important function at higher temperatures, since an *rbfA* mutant shows a twofold-lower growth rate than an *rbfA*$^+$ strain at 42°C (9). RbfA is important for the maturation of the 30S ribosomal subunits, possibly by stabilizing the 5′-terminal helix of 16S rRNA (4, 9). Overexpression of *rbfA* suppresses a cold-sensitive mutation in this 16S rRNA helix (9). Previously, we isolated 23 mutations that increase the growth rate of a Δ*rimM* mutant and that were shown to be tightly linked to the *rbfA* gene (4) of the *metY-nusA-infB* operon (Fig. 1A). This operon contains, in the direction of transcription, the *metY* gene encoding a minor form of the initiator tRNA, the *p15a* open reading frame, the *nusA* gene for the transcriptional elongation factor NusA (8, 15, 16, 45), the *infB* gene encoding the translation initiation factor IF2 (38, 43), the *rbfA* gene (9, 47), and the *truB* gene for the tRNA(Ψ55) synthase (34, 47). The operon is transcribed from two promoters located upstream from *metY*, P_{-1} (12) and P_1 (16, 27), and a minor promoter between *metY* and *p15a*, P_2 (40). The *rpsO* and *pnp* genes located downstream from *truB* and encoding the ribosomal protein S15 and polynucleotide phosphorylase, respectively (39, 41), are also transcribed from these promoters (47); however, the major promoter for these two genes is that just upstream from *rpsO* (42). The cleavage by RNase III at sites between *metY* and *p15a* on the polycistronic mRNA initiates the rapid degradation of the downstream RNA (40). Internal transcriptional terminators are found between *metY* and *p15a* (16, 40) and between *infB* and *rbfA* (47). NusA negatively feedback regulates the expression of the *metY-nusA-infB* operon, and the two terminators between *metY* and *p15a* were suggested to be the regulatory target (30, 37, 40). In this paper, we describe the identification of 14 suppressor mutations in the *metY-nusA-infB* operon, which increase the growth rate of a *rimM* mutant by increasing the expression of the *rbfA* gene. Nine of the mutations were localized to the *nusA* gene and found to result in a deficiency in feedback regulation at the two terminators between *metY* and *p15a* and also at the terminator just upstream from *rbfA*. Of the other mutations, three had the transcriptional terminator between *infB* and *rbfA* deleted; one was an insertion of IS*2* in *infB*, creating a new promoter for *rbfA*; and one was a duplication, placing a second copy of *rbfA* downstream from a putative promoter for *yhbM*.

MATERIALS AND METHODS

Bacterial strains and plasmid constructions. The strains and plasmids used are listed in Table 1. The low-copy-number *lacZ* fusion vector pGOB100 was constructed in the following way. First, a 300-bp fragment containing the $rrnBt_1$ and $rrnBt_2$ transcriptional terminators was amplified by PCR using the oligonucleotides 5′-TTTTGGTACCGATGGTAGTGTGG-3′ and 5′-TTTTGGATCC GTAGATATGACGACAGG-3′, trimmed with *Kpn*I and *Bam*HI and inserted into the low-copy-number vector pCL1921. To facilitate later constructions of

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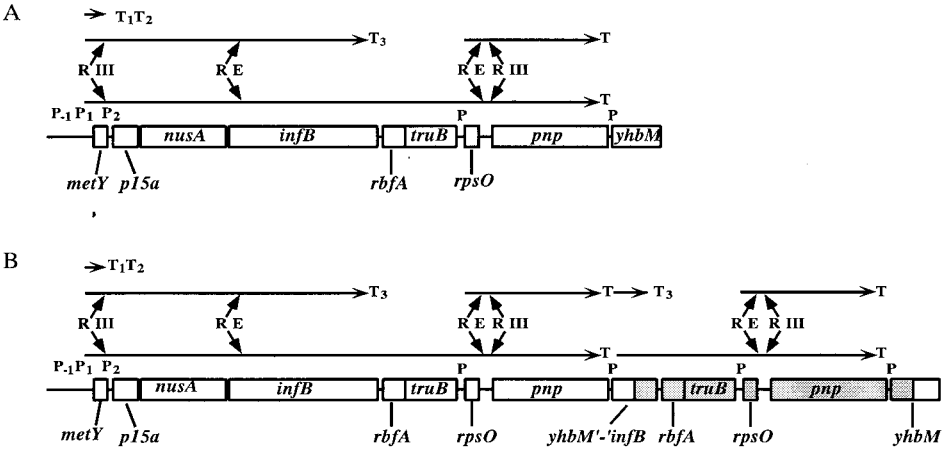

FIG. 1. Genetic organization of the *metY-nusA-infB* operon region on the chromosomes of wild-type *E. coli* (A) and strains that contain a duplication covering the 3′ part of *infB* to the 5′ half of *yhbM* (B; shaded region). P_{-1}, P_1, P_2, and P indicate the locations of promoters; T, T_1, T_2, and T_3 indicate different transcriptional terminators; while R E and R III show sites for the RNA-processing enzymes RNase E and RNase III, respectively. The horizontal arrows represent transcriptional products. For references and explanations of gene symbols, see the introduction.

lacZ fusions, the unique *Bam*HI site of the plasmid obtained was removed by inserting the oligonucleotide 5′-GATCGTCGAC-3′. This plasmid was named pMW348. Next, the unique *Eag*I site downstream from *lacZ* in the fusion vector pTL61T was converted to a *Kpn*I site by inserting the oligonucleotide 5′-GGC CAGGTACCT-3′. Finally, the *Eco*RI-*Kpn*I *lacZ* fragment of the resulting plasmid was cloned into plasmid pMW348.

Two different transcriptional fusions between *metY* and *lacZ* were constructed in pGOB100: DNA fragments containing either P_{metY}-*metY* and the terminators $metYt_1$ and $metYt_2$ between *metY* and *p15a* or P_{metY}-*metY* without the terminators were amplified by PCR using the upstream oligonucleotide 5′-TTTTGAATTC AACAAATGAAAGTGAAC-3′ and the downstream oligonucleotides 5′-TTT TGGATCCGCAGTGTGGATGTGCGACC-3′ and 5-TTTTGGATCCGAA CCCTATAACCGCAACTG-3′, respectively, trimmed with *Eco*RI-*Bam*HI and cloned into pGOB100. The *Eco*RI site of the two resulting plasmids was converted to an *Sph*I site using the oligonucleotide 5′-AATTGCATGC-3′, yielding plasmids pGOB115 and pGOB116, respectively. To enable the introduction of these two *lacZ* fusions into the *lac* operon region of the chromosome, an 880-bp fragment containing most of *lacI* was amplified by PCR using the oligonucleotides 5′-TTTTAAGCTTCTCTTATCAGACCGTTTCC-3′ and 5′-TTTTGGAT CCAGTTGCAGCAAGCGGTCC-3′, trimmed with *Hin*dIII and *Bam*HI, and inserted into the temperature-sensitive vector pMAK700. Into the resulting plasmid, the *trmD* operon transcriptional terminator, *rplSt*, was cloned on a *Bam*HI-*Sph*I fragment consisting of two complementary oligonucleotides: 5′-G ATCGGGCTGGCCAATTGGCTGGCCCTTTTTTGCATG-3′ and 5′-CAAA AAAGGGCCAGCCAATTGGCCAGCCC-3′. Finally, into the *Sph*I site of this plasmid, pGOB119, the two transcriptional fusions on plasmids pGOB115 and pGOB116 were inserted, yielding plasmids pGOB121 and pGOB122, respectively. In a similar way, two control constructions were made: a derivative (pGOB117) of pGOB122 which did not contain any DNA from the *metY* region and a derivative (pGOB118) of pGOB121 which lacked the *metY* promoter fragment and only contained the 245-bp $metYt_1$-$metYt_2$ terminator fragment.

To combine the *metY* promoter fragment with the *trmD* operon transcriptional terminator, *rplSt*, a 307-bp fragment containing *rplSt* was amplified by PCR using the oligonucleotides 5′-TTTTGGATCCGTGAGCGTACTGGTAAGG-3′ and 5′-TTTTGGATCCAAACGGGCGAATGTCGTGG-3′, trimmed with *Bam*HI, and inserted into plasmid pGOB122, yielding plasmid pGOB126.

To study the transcriptional read-through of the $metYt_1$ and $metYt_2$ terminators when transcription was initiated from P_{tet} of pBR322, two different fusions of P_{tet} and *lacZ* were constructed. First, a fragment carrying P_{tet} was amplified by PCR using the oligonucleotides 5′-AGATCCAGTTCGATGTAACC-3′ and 5′-TTTTGGATCCAATTTAACTGTGATAAACTACC-3′, cleaved with *Eco*RI and *Bam*HI, and inserted into plasmid pGOB100, yielding plasmid pMW368. A 262-bp $metYt_1$-$metYt_2$ terminator fragment was amplified by PCR using the oligonucleotides 5′-TTTTGGATCCTTCTGGAAAGTGCTCC-3′ and 5′-TTTT GGATCCGCAGTGTGGATGTGCGACC-3′, trimmed with *Bam*HI, and inserted into pMW368, yielding plasmid pMW381. The *Eco*RI sites of plasmids pMW368 and pMW381 were converted to an *Sph*I site using the oligonucleotide 5′-AATTGCATGC-3′, yielding plasmids pGOB129 and pGOB127, respectively. The transcriptional fusions on these two plasmids were cloned into the *Sph*I site of plasmid pGOB119, yielding plasmids pGOB134 and pGOB131, respectively.

The constructions on the temperature-sensitive plasmids pGOB117, pGOB118, pGOB121, pGOB122, pGOB126, pGOB131, and pGOB134 were integrated into the *lacI-lacZ* region of the chromosome of strain MW100 following the allelic replacement procedure described by Hamilton et al. (14), yielding strains GOB438, GOB440, GOB434, GOB435, GOB838, GOB840, and GOB842, respectively. That the constructions had replaced the wild-type *lacI-lacZ* region was confirmed by PCR.

Strains MW208 and MW210 were used as donors for the transfer of transcriptional fusions of P_{rpsP} and *lacZ* to different *nusA* mutants (Table 1). The construction of strains MW208 and MW210 will be published elsewhere. The two strains carry two copies each of the chromosomal region normally containing the *ffh*$^+$ *rpsP*$^+$-*rimM*$^+$-*trmD*$^+$-*rplS*$^+$ *yfiB*′ genes. Between the two copies is the *nptI* gene, conferring resistance to kanamycin and neomycin. In the right-hand copy of the duplicated region, fusions of P_{rpsP} and *lacZ* have been integrated, replacing the segment containing *rpsP*$^+$-*rimM*$^+$-*trmD*$^+$-*rplS*′. The wild-type *rpsP*$^+$-*rimM*$^+$-*trmD*$^+$-*rplS* operon contains, between P_{rpsP} and *rpsP*, a terminatorlike structure which is active in vitro (6) and probably functions as a transcriptional attenuator. In strain MW208, the fusion point is downstream from the transcriptional attenuator, while in strain MW210, it is upstream from the attenuator.

In order to examine the effect of induced synthesis of the wild-type NusA protein on the read-through of the two terminators between *metY* and *p15a*, plasmid pJML007 was constructed. A DNA fragment containing the *nusA* gene was amplified by PCR using the oligonucleotides 5′-TTTTGAATTCCCCACTTT TAATAGTCTGG-3′ and 5′-TTTTGGTACCTGTTCCTTCCTGCTACAG-3′, trimmed with *Eco*RI and *Kpn*I, and inserted into the expression vector pBAD30.

To investigate whether the *nusA* gene was essential at 37°C, an in-frame deletion of *nusA* was constructed (see Fig. 7). The region upstream from *nusA* was amplified by PCR using the oligonucleotides 5′-TTTTGGATCCATTCAA CAAATGAAAGTGAAC-3′ and 5′-TTTTGTCGACGGCTTCAACTACAGC-3′, while the region downstream from *nusA* was amplified with the oligonucleotides 5′-TTTTGTCGACCGTAATATTTGCTGGTTCGG-3′ and 5′-GCATC ACACCGTCGTCGG-3′. The two DNA fragments were cleaved with *Bam*HI-*Sal*I and *Sal*I-*Pst*I, respectively, and ligated to the *Bam*HI-*Pst*I-digested plasmid vector pMAK705. The *nusA* deletion on the resulting plasmid, pJML001, was substituted for the wild-type *nusA* gene on the chromosme of strain MW100 following the allelic replacement procedure described by Hamilton et al. (14). The resulting strain, JML012, contained the *nusA* deletion on the chromosome and the wild-type *nusA* gene on the temperature-sensitive plasmid (see Fig. 7B), as confirmed by PCR analyses.

Media and growth conditions. Rich medium was either rich morpholinepropanesulfonic acid (MOPS) (33) or Luria-Bertani (LB) (1) medium supplemented with medium E plus vitamin B_1 and 0.4% glucose (52). Cultures were grown at 37°C, and growth was monitored at 600 nm using a Shimadzu UV-1601 spectrophotometer.

Assay of β-galactosidase. The β-galactosidase activity was measured after permeabilization of whole cells with toluene as described previously (25).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Origin or reference[a]
Strains		
GOB375	MW100 *argG2424*::miniTn*10*Cm	
GOB383	PW100 *argG2424*::miniTn*10*Cm	
GOB388	MW100 *nusA98 argG2424*::miniTn*10*Cm	
GOB390	GOB388 $argG^+$	
GOB417	GOB375 *nusA97* $argG^+$	
GOB421	GOB375 *nusA93* $argG^+$	
GOB423	GOB375 *nusA95* $argG^+$	
GOB425	GOB375 *nusA92* $argG^+$	
GOB426	GOB375 *nusA94* $argG^+$	
GOB427	GOB375 *nusA91* $argG^+$	
GOB428	GOB375 *nusA96* $argG^+$	
GOB434	MW100 *lacI′-rplSt*-P_{metY}-*metY-metY*t_1t_2-*lacZ*	
GOB435	MW100 *lacI′-rplSt*-P_{metY}-*metY-lacZ*	
GOB438	MW100 *lacI′-rplSt-lacZ*	
GOB440	MW100 *lacI′-rplSt-metY*t_1t_2-*lacZ*	
GOB492	GOB434 *nusA98 argG2424*::miniTn*10*Cm	
GOB494	GOB434 *argG2424*::miniTn*10*Cm	
GOB496	GOB435 *nusA98 argG2424*::miniTn*10*Cm	
GOB498	GOB435 *argG2424*::miniTn*10*Cm	
GOB600	GOB492 Δ*rimM102 yfiB*::*nptI*	
GOB604	GOB494 Δ*rimM102 yfiB*::*nptI*	
GOB608	GOB496 Δ*rimM102 yfiB*::*nptI*	
GOB612	GOB498 Δ*rimM102 yfiB*::*nptI*	
GOB699	GOB434 *nusA92 argG2424*::miniTn*10*Cm	
GOB702	GOB435 *nusA92 argG2424*::miniTn*10*Cm	
GOB734	GOB434 *nusA96 argG2424*::miniTn*10*Cm	
GOB736	GOB435 *nusA96 argG2424*::miniTn*10*Cm	
GOB742	GOB434 *nusA93 argG2424*::miniTn*10*Cm	
GOB744	GOB435 *nusA93 argG2424*::miniTn*10*Cm	
GOB750	GOB434 *nusA94 argG2424*::miniTn*10*Cm	
GOB752	GOB435 *nusA94 argG2424*::miniTn*10*Cm	
GOB758	GOB434 *nusA95 argG2424*::miniTn*10*Cm	
GOB760	GOB435 *nusA95 argG2424*::miniTn*10*Cm	
GOB766	GOB434 *nusA91 argG2424*::miniTn*10*Cm	
GOB768	GOB435 *nusA91 argG2424*::miniTn*10*Cm	
GOB788	GOB792 *nusA98*	
GOB790	GOB794 *nusA98*	
GOB792	MW100 $nusA^+$ DUP[*ffh*$^+$ to *yfiB′*]*nptI*[*ffh*$^+$ (P+A)$_{rpsP}$-*lacZ* Δ(*rpsP-trmD*) *′rplS yfiB′*]	
GOB794	MW100 $nusA^+$ DUP[*ffh*$^+$ to *yfiB′*]*nptI*[*ffh*$^+$ P_{rpsP}-*lacZ* Δ(*rpsP-trmD*) *′rplS yfiB′*]	
GOB796	GOB792 *nusA93*	
GOB798	GOB794 *nusA93*	
GOB800	GOB792 *nusA95*	
GOB802	GOB794 *nusA95*	
GOB804	GOB792 *nusA94*	
GOB806	GOB794 *nusA94*	
GOB808	GOB792 *nusA91*	
GOB810	GOB794 *nusA91*	
GOB812	GOB792 *nusA96*	
GOB814	GOB794 *nusA96*	
GOB816	GOB792 *nusA92*	
GOB818	GOB794 *nusA92*	
GOB821	GOB434 *nusA97 argG2424*::miniTn*10*Cm	
GOB823	GOB435 *nusA97 argG2424*::miniTn*10*Cm	
GOB838	MW100 *lacI′-rplSt*-P_{metY}-*metY*-T_{rplS}-*lacZ*	
GOB840	MW100 *lacI′-rplSt*-P_{tet}-*metY*t_1t_2-*lacZ*	
GOB842	MW100 *lacI′-rplSt*-P_{tet}-*lacZ*	
GOB868	GOB838 *nusA94 argG2424*::miniTn*10*Cm	
GOB870	GOB840 *nusA94 argG2424*::miniTn*10*Cm	
GOB872	GOB842 *nusA94 argG2424*::miniTn*10*Cm	
JML012	MW100 Δ*nusA*/p*metY-pl5a-nusA-infB′*	
JML087	MW100 Δ*nusA*/pJML007	
JML125	MW100 *sdr-53* (*infB*::IS*2*) *argG2424*::miniTn*10*Cm	
JML126	MW100 *sdr-40* (Δ*infB*t_3) *argG2424*::miniTn*10*Cm	
JML127	MW100 *sdr-44* (Δ*infB*t_3) *argG2424*::miniTn*10*Cm	
JML128	MW100 *sdr-54* (Δ*infB*t_3) *argG2424*::miniTn*10*Cm	
MW37	Hfr P4X Δ*rimM102 yfiB*::*nptI*	36
MW38	Hfr P4X *rimM*$^+$ *yfiB*::*nptI*	36
MW100	Hfr P4X	54
PW093	MW37 *sdr-27* = *nusA97* (ΔA in codon 412)	4
PW100	MW37 *sdr-34* = *nusA98* (GGC to GAC in codon 181)	4
PW101	MW37 *sdr-35* = *nusA93* (ATC to AAC in codon 114)	4

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Genotype	Origin or reference[a]
PW104	MW37 *sdr-38* = *nusA92* (ATC to AAC in codon 49)	4
PW105	MW37 *sdr-39* = *nusA94* (GTG to GAG in codon 142)	4
PW106	MW37 *sdr-40* = Δ*infBt*$_3$	4
PW107	MW37 *sdr-41* = *nusA92* (ATC to AAC in codon 49)	4
PW109	MW37 *sdr-43* = DUP (′*infB* to *yhbM*′)	4
PW110	MW37 *sdr-44* = Δ*infBt*$_3$	4
PW114	MW37 *sdr-48* = *nusA96* (ACT to CCT in codon 198)	4
PW115	MW37 *sdr-49* = *nusA95* (GTC to GAC in codon 197)	4
PW116	MW37 *sdr-50* = *nusA91* (ATT to AAT in codon 23)	4
PW119	MW37 *sdr-53* = *infB*::IS*2*	4
PW120	MW37 *sdr-54* = Δ*infBt*$_3$	4
Plasmids		
pBAD30	*bla cat*′ *araC*	13
pCL1921	Str^r Spc^r	21
pGOB117	*rep*(Ts) *cat* ′*lacI*′-*rplSt-lacZ-rrnBt*$_1$*t*$_2$	
pGOB118	*rep*(Ts) *cat* ′*lacI*′-*rplSt-metYt*$_1$*t*$_2$-*lacZ-rrnBt*$_1$*t*$_2$	
pGOB121	*rep*(Ts) *cat* ′*lacI*′-*rplSt*-P$_{metY}$-*metY-metYt*$_1$*t*$_2$-*lacZ-rrnBt*$_1$*t*$_2$	
pGOB122	*rep*(Ts) *cat* ′*lacI*′-*rplSt*-P$_{metY}$-*metY-lacZ-rrnBt*$_1$*t*$_2$	
pGOB126	*rep*(Ts) *cat* ′*lacI*′-*rplSt*-P$_{metY}$-*metY*-T$_{rplSt}$-*lacZ-rrnBt*$_1$*t*$_2$	
pGOB131	*rep*(Ts) *cat* ′*lacI*′-*rplSt*-P$_{tet}$-*metYt*$_1$*t*$_2$-*lacZ-rrnBt*$_1$*t*$_2$	
pGOB134	*rep*(Ts) *cat* ′*lacI*′-*rplSt*-P$_{tet}$-*lacZ-rrnBt*$_1$*t*$_2$	
pJML001	*rep*(Ts) *cat* P$_{metY}$-*metY-p15a*-Δ*nusA-infB*′	
pJML007	*bla cat*′ *araC* P$_{BAD}$-*nusA*	
pMAK700	*rep*(Ts) *cat*	14
pMAK705	*rep*(Ts) *cat*	14
pTL61T	*bla lacZ*	22

[a] Unless otherwise noted, the origin was this study.

PCR amplification of chromosomal DNA and DNA sequencing. Regions of the *E. coli* chromosome were amplified by PCR from colonies resuspended in H_2O (26, 44). *Pfu* DNA polymerase from Stratagene cloning systems, La Jolla, Calif., was used if the obtained fragments were to be cloned into plasmids, and *Taq* DNA polymerase from Roche Diagnostics Scandinavia AB, Bromma, Sweden, was used in all other cases. The obtained fragments were separated on agarose gels, cut out, and purified using GENE-CLEAN from Bio 101 Inc., La Jolla, Calif. DNA sequencing of PCR fragments and plasmid DNA was done with a Thermo Sequenase II dye terminator cycle-sequencing premix kit from Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England, using an ABI 377 XL DNA Sequencer from PE Applied Biosystems, Stockholm, Sweden.

Northern blot analysis. Total RNA was prepared according to the method of von Gabain et al. (53) and subjected to Northern blot analysis essentially as described by Sambrook et al. (46). That equal amounts of the RNAs from the different strains grown in LB medium were used was determined both by spectrophotometric measurements at 260 nm and by ethidium bromide staining of aliquots of the RNA electrophoresed on agarose gels. DNA fragments used as probes were purified as described above and labeled with [α-^{32}P]dATP using the Megaprime DNA labeling system from Amersham Pharmacia Biotech.

Primer extension analysis. Total RNA was prepared as described by von Gabain et al. (53). Primer extension was performed using 3.75 μg of RNA, ^{32}P end-labeled primer (5′-CAGGTGGAAGGGCTGTTCAC-3′), and AMV reverse transcriptase from Roche Diagnostics Scandinavia AB.

Analysis of proteins by 2-D gel electrophoresis. Steady-state cultures of bacterial cells were grown in rich MOPS medium at 37°C to an optical density at 600 nm of 0.5, labeled for 15 min with 250 μCi of [^{35}S]methionine each (>1,000 Ci/mmol), and chased with 0.167 ml of 0.2 M methionine for 3 min. Extracts were prepared essentially as described by VanBogelen and Neidhardt (51). O'Farrell two-dimensional (2-D) polyacrylamide gels (35) were used to analyze the protein expression pattern. One million counts per minute was loaded onto each first-dimension isoelectric focusing gel (Millipore Intertech, Bedford, Mass.) containing ampholines 3 to 10 and Duracryl acrylamide from Oxford Glycosystems. The first dimension was run as described by the manufacturer, whereas the second dimension was 10 to 17.5% gradient polyacrylamide slab gels containing sodium dodecyl sulfate. The gels were dried and exposed to a PhosphorImager screen and analyzed using ImageQuant software from Molecular Dynamics, Inc.

RESULTS

Identification of 14 suppressor mutations linked to *rbfA*. Previously, we isolated several suppressor mutations (*sdr-27* to *sdr-55*; for suppressor to deletion of *rimM*) that increased the growth rate and translational efficiency of a Δ*rimM102* mutant at 37°C (4). Twenty-three of the mutations were shown to be tightly linked to the *metY-nusA-infB* operon. For one of these strains, PW109 (Δ*rimM102 sdr-43*), we showed that an increased synthesis of the cold shock protein RbfA encoded by the fifth gene of the *metY-nusA-infB* operon (Fig. 1A) was responsible for the suppression at 37°C. The results from Northern blot experiments with probes corresponding to different parts of the *metY-nusA-infB* operon (4; G. O. Bylund and P. M. Wikström, unpublished results) prompted us to examine, by Southern hybridization, whether *sdr-43* was a duplication that covered *rbfA* and *truB*. The results from this experiment (data not shown) demonstrated that the *sdr-43* strain PW109 contained two copies of the *rbfA* gene and that the 5′ half of the *yhbM* gene downstream from *pnp* had been joined to the 3′ part of *infB* (Fig. 1B). The proposed hybrid region was successfully PCR amplified with a downward-facing *yhbM* primer and an upward-facing *infB* primer. The DNA sequence of the obtained PCR product showed that position 369 of *yhbM* had been fused to position 2295 of *infB* (data not shown). A putative promoter for *yhbM* that would explain two very abundant mRNA species (2.6 and 0.9 kb in length) that are present in *sdr-43* strains but not in *sdr*$^+$ strains (4) was identified 21 bp downstream from the transcriptional terminator for *pnp*. The shorter mRNA probably results from termination at the transcriptional terminator just upstream from *rbfA*, since it hybridizes to an *infB* probe but not to an *rbfA* probe, whereas the longer mRNA also contains the *rbfA* and *truB* genes and therefore seems to be responsible for the suppression (4).

Since the suppressor mutation in strain PW109 increased the synthesis of RbfA (4), we found it conceivable that the other suppressor mutations linked to the *metY-nusA-infB* operon

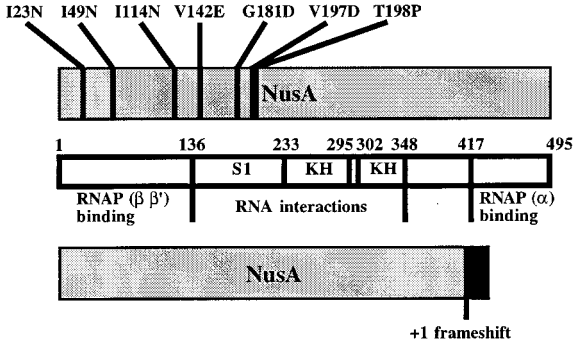

FIG. 2. Locations and natures of different alterations in NusA that suppress the slow growth of a Δ*rimM102* mutant. The linear functional map of NusA shown has been modified from reference 23, integrating information from reference 24. S1 and KH represent different motifs found to be important for binding to RNA (2, 11, 49).

would also do so. We reasoned that three regions were likely to contain suppressor mutations which could increase expression of *rbfA*: (i) the region immediately upstream from *rbfA*, including the transcriptional terminator $infBt_3$ between *infB* and *rbfA*; (ii) the region between *metY* and *p15a*, containing an internal promoter, an RNase III site the processing at which has been shown to decrease the stability of the downstream part of the mRNA (40), and two internal transcriptional terminators, $metYt_1$ and $metYt_2$; and (iii) the *nusA* gene, since the NusA protein feedback regulates the expression of the *metY-nusA-infB* operon (7, 30, 37, 40). Therefore, we sequenced one or more of these candidate regions in 13 of the suppressor strains. In three of the suppressor strains, the major part of the $infBt_3$ terminator between *infB* and *rbfA* had been deleted (strain PW106, nucleotides [nt] −41 to −25; strain PW110, nt −40 to −30; and strain PW120, nt −39 to −30, relative to the *rbfA* start codon). Strain PW119, which was cold sensitive for growth, contained an insertion of IS*2*, just before the penultimate codon of *infB*. Nine of the suppressor strains were found to have mutations in *nusA* (Table 1 and Fig. 2). Two of these strains contained the same mutation (*nusA92*) in codon 49, substituting asparagine for isoleucine. Of the other seven mutations, six also resulted in single-amino-acid substitutions in the N-terminal half of NusA, while one was a deletion of a single base pair, resulting in replacement of the last 84 amino acids of NusA with 23 amino acids encoded by the +1 reading frame. One of the mutations (*nusA98*) was identical to a previously isolated conditional-lethal mutation, *nusA11* (28, 31), substituting aspartate for glycine in position 181 (8, 17).

The *nusA* mutations increase the amounts of two *metY-nusA-infB* operon mRNAs. Previously, we found that the suppressor strain PW100 (Δ*rimM102 sdr-34*), here shown to contain a mutation in *nusA* (*nusA98*), expressed dramatically increased

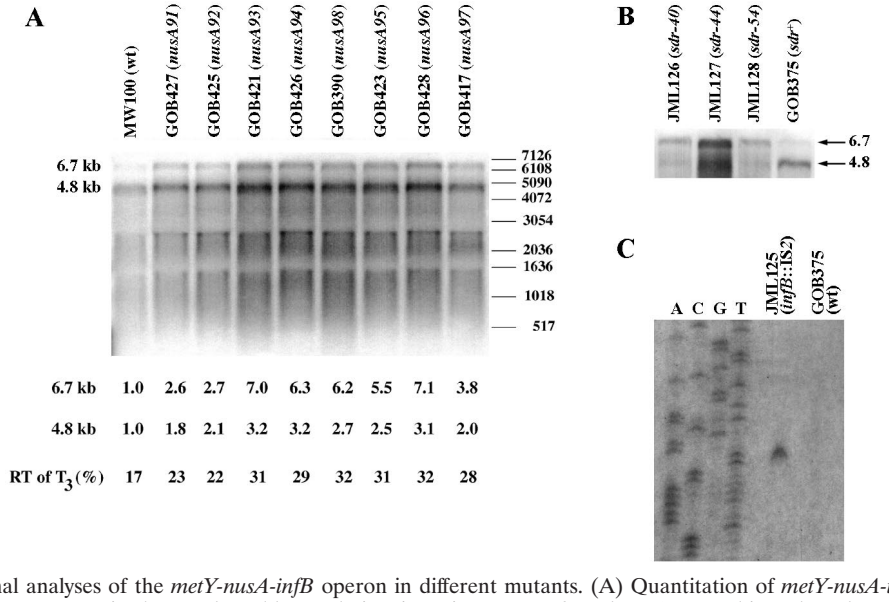

FIG. 3. Transcriptional analyses of the *metY-nusA-infB* operon in different mutants. (A) Quantitation of *metY-nusA-infB* operon mRNAs in wild-type (wt) and *nusA* mutant strains by Northern blot analysis. Five micrograms of total RNA was subjected to electrophoresis in an agarose gel containing formaldehyde, transferred to a Hybond N filter, and probed with a radiolabeled PCR fragment corresponding to the *p15a* gene. The strains used (with the relevant genetic markers in parentheses) are indicated above the respective lanes. The sizes of the ^{32}P-end-labeled fragments of the 1-kb DNA ladder (GIBCO BRL Life Technologies Inc., Gaithersburg, Md.) are indicated. The 6.7-kb transcript results from read-through of the $metYt_1$ and $metYt_2$ terminators between *metY* and *p15a* and the $infBt_3$ terminator just upstream from *rbfA*, while the 4.8-kb transcript terminates at the $infBt_3$ terminator (Fig. 1). The amounts of these transcripts (determined by quantitation of the radioactivity using a PhosphorImager from Molecular Dynamics, Inc.) in the different *nusA* mutants were normalized to those for the *nusA*$^+$ strain MW100. The read-through (RT) of the $infBt_3$ terminator was calculated as the amount of radioactivity in the 6.7-kb band divided by the sum of the radioactivity in the 4.8- and 6.7-kb bands. (B) Northern blot analysis showing the effect of deletions in the $infBt_3$ transcriptional terminator on the amount of the 6.7-kb transcript relative to that of the 4.8-kb transcript. (C) Identification of the 5′ end of the mRNA resulting from transcription initiation at a new promoter created by the insertion of IS*2* in *infB*. Primer extension analyses of mRNA and DNA sequencing of a PCR fragment covering the 3′ part of *infB* and the 5′ part of *rbfA* from a wild-type strain were performed using a ^{32}P-end-labeled primer binding to positions −54 to −73 relative to the start codon of *rbfA*. The primer extension product obtained for strain JML125 (*infB*::IS*2*) corresponds to an mRNA 5′ end at the A 6 nt downstream from the −10 region of the proposed promoter.

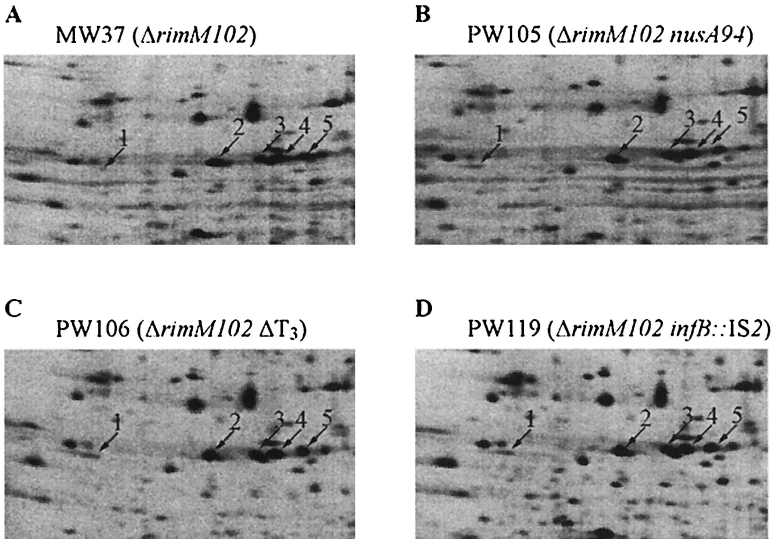

FIG. 4. Synthesis of individual proteins at 37°C in the Δ*rimM102* mutant and three different suppressor strains. Total cell extracts of the indicated strains labeled with [^{35}S]methionine were separated on 2-D gels. Only the relevant part of each gel is shown. The indicated proteins are as follows: 1, RbfA; 2, H-NS; 3, 4, and 5, ribosomal protein S6. The position of RbfA on the gels was determined previously (4), and the identities of the other proteins were obtained by comparing the gels with those of VanBogelen et al. (50).

levels of two *metY-nusA-infB* operon mRNA species, 4.8 and 6.7 kb in size, compared to those in the Δ*rimM102* mutant MW37 (4). The *nusA98* mutation is identical to *nusA11*, which has been shown to reduce transcription termination (18, 28, 29). Therefore, we wanted to examine whether the different *nusA* mutations isolated here increased the read-through of transcriptional terminators internal to the *metY-nusA-infB* operon, leading to an increased synthesis of RbfA, which would then explain the suppression of the slow growth of the Δ*rimM102* mutant MW37. The difference in expression of the 4.8- and 6.7-kb transcripts between strains PW100 (Δ*rimM102 nusA98*) and MW37 (Δ*rimM102*) might be partly a secondary effect resulting from the 2.5-fold growth rate difference between the two strains (the specific growth rates, $k = \ln 2/g$, where g is the mass doubling time in hours, were 0.92 and 0.37, respectively, in LB medium). Therefore, to assess any direct effects of the different *nusA* mutations on the read-through of *metY-nusA-infB* operon transcriptional terminators, the amounts of the 4.8- and 6.7-kb mRNAs were determined for *rimM*$^+$ strains containing the different *nusA* mutations and showing only minor growth rate differences at 37°C. The levels of the 4.8- and 6.7-kb mRNAs as determined by using the region corresponding to the *p15a* gene (Fig. 1A) as a probe in a Northern blot experiment were higher in all of the *nusA* mutants than in the *nusA*$^+$ strain MW100 (Fig. 3A). The shorter of the two mRNAs probably corresponds to an RNase III-processed form of the initial transcript of 5.0 kb, which starts upstream from *metY* and terminates at the *infBt*$_3$ terminator just before *rbfA* (4, 40, 47). The longer transcript results from read-through of the *infBt*$_3$ terminator (4), and its size suggests that the 3′ end is between *rpsO* and *pnp*. The larger amounts of the 4.8-kb transcript in the *nusA* mutants relative to the *nusA*$^+$ strain suggest that the *nusA* mutations increased the read-through of the *metYt*$_1$ and *metYt*$_2$ terminators between *metY* and *p15a*, although other explanations, such as increased promoter activities, could not be excluded. However, the relative differences between the amounts of the 6.7-kb transcript for each of the *nusA* mutants and that in the *nusA*$^+$ strain were higher (2.6- to 7.1-fold) than those for the 4.8-kb transcript (1.8- to 3.2-fold), clearly indicating that all of the *nusA* mutations increased the read-through of the *infBt*$_3$ terminator preceding *rbfA*. In fact, the calculated read-through of that terminator increased 1.3- to 1.9-fold due to the *nusA* mutations (Fig. 3A). Thus, NusA is important for transcription termination at least at the *infBt*$_3$ terminator, but likely also at the *metYt*$_1$ and *metYt*$_2$ terminators between *metY* and *p15a*. Further, we note that there is a correlation between the degree of suppression and mRNA expression levels, since the *nusA* mutations of the slowest-growing suppressor strains, PW107 (*nusA92*) and PW116 (*nusA91*) ($k = 0.53$ and 0.66, respectively) increased the read-through of the *infBt*$_3$ terminator and the amount of the 6.7-kb transcript to a lesser extent than did the suppressor mutations in the faster-growing strains, PW101 (*nusA93*), PW105 (*nusA94*), PW100 (*nusA98*), PW115 (*nusA95*), PW114 (*nusA96*), and PW093 (*nusA97*), which had specific growth rates, k, between 0.79 and 0.92.

The suppressor mutations increase the amount of the RbfA protein. Conceivably, the three suppressor mutations deleting the major part of the terminator *infBt*$_3$ between *infB* and *rbfA* increased the amount of the 6.7-kb read-through transcript relative to that of the 4.8-kb transcript resulting from termination at this terminator (Fig. 3B). As mentioned above, one of the suppressor strains contained an insertion of IS*2* in *infB*. There are a number of examples where IS*2* activates the transcription of genes located downstream from the insertion point, probably by creating hybrid promoters (10). We note that in the *infB* sequence there is a four-out-of-six match (underlined) (TACCAT) to the consensus sequence for a −10 promoter region 17 bp downstream from a postulated −35 region in the left end of the IS*2* insertion (10). To examine whether the proposed promoter could initiate transcription, mRNA from strain JML125 containing the *infB*::IS*2* mutation

was subjected to primer extension analysis using a primer binding upstream from the $infBt_3$ terminator. A primer extension product corresponding to an mRNA 5′ end 6 nt downstream from the −10 hexamer of the proposed promoter was obtained for strain JML125, whereas no primer extension product was seen for the control strain, GOB375 (Fig. 3C), indicating that the IS*2* insertion in *infB* had created a new promoter for *rbfA*. To examine whether the suppressor mutations increased the synthesis of the RbfA protein, total protein extracts from suppressor mutants were analyzed by 2-D protein gel electrophoresis. The amounts of RbfA in the suppressor mutants PW105 (Δ*rimM102 nusA94*), PW106 (Δ*rimM102* Δ$infBt_3$), and PW119 (Δ*rimM102 infB*::IS*2*) were severalfold higher than in the suppressor-free Δ*rimM102* mutant MW37 (Fig. 4). Thus, these findings indicate that the suppressor mutations increasing the growth rate of the Δ*rimM102* mutant MW37 were obtained because they increase the synthesis of RbfA.

The *nusA98* mutant is deficient in NusA-mediated transcriptional feedback regulation at the terminators between *metY* and *p15a*. Previously, the $metYt_1$ and $metYt_2$ terminators between *metY* and *p15a* were suggested to be the target for the NusA-mediated negative-feedback regulation of transcription of the *metY-nusA-infB* operon (30, 37). However, formally the possibility that the region upstream from the two terminators, which contains promoters and the *metY* gene, was the site of regulation could not be excluded. Furthermore, other results indicated that the regulatory site is located further downstream (7). To investigate whether the $metYt_1$ and $metYt_2$ terminators were a target for the NusA-mediated regulation, the effect of increased synthesis of NusA from an expression vector on the read-through of the terminators was examined. Transcriptional fusions between *metY* with or without the terminators and *lacZ* were constructed and integrated into the *lacI-lacZ* region of the chromosome of a $rimM^+$ strain (Fig. 5; see Materials and Methods), and the effect of arabinose-induced synthesis of wild-type NusA from the P_{BAD} promoter in plasmid pJML007 on the activity of β-galactosidase was measured. The read-through of the two terminators was approximately 25% when no arabinose was added or when the expression vector did not contain the *nusA* gene, as judged from a comparison of the β-galactosidase activity of the terminator-containing *lacZ* fusion with that of the fusion lacking the two terminators (Table 2). The expression of the terminator-containing *lacZ* fusion of strain GOB492 dropped almost twofold, whereas that of the *lacZ* fusion lacking the two terminators (strain GOB496) was not significantly affected when NusA synthesis was induced with 0.2% arabinose. This suggests that the target for the NusA-mediated feedback regulation of the *metY-nusA-infB* operon expression indeed is the terminators between *metY* and *p15a*. Further, the read-through was 1.7-fold higher in the *nusA98* mutant than in the $nusA^+$ strain at 37°C when synthesis of the wild-type NusA protein from plasmid pJML007 was not induced, indicating that the mutant NusA protein is deficient in feedback regulation at the terminators (Table 2). However, induction of wild-type NusA protein synthesis with 0.2% arabinose in the *nusA98* mutant restored termination to wild-type levels. The higher β-galactosidase activity (i.e., enzyme activity per unit of optical density of the culture) of the fusion lacking the terminator in the *nusA98* strains relative to that in $nusA^+$ strains seemed to result from spontaneous lysis of the *nusA98* strains (leading to an underestimation of the cell culture density). However, an increased lysis was also observed for the *nusA98* strains that carried the *lacZ* fusion containing the $metYt_1$ and $metYt_2$ terminators, and thus, the calculated transcriptional read-through was not affected by this lysis.

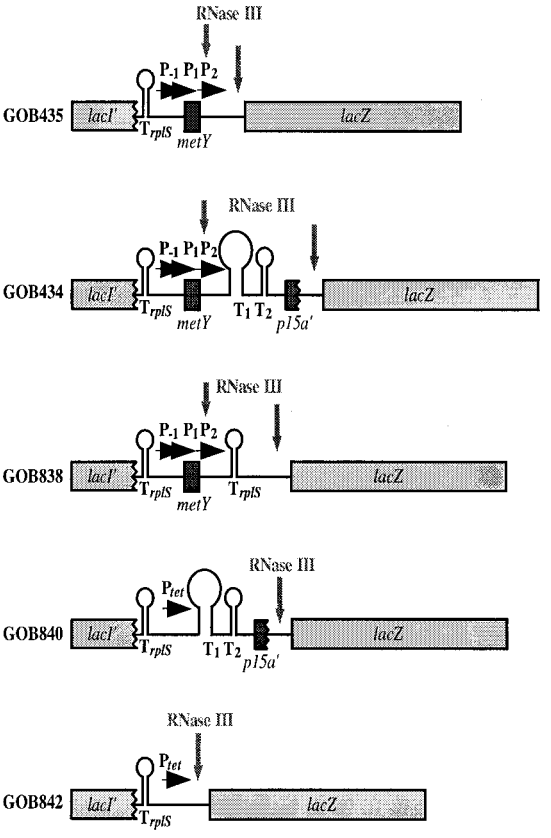

FIG. 5. Transcriptional fusions integrated into the *lacI-lacZ* region of the chromosome. The fusion point in strain GOB434 is 245 bp downstream from that in strain GOB435. P_{-1}, P_1, and P_2 indicate promoters of the *metY* operon, and P_{tet} is the promoter for the tetracycline resistance gene of plasmid pBR322; T_1 and T_2 indicate the terminators between *metY* and *p15a*, and T_{rplS} is the terminator of the *trmD* operon. Two different RNase III-processing sites are indicated, one (left-hand arrow) native to the region between *metY* and *p15a* and the other (right-hand arrow) present in the *lacZ* fusion vector used (22). For a description of the construction of the different fusions, see Materials and Methods.

To investigate whether the effect of the *nusA98* mutation on the read-through of the terminators between *metY* and *p15a* was different in a Δ*rimM102* and in a $rimM^+$ background, the expression of the *lacZ* fusions was also measured in strains containing the Δ*rimM102* mutation. The expression of the *lacZ* fusions in the Δ*rimM102* strains was slightly lower than that in the $rimM^+$ strains; however, the read-through of the terminators was not dependent on the allelic state of *rimM* either in $nusA^+$ or in *nusA98* strains (Table 3).

The new *nusA* mutations increase the read-through of the terminators between *metY* and *p15a*. To examine whether the new *nusA* mutations isolated here also increased the read-through of the $metYt_1$ and $metYt_2$ terminators between *metY* and *p15a*, the different *nusA* mutations were introduced into the *lacZ* fusion strains and the β-galactosidase activity was

TABLE 2. NusA-mediated transcriptional feedback regulation at the terminators between *metY* and *p15a*[a]

Relevant genotype	β-Galactosidase activity				Read-through[b]	
	P		P + T			
	−	+	−	+	−	+
nusA$^+$/pJML007(*nusA*$^+$)	5,782 (5,804, 5,760)	6,050 (6,461, 5,638)	1,472 (1,490, 1,453)	860 (874, 846)	0.25	0.14
nusA$^+$/pBAD30	5,952 (5,938, 5,966)	5,599 (5,720, 5,318)	1,498 (1,537, 1,460)	1,410 (1,454, 1,367)	0.25	0.25
nusA98/pJML007(*nusA*$^+$)	7,408 (8,070, 6747)	6,363 (6,352, 6,374)	3,112 (3,164, 3060)	1,476 (1,527, 1,425)	0.42	0.23
nusA98/pBAD30	7,782 (8,148, 7,417)	7,521 (7,860, 7,182)	3,298 (3,434, 3,161)	2,991 (3,070, 2,912)	0.42	0.40

[a] Expression of the P_{metY}-*metY-lacZ* (P) and P_{metY}-*metY-metYt*$_1$*t*$_2$-*lacZ* (P + T) fusions is shown in Miller units. The average expression from two independent experiments is shown for each strain, with the actual values from the respective experiment within parentheses. pBAD30 is the plasmid vector control for pJML007 (*nusA*$^+$). + indicates that a concentration of 0.2% arabinose was used for induction of the pBAD promoter on the two plasmids; − indicates no arabinose.
[b] Calculated as (P + T)/P.

measured. The eight different *nusA* mutations had only minor effects on the expression of the fusion containing the *metY* promoter fragment (data not shown); however, they increased the read-through of the terminators between *metY* and *p15a* 1.3- to 1.9-fold (Fig. 6A). This is in agreement with the higher levels of the 4.8-kb mRNA in the different *nusA* mutants relative to that in the *nusA*$^+$ strain (Fig. 3A). To investigate whether the different *nusA* mutations also affected the termination at a completely different internal terminator, the read-through of the *trmD* operon attenuator (5, 6) was measured. The *nusA* mutations were introduced into strains containing either a fusion of the *trmD* operon promoter P_{rpsP} and *lacZ* or P_{rpsP}, the attenuator, and *lacZ*. The read-through of the attenuator was calculated as the ratio of the β-galactosidase activity of the fusion containing the attenuator to that of the fusion lacking the attenuator. Evidently, the *nusA* mutations had little or no effect on the read-through of the *trmD* operon attenuator (Fig. 6B). This suggests either that the different *nusA* mutations were specific for the terminators within the *metY-nusA-infB* operon or, perhaps more likely, that NusA does not enhance termination at the *trmD* operon attenuator.

NusA-mediated feedback regulation is not promoter or terminator specific. To investigate whether the observed NusA-mediated feedback regulation of transcription termination was promoter or terminator specific, we made two different chimeric promoter-terminator constructions fused to *lacZ* and quantified the read-through in *nusA*$^+$ and *nusA94* strains. First, the terminator, *rplSt*, of the *trmD* operon was substituted for the fragment containing the $metYt_1$ and $metYt_2$ terminators in the fusion described earlier (Fig. 5). From measurements of the β-galactosidase activities of the different constructions, the transcriptional read-through of $metYt_1$ and $metYt_2$ was found to be more than twofold higher than that of *rplSt* (Table 4), demonstrating that the *rplSt* terminator is more efficient than the $metYt_1$ and $metYt_2$ terminators. However, the effect of the *nusA94* mutation on the read-through of the *rplSt* terminator was as pronounced as that on the read-through of the $metYt_1$-$metYt_2$ terminators (Table 4), showing that the NusA-mediated transcriptional termination was not absolutely dependent on

TABLE 3. Effect of the *nusA98* mutation on the read-through of transcriptional terminators between *metY* and *p15a* in a Δ*rimM* mutant[a]

Relevant genotype	β-galactosidase activity		Read-through[b]
	P	P + T	
Δ*rimM102 nusA*$^+$	4,204 (3,712–4,681)	1,024 (998–1,070)	0.24
Δ*rimM102 nusA98*	4,455 (3,324–5,346)	1,898 (1,658–2,338)	0.43
rimM$^+$ *nusA*$^+$	5,063 (4,726–5,584)	1,226 (1,123–1,293)	0.24
rimM$^+$ *nusA98*	6,491 (5,698–7,282)	2,638 (2,446–2,767)	0.41

[a] Expression of the P_{metY}-*metY-lacZ* (P) and P_{metY}-*metY-metYt*$_1$*t*$_2$-*lacZ* (P + T) fusions is shown in Miller units. The average expression shown is from four independent experiments for the Δ*rimM102* strains and from five independent experiments for the *rimM*$^+$ strains. The variation in expression is shown within parentheses. The expression of a control construction (strain GOB438) lacking the *metY* insert and the cloning cassette was 17 Miller units, while a similar construction (strain GOB440) containing the $metYt_1$ and $metYt_2$ terminators expressed 3 Miller units of β-galactosidase activity in a Δ*rimM102 nusA*$^+$ strain.
[b] Calculated as (P + T)/P.

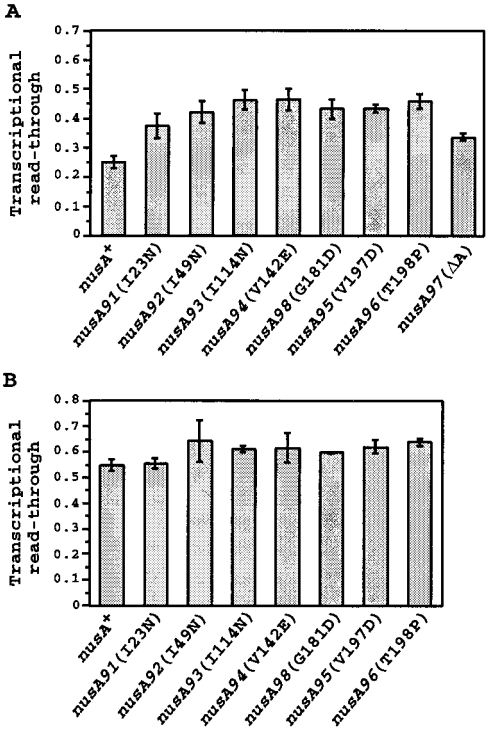

FIG. 6. Transcriptional read-through of terminators in different *nusA* mutants. The read-through of the terminators T_1 and T_2 between *metY* and *p15a* (A) and that of the attenuator upstream from *rpsP* (B) were calculated as the ratio of the β-galactosidase activity from a *lacZ* fusion containing the respective terminator(s) to the activity from a fusion lacking the terminator(s) in the genetic backgrounds indicated. The standard deviations are shown as error bars.

TABLE 4. NusA-mediated feedback regulation of transcriptional termination in different chimeric promoter-terminator constructions

Promoter	Terminator	Transcriptional read-through[a]		
		nusA$^+$	*nusA94*	*nusA94*/*nusA*$^+$
P_{metY}	$metYt_1t_2$	0.25 (0.22–0.27)	0.42 (0.40–0.43)	1.7
P_{metY}	*rplSt*	0.089 (0.070–0.12)	0.20 (0.20–0.21)	2.2
P_{tet}	$metYt_1t_2$	0.035 (0.033–0.037)	0.073 (0.063–0.081)	2.1

[a] Transcriptional read-through was calculated as the ratio of the β-galactosidase activity of P_{metY}-$metYt_1t_2$-*lacZ* to that of P_{metY} *lacZ*, that of P_{metY}-T_{rplSt}-*lacZ* to that of P_{metY}-*lacZ*, and that of P_{tet}-$metYt_1t_2$-*lacZ* to that of P_{tet}-*lacZ* in *nusA* as well as *nusA94* strains. The values presented are the averages from three independent experiments, with the variation shown within parentheses.

the $metYt_1$ and $metYt_2$ terminators. To examine whether the feedback regulation at the $metYt_1$ and $metYt_2$ terminators requires the presence of the native promoters for the *metY-nusA-infB* operon, P_{tet} from pBR322 was substituted for the fragment containing the P_{-1}, P_1, and P_2 promoters in the fusions between *metY*, with or without the $metYt_1$ and $metYt_2$ terminator fragment, and *lacZ* described above (Fig. 5). The effect of the *nusA94* mutation on the read-through of the $metYt_1$ and $metYt_2$ terminators was comparable for the fusions containing either the P_{tet} promoter or the fragment with the *metY* promoters (Table 4). Thus, the NusA-mediated feedback regulation at the $metYt_1$ and $metYt_2$ terminators is not dependent on the presence of the *metY* operon promoters. Interestingly, the read-through of the $metYt_1$ and $metYt_2$ terminators was severalfold higher in both *nusA*$^+$ and *nusA94* strains when the native *metY* promoter fragment was present than when the P_{tet} promoter fragment was used.

The *nusA* gene is essential at 37°C. A deletion of the *nusA* gene is lethal at 42°C in a *rho*$^+$ strain but not in *rho* mutants (55). Also, the *nusA*(Am113) amber mutation is lethal at 42°C in a strain with a temperature-sensitive *supF6* tRNA suppressor (32). Further, *rho*$^+$ strains with the *nusA11* missense mutation cannot grow at 42°C (28, 31). All of the *nusA* mutations isolated here were also found to confer a temperature-sensitive phenotype (data not shown). Since the *nusA* mutations were selected as fast-growing derivatives of a Δ*rimM102* mutant at 37°C, it was surprising that all were temperature sensitive. This made us consider the possibility that the NusA protein, or at least its function in transcription termination, was essential at 42°C but not at 37°C. Therefore, we constructed an in-frame deletion of *nusA* in the plasmid vector pMAK705 (Cm^r) containing a temperature-sensitive replicon (Fig. 7). Clones carrying cointegrates between the recombinant plasmid pJML001 and the chromosome were selected for at 44°C on chloramphenicol plates. The obtained clones were grown at 30°C to select for resolution of the cointegrates. (Cells containing cointegrates grow slowly at 30°C due to replication of the chromosome from the plasmid replicon.) One of these segregants (JML012) was shown by PCR to carry the *nusA* deletion on the chromosome and the wild-type *nusA* gene on the plasmid (Fig. 7). The plating efficiency of JML012 at 37°C was only 10% of that at 30°C in a viable-count experiment (data not shown). The surviving colonies were Cm^r, suggesting that they still contained the complementing plasmid, either free in the cytoplasm or as a cointegrate. Upon restreaking of these colonies, most continued to show a low plating efficiency and exhibited a heterogeneous growth phenotype, indicating a further loss of the plasmid with a concomitant death of the cells. Colonies that grew well at 37°C were Cm^r and grew poorly at 30°C, typical for clones with the plasmid integrated into the chromosome. These findings suggested that the NusA protein is also essential at 37°C. This was also corroborated by an experiment in which the expression of *nusA*$^+$ was from the P_{BAD} promoter in plasmid pJML007; the temperature-sensitive plasmid (Cm^r) that carries *nusA*$^+$ in strain JML012 with *nusA* deleted on the chromosome (Fig. 7) was replaced by pJML007 (Cb^r) containing *nusA*$^+$ under the control of the arabinose-inducible P_{BAD} promoter by transformation and selection for Cb^r at 44°C in the presence of 0.2% arabinose. One Cm^s transformant, JML087, was tested for the ability to grow at 30, 37, and 44°C on plates lacking or containing arabinose. At all three temperatures, strain JML087 grew only in the presence of arabinose, demonstrating that *nusA* is essential at these temperatures.

DISCUSSION

In this report, we describe the identification of 14 mutations in the *metY-nusA-infB* operon that suppress the slow growth of a Δ*rimM102* mutant. These suppressor mutations increase the expression of *rbfA*, encoded by the fifth gene of the *metY-nusA-infB* operon, in accordance with the previous finding that overexpression of RbfA suppresses the slow growth and transla-

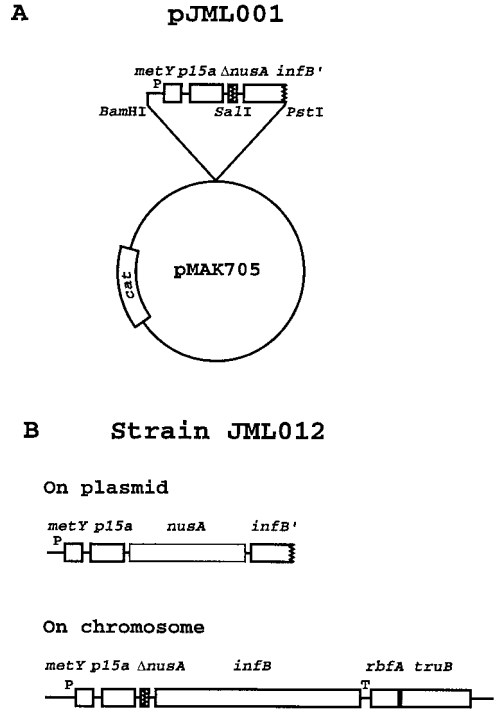

FIG. 7. Deletion of the chromosomal *nusA* gene. (A) Plasmid pJML001 carries a *metY-nusA-infB* operon fragment containing an in-frame deletion of *nusA* cloned into the temperature-sensitive allelic replacement vector pMAK705. (B) Genetic organization of the chromosomal *metY-nusA-infB* operon containing the *nusA* deletion and that of the complementing *nusA*$^+$ plasmid after resolution of cointegrates formed between plasmid pJML001 and the chromosome of the wild-type strain, MW100. P represents the P_{-1} and P_1 promoters, and T represents the $infBt_3$ terminator.

tional deficiency of the Δ*rimM102* mutant (4). RimM and RbfA are both crucial for efficient maturation of the 30S ribosomal subunits (4). The plethora of suppressor mutations that increase the expression of *rbfA* emphasizes the importance of elevated levels of RbfA in strains lacking RimM and seems to connect the function of RbfA to that of RimM in ribosome maturation.

Nine of the isolated suppressor mutations were localized to the *nusA* gene encoding the transcriptional elongation factor NusA, important for termination and antitermination of transcription. Transcription of the *metY-nusA-infB* operon can be repressed by overexpression of *nusA* from plasmids and derepressed by *nusA* mutations (30, 37). It was suggested that the two transcriptional terminators ($metYt_1$ and $metYt_2$) between *metY* and *p15a* were the target for the autoregulation (30, 37). The addition of NusA to an in vitro transcription system completely prevented the read-through of the $metYt_1$ terminator by the RNA polymerase (40). Similarly, in vitro transcription of the *nusA* gene was inhibited by a plasmid expressing *nusA*; however, in this case it was concluded that the two terminators were not the target for the regulation (7). Here we demonstrate that one target for NusA-mediated autoregulation in vivo resides within a 245-nt region that contains the $metYt_1$ and $metYt_2$ terminators. Previously, a 7- to 10-fold plasmid-mediated overexpression of *nusA* was found to reduce the expression of chromosomal *metY-nusA-infB* operon *lacZ* fusions by 50% (37). A similar reduction in the read-through (from 0.25 to 0.14) of the terminators was obtained when we induced the expression of a plasmid-carried copy of *nusA* from a P_{BAD} promoter with 0.2% arabinose in a $nusA^+$ strain. This amount of arabinose also reduced the read-through to the same extent (from 0.42 to 0.23) in the *nusA98* mutant (and restored termination to wild-type levels), indicating that the amount of NusA produced from the plasmid under these conditions was similar to that expressed from the $nusA^+$ gene on the chromosome. Thus, as little as an approximately twofold overexpression of *nusA* is sufficient to repress efficiently the read-through of the terminators.

The effect of the different *nusA* mutations on the transcriptional read-through at the $metYt_1$ and $metYt_2$ terminators varied from 1.3- to 1.9-fold (Fig. 6A), as calculated from the results obtained with *lacZ* transcriptional fusions. Similarly, the effect of the mutations on the read-through at the $infBt_3$ terminator just upstream from *rbfA* was 1.3- to 1.9-fold, as judged from the quantification of the amounts of the 4.8- and 6.7-kb transcripts detected in the Northern blotting experiment (Fig. 3A). Interestingly, the relative derepression (1.8- to 3.2-fold) in the *nusA* mutants of the 4.8-kb transcript, which requires the read-through of the $metYt_1$ and $metYt_2$ terminators, was higher than the 1.3- to 1.9-fold increase in read-through of these terminators. Possibly, this difference could be accommodated by invoking other sites on the mRNA at which NusA could promote transcriptional termination in accordance with in vitro results that suggest that NusA acts downstream from the $metYt_1$ and $metYt_2$ terminators (7). Alternatively, the difference could result from an increased *metY* promoter activity in the *nusA* mutants; however, we find this explanation unlikely, since the difference in expression of the *metY-lacZ* fusion lacking the $metYt_1$ and $metYt_2$ terminators between the $nusA^+$ strain and the *nusA* mutants was less than 10% in the experiments shown in Fig. 6A (data not shown).

The effect of the *nusA94* mutation on the read-through of different terminators in some chimeric promoter-terminator constructions was dependent neither on the native promoters nor on the native terminators of the *metY-nusA-infB* operon, suggesting that this mutation has a general effect on transcription termination. Similarly, the *nusA11* mutation, identical to *nusA98* described here, has been shown to decrease termination efficiency at different terminators (18, 28, 29). However, neither of the *nusA* mutations studied here seemed to affect the read-through of the *trmD* operon attenuator. Conceivably, NusA might not be involved in termination at this terminator structure because of the short distance between the promoter and the attenuator (5), which could decrease the possibility for NusA to bind to the RNA polymerase before it reaches the terminator structure. Interestingly, the read-through of the $metYt_1$ and $metYt_2$ terminators was severalfold higher when transcription initiation was from the native *metY* promoter fragment than when it was from the P_{tet} promoter in both a wild-type and a *nusA94* mutant strain. Thus, the *metY* promoter fragment (including the *metY* gene) seems to have an NusA-independent antitermination function.

Sequence and structural alignments have suggested that NusA contains similarities to the proposed RNA binding domains S1 (2) and KH (11), which seem important for interactions with RNA during termination and antitermination (23). Four of the amino acid substitutions in NusA studied here, V142E (*nusA94*), G181D (*nusA98*), V197D (*nusA95*), and T198P (*nusA96*), are in the region of homology to the S1 RNA binding domain, supposedly explaining their negative effect on the NusA-mediated feedback regulation at the terminators between *metY* and *p15a* and at that just upstream from *rbfA*. We note that the V142E substitution is in one of the most conserved positions of the S1 region whereas the G181D, V197D, and T198P substitutions are in less conserved positions (2). However, since all four substitutions result in dramatic changes of the amino acid side chain in the respective position, they might have altered the structure of the entire S1 homology region. Two other substitutions in the same region, L183R and R199A, cause defects in the interaction between NusA and the *nut* site RNA (24), corroborating the importance of this region of NusA in termination and antitermination of transcription.

The 79 C-terminal amino acids of NusA prevent the RNA binding regions of NusA from interacting with RNA. However, interactions between the C-terminal domain of the α subunit of the RNA polymerase and the 79 C-terminal amino acids of NusA seem to allow the RNA binding regions of NusA to bind to RNA (24). Since NusA lacking the 79 C-terminal amino acids binds RNA alone and is proficient in transcription termination (23, 24), it is surprising that the *nusA97* mutation, which results in the substitution of 23 amino acids encoded by the +1 reading frame for the C-terminal 84 amino acids of NusA (due to a deletion of 1 nt in codon 412), confers a reduced termination at the internal terminators of the *metY-nusA-infB* operon. We note that the truncated NusA protein seems to be more affected in its ability to promote termination at the $infBt_3$ terminator just upstream from *rbfA* than at the $metYt_1$ and $metYt_2$ terminators between *metY* and *p15a* (cf. Fig.

3A and 6A). Possibly, the 23 new amino acids added to the C-terminally truncated NusA interfere with the ability of NusA to interact with some terminators.

NusA is essential for bacterial growth at temperatures above 42 and below 32°C (28, 31, 32, 48, 55). Since all of the *nusA* mutations isolated here conferred temperature-sensitive phenotypes, we considered it possible that NusA was not essential at 37°C. However, here we show that NusA is also essential at this temperature by controlling the expression of *nusA* from an inducible promoter. Further, we discovered that the temperature sensitivity of the *nusA* mutants studied here could be partially suppressed by increasing the sodium chloride concentration in the medium from 0.5 to 1 to 2% (data not shown). However, the termination function of NusA at 37°C was not restored by increased levels of sodium chloride, as demonstrated by efficient suppression of the slow growth of a Δ*rimM102* mutant at increased levels of sodium chloride (data not shown). We suggest that the temperature sensitivity conferred by the *nusA* mutations is the result of increased degradation of the mutant NusA proteins at high temperature and that this proteolysis can be inhibited by exogenous salt, as suggested for many other temperature-sensitive mutants (20). However, we cannot exclude the possibility that the *nusA* mutations isolated here reduce termination at a terminator(s) that is essential at high temperatures and that the effect of this reduced termination can be suppressed by increased concentration of salt by some unknown mechanism.

The high frequency of *nusA* mutations among the suppressors of the Δ*rimM102* mutation together with the straightforward complementation of obtained mutations by arabinose-induced expression of wild-type NusA from plasmid pJML007 make this an excellent system for isolating several new termination-deficient *nusA* mutants.

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