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Zinc-Responsive Regulation of Alternative Ribosomal Protein Genes in *Streptomyces coelicolor* Involves Zur and σ^{R}▽†

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Streptomyces coelicolor **contains paralogous versions of seven ribosomal proteins (S14, S18, L28, L31, L32, L33, and L36), which differ in their potential to bind structural zinc. The paralogues are termed C^+ or C^- on the basis of the presence or absence of putative cysteine ligands. Here, mutational studies suggest that the C^- version of L31 can functionally replace its C^+ paralogue only when expressed at an artificially elevated level. We show that the level of expression of four transcriptional units encoding C^- proteins is elevated under conditions of zinc deprivation. Zur controls the expression of three transcriptional units (including *rpmG2*, *rpmE2*, *rpmB2*, *rpsN2*, *rpmF2*, and possibly *rpsR2*). Zur also controls the expression of the *znuACB* operon, which is predicted to encode a high-affinity zinc transport system. Surprisingly, the zinc-responsive control of the *rpmG3-rpmJ2* operon is dictated by σ^{R}, a sigma factor that was previously shown to control the response to disulfide stress in *S. coelicolor*. The induction of σ^{R} activity during zinc limitation establishes an important link between thiol-disulfide metabolism and zinc homeostasis.**

Bacterial 70S ribosomes are assembled on mRNA from 30S and 50S subunits during the process of translation initiation. The ribosome is approximately two-thirds RNA and one-third protein. The 30S subunit usually contains 21 proteins and 16S rRNA, whereas the 50S subunit comprises up to 36 proteins together with 23S and 5S rRNA. A major role of ribosomal proteins (R proteins) is to stabilize the rRNA (31), although several proteins play additional roles in ribosome function (8). The production of ribosomes is a major drain on cellular energy and material resources, and so the synthesis of ribosomal components is tightly controlled in most, if not all, bacteria. rRNA synthesis is the major control point, and various mechanisms, for example, ppGpp-dependent stringent control, ensure that synthesis is commensurate with growth requirements and nutrient availability (45). The control of R-protein gene transcription is also subject to stringent control (9, 11), although feedback posttranscriptional control of translation initiation is an additional important control mechanism (12).

Although most bacteria contain multiple copies of rRNA operons, the ~55 R proteins are usually encoded by single, highly conserved genes. However, it was recently discovered that this is not the case for all R proteins. Seven R proteins are found in phylogenetically distinct forms both among different bacteria and within single genomes. These proteins are L31 (*rpmE*), L32 (*rpmF*), L33 (*rpmG*), L36 (*rpmJ*), L28 (*rpmB*), S18 (*rpsR*), and S14 (*rpsN*) (30). Strikingly, the two forms of each protein differ by the presence or absence of predicted (and in the cases of L31, L36, and S14, proven [20, 32, 50]) Zn ribbon motifs that usually consist of two pairs of conserved cysteines. The presence or absence of cysteine zinc ligands in these R proteins has led to their designation as C^+ or C^-, respectively, and these proteins are known collectively as the $C^{+/-}$ group (30). It was proposed previously that an ancient duplication of the ancestral C^+ form of each gene was followed by the evolution of C^- forms and the subsequent alternative loss of C^+ or C^- forms in different lineages (30). However, some genomes maintain both C^+ and C^- forms of certain R-protein genes, in which case the protein products are considered paralogues. For example, the *Escherichia coli* genome encodes mostly C^- R proteins but encodes both C^+ and C^- forms of L31, whereas *Bacillus subtilis* uses either C^+ or C^- R proteins in most cases but contains both forms of S14, L31, and L33 (30). In rapidly growing cells, ribosomes that contain up to seven C^+ R proteins clearly place a major demand on zinc resources. It seems likely, therefore, that zinc limitation was a driving force behind the evolution of the C^- R proteins (42). Indeed, bioinformatic analyses of the promoter regions of paralogous C^- R-protein genes suggested that they might be controlled by zinc-responsive regulators in several bacteria (42). Recently, studies with *B. subtilis* (2, 32) confirmed that zinc deficiency stimulates the expression of the C^- gene *rpmE2* and the incorporation of $L31^{C^-}$ into ribosomes.

Although zinc is an essential metal in bacteria, high levels can be toxic. In *E. coli*, the levels of free zinc are therefore tightly controlled by the action of the zinc-responsive regulators Zur and ZntR, which control uptake and export systems, respectively (7, 15, 44). These regulators have extremely high affinities for zinc, implying that there is essentially no free zinc in the cell (34). The question of how zinc metalloproteins acquire their zinc despite the absence of free zinc in the cytoplasm thus remains a crucial question.

Our studies of the antibiotic-producing actinomycete *Streptomyces coelicolor* have revealed that *rpmE1*, which encodes $L31^{C^+}$, is transcribed from two promoters. The *rpmE1*-proximal promoter $rpmE1p_1$ is active during normal growth, whereas the *rpmE1*-distal promoter $rpmE1p_2$ is controlled by the disulfide

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli strains		
BL21(DE3)pLysS	Overexpression host	48
ET12567(pUZ8002)	Nonmethylating donor for *E. coli-Streptomyces* conjugations	38
S. coelicolor strains		
M145	A3(2) SCP1$^-$ SCP2$^-$	24
M600	A3(2) SCP1$^-$ SCP2$^-$	10
J1915	M145 Δ*glkA119* SCP1$^-$ SCP2$^-$	23
J2139	M600 Δ*sigR*	40
J1915(pSX110-X1)	J1915 Δ*rpmE1*; single-crossover recombinant	This study
S103	J1915 Δ*rpmE2*	This study
S116	M600 Δ*rpmE3*::*apr*	This study
S117	J1915 Δ*rpmE2* Δ*rpmE3*::*apr*	This study
S118(pSX125)	J1915 Δ*rpmE1*(pSX125)	This study
S118(pSX122)	J1915 Δ*rpmE1*(pSX122)	This study
S118(pSX128)	J1915 Δ*rpmE1*(pSX128)	This study
S121	M145 Δ*zur*::*apr*	This study
Plasmids		
pBluescript II SK(+)	Multicopy cloning vector (*bla lacZa*)	Stratagene
pHJL401	SCP2-derived bifunctional plasmid with low copy number (*bla tsr lacZa*)	25
pIJ6650	Conjugative, counterselectable suicide plasmid (*apr glkA lacZa* oriT)	37
pMT3000	pUC18-based cloning vector (*bla lacZa*)	39
pSET152	Conjugative, integrative vector (*apr lacZa* oriT ΦC31 *attP*); ΦC31 based	5
pET20b	*E. coli* T7-based expression vector (*bla*)	Novagen
pSX110	pIJ6650 into which 2.5-kb Δ*rpmE1* allele has been cloned	This study
pSX121	pIJ6650 into which 3-kb Δ*rpmE2* allele has been cloned	This study
pSX122	pSET152::*rpmE1*	This study
pSX125	pHJL401::*rpmE1*	This study
pSX128	pSET152::Φ(*rpmE1′-rpmE2*)	This study

stress response sigma factor σ^R (41). The genome sequence of *S. coelicolor* (4) revealed two genes predicted to encode C$^-$ forms of L31 (*rpmE2* and *rpmE3*), which prompted our investigation into the functional roles of the three L31-encoding genes. In this paper, we show that *rpmE2* and *rpmE3* are alternative, nonessential genes and provide evidence that *rpmE1* is essential. We show that *S. coelicolor* contains paralogous versions of all seven C$^{+/-}$ R proteins and that the C$^-$ genes are controlled by zinc availability. The major regulator is the zinc-responsive regulator Zur, although we also show that the zinc-dependent control of two C$^-$ R-protein genes is dependent on σ^R. Our data reveal regulatory interplay between the responses to zinc deprivation and disulfide stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. coelicolor* A3(2) strains were cultivated on minimal medium, R5, supplemented minimal medium (SMMS) and mannitol-soya agars and modified liquid minimal medium (NMMP) as described previously (24). For liquid growth, strains were grown to mid- to late exponential phase in NMMP by using glucose as the carbon source. Zinc deficiency was achieved by using NMMP without added zinc, along with ultrapure reagents and acid-treated plasticware. Presumably, trace amounts of zinc remained under these conditions because there was no obvious effect on the growth rate up to at least mid- to late exponential phase. $ZnSO_4$ was added as indicated in the figure legends at concentrations of up to 3.5 μM, the level normally added in the preparation of NMMP medium. A list of strains is provided in Table 1.

Construction of plasmids. *rpmE1*, along with 158 bp of upstream DNA including the two native promoters (41), was amplified by PCR by using the primers rpmE5 and rpmE7 (Table 2) and then cloned into EcoRI-BamHI-digested pSET152 and pHJL401 to give pSX122 and pSX125, respectively. To construct pSX128, *rpmE2* was amplified by PCR and then cloned into EcoRV-digested pBluescript II SK(+) to give pSX126. *rpmE1*, along with its upstream transcriptional-translational control region (159 bp), was isolated by PCR as an EcoRI fragment and subcloned into EcoRI-digested pSX126. In this construct, *rpmE1* is adjacent to, and in the same orientation as, *rpmE2*. Inverse PCR mutagenesis with the primers rpmE2-P2-1 and rpmE2-P2-2 (Table 2) was used to fuse the promoters and first four residues of *rpmE1* to *rpmE2* at the fifth codon (see Fig. 2). The *rpmE1-rpmE2* transcriptional-translational fusion, Φ(*rpmE1′-rpmE2*), was subcloned as an EcoRI-BamHI fragment into pSET152 to give pSX128.

Construction of mutant strains. An in-frame Δ*rpmE1* allele was constructed by amplifying 1.3-kb upstream and downstream *rpmE1*-flanking regions by PCR and then combining the fragments into the cloning vector pMT3000. This procedure resulted in an in-frame *rpmE1* allele containing three codons corresponding to the N terminus of the product, two codons corresponding to the C terminus, a central EcoRI site, and flanking BamHI-HindIII sites. The ~2.5-kb Δ*rpmE1* allele was subcloned into the counterselectable gene replacement vector pIJ6650 to give pSX110 and then introduced into *S. coelicolor* J1915 by conju-

TABLE 2. Oligonucleotides used in this study

Name	Sequence
rpmE5	5′ GGGGAATTCCGAGGCGAAGGGCGTCGTCAAC
rpmE7	5′ CGGGGATCCTGCTACTGCGTGGGAAAGG
rpmE2-P2-1	5′ CAAGGTGTCTCCTAGTGTTCC
rpmE2-P2-2	5′ AAGCGCGACATCCACCCCGAGTACCGCCCGGTC
Zur-F	5′ CCGAATTCATATGACCACCGCTGGACCGCCCGTG
Zur-R	5′ CCGGATCCTCATCAACCGCCGGAGGCCCCCGC
znuAF1	5′ CGACGTCGAACTTGTCGGTGTTGC
znuAR1	5′ CGAGATGCTTCCAGAGAATGCTTCAG
F2S1a	5′ ACCGGCACACAGCGGCGATGA
F2S1b	5′ ACCGTCACGGGCACCAGATTC
G3S1b	5′ CAGCCGGTCGGGGTCGTTGCGGC
G3S1c	5′ GTACCGCGCGACGATCTCCCGG

TABLE 3. *S. coelicolor* C^+ R proteins and putative C^- R proteins

R protein	C^+ gene (gene no.)	C^- gene (gene no.)
S14	*rpsN1* (SCO4715)	*rpsN2* (SCO3430)
S18	*rpsR1* (SCO3908)	*rpsR2* (SCO3425)
L28	*rpmB1* (SCO5564)	*rpmB2* (SCO3429)
L31	*rpmE1* (SCO5359)	*rpmE2* (SCO3427)
		rpmE3 (SCO1150)
L32	*rpmF1* (SCO5571)	*rpmF2* (SCO0436)
L33	*rpmG1* (SCO4635)	*rpmG2* (SCO3428)
		rpmG3 (SCO0570)
L36	*rpmJ1* (SCO4726)	*rpmJ2* (SCO0569)

gation from *E. coli* ET12567(pUZ8002) (24). A single-crossover recombinant was isolated and designated J1915(pSX110-X1). Double-crossover Δ*rpmE1* mutants could not be isolated.

An in-frame Δ*rpmE2* allele was constructed by amplifying ~1.5-kb flanking regions and combining them into pMT3000 as described above, giving a fragment with 4 codons corresponding to the N terminus of the product, 21 codons corresponding to the C terminus, a central BamHI restriction site, and flanking BglII sites. The 3-kb BglII fragment was subcloned into pIJ6650 to give pSX121 and then introduced by conjugation into *S. coelicolor* J1915. Initial exconjugants were subjected to counterselection by using 2-deoxyglucose (2-DOG) to remove the integrated plasmid and were screened for the presence of the Δ*rpmE2* allele by PCR; a Δ*rpmE2* mutant was designated S103.

Δ*rpmE3*::*apr* and Δ*zur*::*apr* alleles were constructed in the cosmids StG8A and StC121 (46), respectively, by using a PCR-mediated mutagenesis approach (18). Briefly, in each case a transferable *apr*-oriT resistance cassette was amplified from pIJ773 (18) by using primers designed to delete the DNA between the start and stop codons of each gene upon the recombination of the PCR product into the cosmid. The Δ*rpmE3*::*apr* mutant allele was recombined by a double-crossover event into the M600 genome to generate S116 and into the S103 genome to generate S117. The Δ*zur*::*apr* allele was recombined by a double-crossover event into the genome of M145 to generate a strain designated S121.

RNA preparation and transcription analysis. RNA was isolated from liquid-grown mycelium by using the Kirby mix method as described previously (24). Probes for S1 nuclease mapping were generated by PCR, generally by using a primer corresponding to an internal region of the open reading frame (ORF) and labeled at the 5′ end with [γ-^{32}P]ATP and an unlabeled primer that hybridizes ~400 bp upstream of the ORF. Labeled probes were purified by using a QIAGEN PCR purification kit and then hybridized with 30 μg of total RNA as described previously (24). Following S1 nuclease treatment, protected DNA fragments were separated by denaturing electrophoresis and visualized by using a phosphorimager (Storm; GE Healthcare).

Overproduction and purification of Zur. The primers Zur-F and Zur-R (Table 2) were used to amplify the *zur* ORF by PCR, thereby generating an NdeI site corresponding to the N terminus of the gene product and a BamHI site corresponding to the C terminus for cloning into pET20b (Novagen). pET20b::*zur* was introduced into *E. coli* BL21(DE3)pLysS, and *zur* overexpression was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Cell pellets in buffer A (20 mM Tris-HCl [pH 8], 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol, 1 μM $ZnCl_2$, complete mini EDTA-free proteinase inhibitor cocktail [Roche]) were disrupted by using a French press. Following the removal of cell debris by centrifugation, the soluble extract was subjected to ion exchange chromatography with a HiPrep 16/10 QFF column (GE Healthcare), heparin affinity chromatography with a HiPrep 16/10 heparin FF column (GE Healthcare), and gel filtration with a Sephadex 200 column (GE Healthcare), which resulted in ~95% pure protein. Following the concentration of Zur to ~1.4 mg/ml, purified protein in buffer B (20 mM Tris-HCl [pH 8], 400 mM NaCl, 5% glycerol, 5 mM dithiothreitol, 1 μM $ZnCl_2$) was snap-frozen in small aliquots and stored at −80°C.

EMSAs and DNase I footprint assays. Probes that included the promoter region of *znuA*, *rpmF2*, and the intergenic region of a cluster of seven genes (SCO3425 to SCO3431) that includes five C^- R-protein genes, referred to herein as the RP^{C^-} cluster, were generated by PCR by using the primer pairs znuAF1-znuAR1 (252 bp), F2S1a-F2S1b (203 bp), and G3S1b-G3S1c (472 bp), respectively (Table 2), and then 5′ end labeled with [γ-^{32}P]ATP. Electrophoretic mobility shift assay (EMSA) mixtures contained the following in a final volume of 10 μl: DNA probe (<1 nM), binding buffer (10 mM Tris-HCl [pH 8.0], 5% [vol/vol] glycerol, 50 mM NaCl, 1 μM $ZnCl_2$, 2 μg of bovine serum albumin), purified Zur, and 1 μg of herring sperm DNA (Promega). Following 15 min of incubation at room temperature, the binding reaction mixtures were separated on a 6% Tris-borate (no EDTA) polyacrylamide gel at 4°C. Bands were visualized by using a phosphorimager (Storm; GE Healthcare). Experiments were performed at least twice, and representative data sets are presented.

For DNase I footprinting, the RP^{C^-} cluster, *rpmF2*, and *znuA* templates were prepared by PCR by using the oligonucleotides described above. To label one strand, the primers G3S1b (with a sequence corresponding to an internal region of *rpmG2*), F2S1a (with a sequence corresponding to a region upstream from *rpmF2*), and znuAR1 (with a sequence corresponding to an internal region of *znuA*) were end labeled with [γ-^{32}P]ATP. Binding reactions were performed with a final volume of 25 μl by using 30 ng of template DNA and various concentrations of Zur. DNase I digestion was carried out according to the method of Takano et al. (49). To determine the extent of protection conferred by Zur, dideoxynucleotide sequencing reactions were performed by using a Sequenase 2.0 DNA sequencing kit (GE Healthcare) and the above-described 5′-end-labeled primers.

RESULTS

***S. coelicolor* encodes C^+ and C^- versions of seven ribosomal proteins.** An analysis of the annotated *S. coelicolor* genome sequence revealed that both C^+ and C^- forms of all seven of the $C^{+/-}$ group R proteins are encoded (Table 3). In most cases, a single C^+ and a single C^- gene are present. However, pairs of closely related C^- genes for L31 and L33 are present, along with single C^+ genes for these proteins. We hypothesized that, under standard growth conditions, the C^+ protein forms would act as the primary R proteins, a prediction that is supported by the genomic context of each gene. Of the seven C^+ genes, six are closely linked to other genes with predicted essential roles in translation (data not shown). In contrast, many of the C^- R-protein genes are linked to other C^- R-protein genes but not to other genes involved in translation. The C^- R-protein genes are organized among four loci (Fig. 1). Of particular interest is the RP^{C^-} cluster. As well as the C^- R-protein genes, the RP^{C^-} cluster includes SCO3426, encoding a protein related to YciC from *B. subtilis* (28% identity), which is thought to play a role in zinc homeostasis (13, 15). The remaining gene in the RP^{C^-} cluster, SCO3431, lies downstream of *rpsN2* and encodes a putative membrane-spanning protein with no predicted function. The annotated start codon of SCO3431 overlaps with the stop codon of *rpsN2*, although note

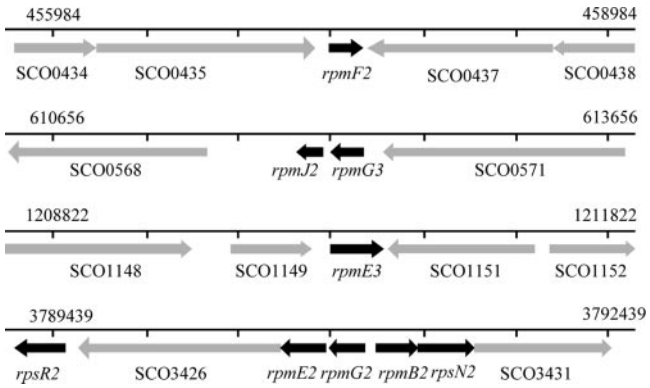

FIG. 1. Genome organization of the genes that encode C^- R proteins in *S. coelicolor*. In each case, a ~3-kb region of the annotated genome is shown. Arrows indicate the directions of transcription. Black arrows represent the C^- R-protein genes.

that an alternative start codon is located 60 bp downstream, which is consistent with the proposed start codon in *Streptomyces avermitilis*. It is therefore presently unclear whether *rpsN2* and SCO3431 are cotranscribed. Elsewhere in the genome, two further C^- genes (*rpmG3* and *rpmJ2*) are organized as a likely operon (SCO0569-SCO0570), and *rpmE3* may be organized in an operon downstream from a gene with no known function. Finally, *rpmF2* appears to be transcribed as a monocistronic mRNA (see below). The sequences of the seven types of *S. coelicolor* putative C^- R protein are aligned with those of their C^+ counterparts from *S. coelicolor* as well as those of homologues from other bacteria in Fig. S1 in the supplemental material.

***rpmE1* appears to be essential.** Our initial functional studies of the $C^{+/-}$ group of R proteins focused on the three genes that encode L31 paralogues. The gene that encodes L31, *rpmE*, appears on several compiled lists of essential bacterial genes (16). However, it was conceivable that each *rpmE* gene in *S. coelicolor* might be individually redundant if the $L31^{C^-}$ and $L31^{C^+}$ paralogues were functionally equivalent. To test this, an attempt was made to delete the $L31^{C^+}$ gene, *rpmE1*. An *rpmE1* in-frame deletion allele was recombined via a single crossover into the *S. coelicolor* J1915 (Δ*glkA*) genome to generate a strain designated J1915(pSX110-X1). pSX110 contains the glucose kinase gene *glkA*, which enables the positive selection of double-crossover recombinants on medium containing the toxic glucose analogue 2-DOG (24). More than 20 double-crossover isolates resistant to 2-DOG and sensitive to apramycin were screened for the presence of the Δ*rpmE1* allele by Southern analysis or PCR, but each had reverted to the wild type, suggesting that *rpmE1* is essential. In support of this conclusion, the introduction of a functional copy of *rpmE1* into J1915(pSX110-X1) on a low-copy-number plasmid (pSX125) permitted the subsequent replacement of the chromosomal wild-type gene with the Δ*rpmE1* allele. The resulting strain was designated S118(pSX125), and the chromosomal structure was confirmed by Southern blot analysis (data not shown). These data imply that under the growth conditions used, *rpmE2* and *rpmE3* cannot compensate for the loss of *rpmE1*.

***rpmE2* and *rpmE3* are nonessential.** To investigate the functions of *rpmE2* and *rpmE3*, single and double mutants were constructed. An in-frame Δ*rpmE2* allele was introduced into the J1915 genome to generate a strain designated S103, an *rpmE3*::*apr* allele was recombined into the *S. coelicolor* M600 genome to generate S116, and a Δ*rpmE2* Δ*rpmE3*::*apr* mutation in the J1915 background was created to generate S117. The three strains, S103, S116, and S117, were easily obtained and appeared to be identical to their parental counterparts in terms of growth, sporulation, and antibiotic production. In addition, no mutant appeared to be disadvantaged compared to the parental strains when growing in medium lacking added zinc. We therefore conclude that *rpmE1* encodes the primary, essential L31 R protein in *S. coelicolor* and that *rpmE2* and *rpmE3* are nonessential. A similar conclusion was reached for *B. subtilis* (2).

Expression of *rpmE2* partially rescues an *rpmE1* null mutant. The failure of *rpmE2* or *rpmE3* to suppress a Δ*rpmE1* mutation might be because $L31^{C^-}$ and $L31^{C^+}$ are not functionally equivalent or because *rpmE2* and *rpmE3* are not expressed at levels sufficient for ribosome biogenesis. To test the latter possibility, we constructed a transcriptional-translational fusion between *rpmE1* and *rpmE2*. The *rpmE2* reading frame, from codon 5 onward, was fused to *rpmE1* after the fourth codon in the integrative plasmid pSX128. The transcriptional-translational fusion, Φ(*rpmE1′-rpmE2*), was introduced into *S. coelicolor* S118(pSX125), which allowed the subsequent loss of pSX125 (pHJL401::*rpmE1*). As a control, pSX122, which includes *rpmE1* along with its promoter region, was integrated into the S118 genome (Fig. 2). S118(pSX122) was indistinguishable from the parental strain J1915, whereas S118(pSX128) grew and sporulated poorly and formed irregular colonies. In addition, S118(pSX128) overproduced the pigmented antibiotic actinorhodin on SMMS agar medium. Therefore, it appears that *rpmE2* cannot fully compensate for the loss of *rpmE1*, even when expressed in association with the *rpmE1* transcription and translation initiation signals. Nonetheless, these data suggest that $L31^{C^-}$ encoded by *rpmE2* is able to partially provide the essential L31 function. The ability of *rpmE3*-encoded $L31^{C^-}$ to functionally replace $L31^{C^+}$ remains unknown.

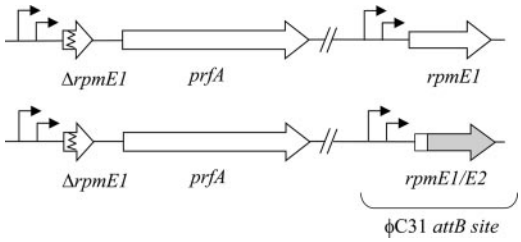

FIG. 2. Expression of *rpmE2* in association with the transcription and translation initiation signals of *rpmE1*. The Φ(*rpmE1′-rpmE2*) fusion [bottom; strain S118(pSX128)] or the *rpmE1* control [top; strain S118(pSX122)] gene was introduced into the chromosome by using the integrative vector pSET152, which integrates at the ΦC31 *attB* site. Both constructs allowed the in-frame deletion of *rpmE1* at the natural locus.

Zinc regulation of C^- R-protein genes. Initial S1 nuclease mapping experiments using RNA extracted from NMMP-grown cultures to investigate the divergent RP^{C^-} cluster promoters (*rpmG2p* and *rpmB2p*), as well as the promoter regions upstream of *rpmG3*, *rpmE3*, and *rpmF2*, failed to reveal transcription activity. To test the hypothesis that C^- R proteins might replace C^+ proteins during zinc depletion, RNA was extracted from *S. coelicolor* M145 grown in NMMP from which $ZnSO_4$ had been initially omitted and then added back in at concentrations in the range of 0 to 3.5 μM. Transcripts were detected for four of the five promoter regions under conditions of zinc depletion but were absent at higher levels of zinc. For the leftward arm of the RP^{C^-} cluster, a single *rpmG2* promoter was detected, with the +1 transcriptional start site coinciding with the first base of the *rpmG2* start codon (Fig. 3; also, see Fig. 6). The coincidence of transcription and translation start sites is generally a rare phenomenon in bacteria but appears to be quite prevalent in *Streptomyces* (21). For the rightward arm of the RP^{C^-} cluster, two closely spaced 5′ ends were detected ~70 bp upstream of the *rpmB2* start codon. The *rpmG2* and *rpmB2* transcript 5′ ends are ~20 bp apart, indicating that the promoters overlap (Fig. 3; also, see Fig. 6). The *rpmG3-rpmJ2* operon was induced under conditions of zinc depletion from a

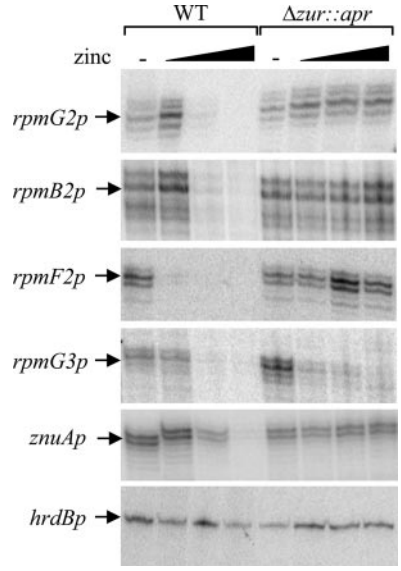

FIG. 3. Zinc-dependent control of the *rpmG2*, *rpmB2*, *rpmF2*, *rpmG3*, and *znuA* promoters and possible involvement of *zur* in regulation. Wild-type (WT; M145) and Δ*zur*::*apr* (S121) strains were grown in NMMP liquid medium containing various concentrations of added $ZnSO_4$ (0 [−], 0.9, 1.7, and 3.5 μM) to mid- to late exponential phase prior to RNA extraction. Transcripts derived from each promoter were assayed by S1 nuclease mapping by using promoter-specific probes radiolabeled at one end. Constitutive *hrdBp* controls the expression of the principle sigma factor σ^{HrdB} and was used as a positive control. Note that the zinc-dependent regulation of *rpmG3* is maintained in S121.

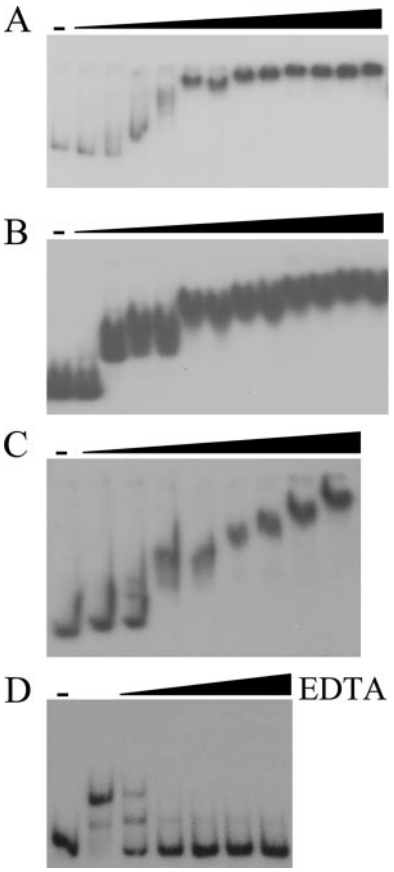

FIG. 4. EMSAs using DNA fragments containing the RP^{C-} cluster intergenic region (A), the *rpmF2p* region (B and D), or the *znuA* promoter region (C). (A to C) Assay mixtures contained either no added Zur (−) or increasing concentrations of Zur, as follows: 0.003, 0.034, 0.085, 0.175, (0.255), 0.340, (0.51), (0.68), 0.85, (1.02), (1.36), 1.7, and 3.4 μM. The concentrations in parentheses were not used for the *znuA* probe, and the 3.4 μM concentration was used only for the *znuA* probe. (D) Assay mixtures contained either no added Zur (−) or 0.85 μM Zur. In assay mixtures containing Zur, EDTA was added at increasing concentrations, as follows: 0, 5 μM, 50 μM, 0.5 mM, 1.25 mM, and 2.5 mM.

single promoter located ~60 bp upstream of the start codon (Fig. 3; also, see Fig. 6). Finally, *rpmF2* was induced under conditions of zinc depletion from a single promoter located 22 bp upstream of the start codon. The remaining C^- R-protein gene, *rpmE3*, was not expressed under any of the growth conditions tested.

Zur controls the expression of the RP^{C-} cluster and *rpmF2*. The increased transcription of C^- R-protein genes when *S. coelicolor* was growing in zinc-depleted medium suggested that a zinc-responsive regulator controls their expression. A likely candidate was the product of SCO2508, which is homologous to Zur proteins in other bacteria (e.g., 29% identical to *B. subtilis* Zur) and which is henceforth designated Zur. In support of its potential role as a zinc regulator, the *zur* gene lies immediately downstream from three genes that encode predicted components of a ZnuABC high-affinity zinc uptake system (SCO2505 to SCO2507, or *znuACB*) (44). The *zur* reading frame was deleted in M145 to generate a strain designated S121 (Δ*zur*::*apr*). S1 nuclease mapping revealed that the promoters *rpmG2p*, *rpmB2p*, and *rpmF2p* were deregulated in S121 compared to the parent, with significant activity detected even in the presence of 3.5 μM zinc (Fig. 3). However, *rpmG3p* retained its association with zinc control, suggesting that an alternative regulator may modulate its expression. The genomic location of *zur* suggested that it controls the expression of the *znuACB* operon, and this situation was confirmed by S1 nuclease mapping (Fig. 3). The single *znuA* promoter was induced under conditions of zinc limitation and deregulated in the *zur* mutant (Fig. 3). Like those of *rpmG2p*, the *znuAp* transcription and translation initiation sites coincide (Fig. 3; also, see Fig. 6).

Zur binds to the RP^{C-} cluster, *rpmF2*, and *znuA* promoter regions. To confirm that Zur directly controls *rpmG2p*, *rpmB2p*, *rpmF2p*, and *znuA*, Zur was purified from an overproducing *E. coli* strain and used in EMSAs. DNA fragments containing the RP^{C-} cluster, *rpmF2*, or *znuA* promoter region revealed specific binding (Fig. 4). In each case, increasing concentrations of Zur gave rise to slower-migrating protein-DNA complexes, suggesting that higher-order Zur-DNA complexes can be formed. In each case, the Zur-DNA complexes were resistant to nonspecific DNA but dissociated upon incubation with specific unlabeled DNA, indicating that binding was specific (data not shown). Like that of other members of the Fur family of regulators, the DNA binding activity of previously characterized Zur proteins is diminished when the bound regulatory metal is removed (13, 35, 43). In support of this pattern, the Zur-DNA complexes were dissociated when EDTA

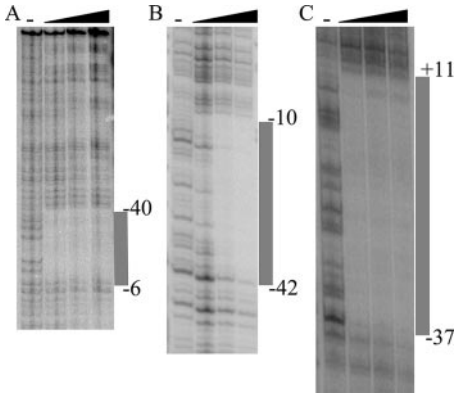

FIG. 5. DNase I footprinting of Zur-operator complexes. Promoter fragments were 5′ end labeled on one strand and mixed with increasing concentrations of Zur (0.14, 0.27, and 0.7 μM) prior to DNase I treatment. (A) RP^{C-} cluster intergenic region; (B) *rpmF2* promoter region; (C) *znuA* promoter region. The extents of the interactions between Zur and the intergenic regions, as determined using dideoxynucleotide sequencing, are indicated by vertical gray bars (data not shown). The positions of the protected regions with respect to the transcription initiation points are indicated; for the RP^{C-} cluster, this position corresponds to the *rpmG2* promoter.

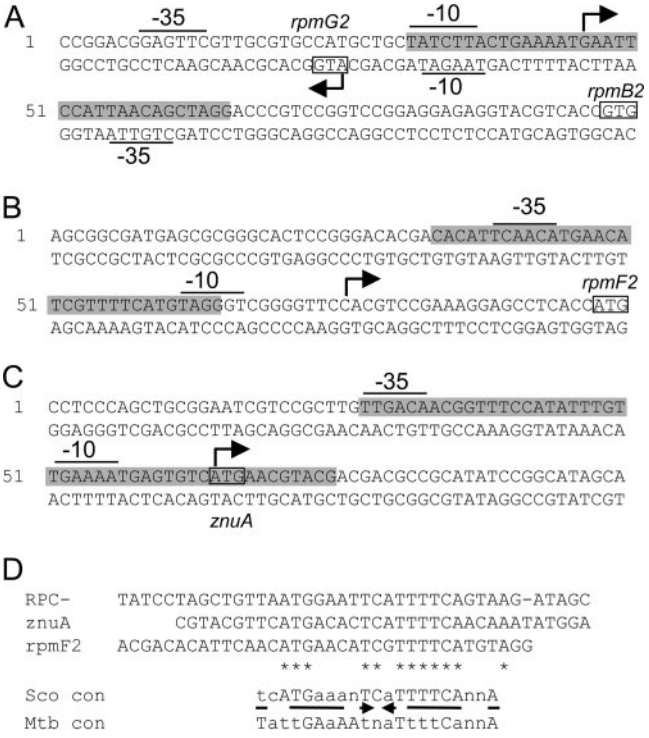

FIG. 6. Transcriptional organization and Zur binding site locations in the *rpmG2*, *rpmB2*, *rpmF2*, and *znuA* promoter regions. Transcriptional start points are indicated by arrows, and the putative −10 and −35 promoter elements are underlined. Boxes indicate predicted translation start points. The extents of Zur protection on the labeled strands are indicated by gray highlighting. (A) RP^{C-} cluster intergenic region. (B) *rpmF2* promoter region. (C) *znuA* promoter region. (D) Alignment of Zur binding sites in *S. coelicolor*, indicating a derived consensus sequence (Sco con), and comparison of this sequence to the consensus binding site sequence for *M. tuberculosis* Zur (Mtb con) (29). Asterisks and uppercase letters in the consensus sequences correspond to nucleotides that are identical among all the Zur binding sites analyzed; n represents any nucleotide.

was included in the binding reaction mixtures (Fig. 4D). DNase I footprinting was used to determine the Zur binding site in each promoter region (Fig. 5 and 6). Zur protected ~35 bp centered 22 bp upstream and 2 bp downstream from the *rpmG2p* and *rpmB2p* transcription initiation sites, respectively (Fig. 6A). Zur protected ~32 bp centered 27 bp upstream from the *rpmF2p* transcription initiation site (Fig. 6B). Zur protected a more extensive stretch (48 bp) of the *znuA* promoter region, an area which overlapped with the transcription initiation site. In each case, multiple binding sites were not detected, which suggests that the level of Zur used fully occupied the binding sites. An alignment of the three Zur binding regions revealed significant homology to one another and to the recently described consensus binding site of *Mycobacterium tuberculosis* Zur (29). Like that in *M. tuberculosis*, the Zur binding site exhibits inverted dyad symmetry (Fig. 6D).

***rpmG3* and *rpmJ2* are controlled by σ^R.** As mentioned above, the level of transcription of *rpmG3-rpmJ2* increased in medium deprived of added zinc, and this regulation was retained in S121 (Δ*zur*::*apr*), suggesting that an alternative zinc-responsive regulator controls transcription. An analysis of the promoter region of *rpmG3* revealed an exact match of the σ^R consensus promoter sequence (GGAAT[18 bp]GTT) (41). σ^R activity is controlled by the cellular thiol-disulfide redox state and can be induced by the thiol oxidant diamide. S1 nuclease mapping confirmed that *rpmG3p* was induced by diamide in the wild-type strain M600 but not in the Δ*sigR* mutant J2139, confirming that σ^R controls this promoter (Fig. 7A). Furthermore, we were unable to detect *rpmG3p* activity in a Δ*sigR* background even under zinc-limited conditions. Experiments using S1 nuclease mapping probes that extended into *rpmJ2* indicated that *rpmG3* and *rpmJ2* form an operon with a single promoter (data not shown). These data suggest that σ^R activity increases during zinc deprivation. To investigate this further, we analyzed the expression of another σ^R target, *trxC*, during zinc depletion. *trxC* encodes a thioredoxin and is transcribed from two promoters, $trxCp_1$ and $trxCp_2$, the former completely dependent on σ^R (37). As expected, S1 nuclease mapping revealed that $trxCp_1$ is induced by zinc deficiency (Fig. 7B). Taken together, these data indicate that the *rpmG3-rpmJ2* operon is regulated by both zinc deficiency and disulfide stress and that σ^R is a novel zinc-responsive regulator in *S. coelicolor.*

DISCUSSION

There is considerable variation among bacteria in the potential for producing members of the $C^{+/-}$ R-protein family (30). Indeed, an analysis of annotated genomes (36) suggests that all three of the following extreme situations exist: organisms can encode only C^+ forms, organisms can encode only C^- forms, and organisms can encode both forms of each member of the $C^{+/-}$ family, the latter being the case for *S. coelicolor*. These extreme situations appear to be rare, with most bacteria at intermediate positions on this three-way scale, producing C^- or C^+ forms of some proteins and both paralogous versions of others. In cases in which $C^{+/-}$ paralogues are present in the

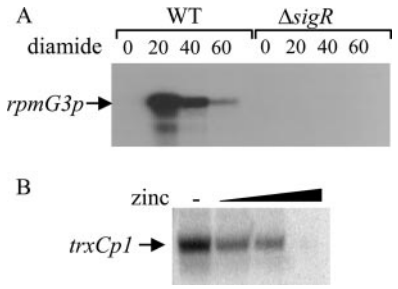

FIG. 7. Transcriptional analysis of the σ^R-controlled promoters *rpmG3p* and *trxCp*$_1$. (A) The *rpmG3-rpmJ2* operon is induced by diamide in a σ^R-dependent manner. Diamide was added at time 0 to a final concentration of 0.5 mM to cultures of wild-type (WT; M600) and *sigR* mutant (J2139) strains. Numbers indicate minutes postaddition. (B) The σ^R-dependent promoter *trxCp*$_1$ in M145 (wild type) was induced under conditions of zinc limitation. The strain was cultured in NMMP liquid medium containing various concentrations of added $ZnSO_4$ (0 −, 0.9, 1.7, and 3.5 μM) to mid- to late exponential phase prior to RNA extraction. Transcripts were assayed by S1 nuclease mapping by using promoter-specific probes radiolabeled at one end.

same organism, data from studies with *B. subtilis* (2, 32) and the experiments reported here suggest that the C^- forms are alternative proteins that are not produced under standard growth conditions. We demonstrated here that most, if not all, of the C^- genes in *S. coelicolor* are not transcribed under standard zinc-replete growth conditions. However, the majority of C^- R-protein genes were induced in response to zinc deprivation, which substantiates the proposal that these genes act to provide a selective advantage when zinc is limited, presumably by reducing the overall cellular demand for zinc (42) and possibly releasing zinc for use by other zinc metalloproteins. Studies with *E. coli* have estimated that each cell contains 2×10^5 to 4×10^5 zinc atoms (34) and up to 70,000 ribosomes at high growth rates (28). Although zinc levels and ribosome numbers in *S. coelicolor* have not been determined, it is clear that a large proportion of cellular zinc would be held by the ribosome. Therefore, the production of C^- R proteins and the quantitative replacement of C^+ proteins are likely to have a major influence on the availability of zinc to other zinc metalloproteins.

While the use of C^- R proteins for ribosomal function presents an apparently clear advantage for organisms under conditions of zinc deprivation, the benefit of maintaining the C^+ proteins is not obvious. One possibility is that the C^- and C^+ forms are not functionally equivalent. We showed here that even when fused to the transcriptional and translational initiation signals of *rpmE1*, the *rpmE2* gene only partially suppressed the apparently lethal phenotype of a Δ*rpmE1* mutation. However, it remains possible that the translational fusion did not confer sufficient $L31^{C^-}$ production for normal growth and development. Further, it is also important to emphasize that some organisms contain only the gene for $L31^{C^-}$, implying that the C^- forms are fully functional, at least in these bacteria. One possible benefit of maintaining the C^+ forms is to allow the ribosome to act as a repository of cellular zinc. Thus, *S. coelicolor* may accumulate zinc in the ribosomes under zinc-replete conditions and be able to release that zinc during zinc deficiency by replacing the C^+ proteins with C^- proteins. This possibility is supported by studies with *B. subtilis* (2) in which it was shown that *B. subtilis* $L31^{C^-}$ can expel $L31^{C^+}$ from ribosomes irrespective of the presence or absence of zinc. The ability to release zinc from ribosomes may be particularly important for a morphologically complex organism such as *S. coelicolor*. During sporulation, *S. coelicolor* produces specialized aerial hyphae that extend away from the substrate and eventually divide to form spores. In aerial hyphae, metal uptake from the environment would be limited, and the hyphae would instead rely on metal storage proteins or the intrahyphal transfer of metals from the substrate mycelium. The use of ribosomal zinc may be important in such a situation. In eukaryotes, metallothioneins are important cysteine-rich proteins that act as repositories for excess zinc, with the potential for donating zinc to zinc metalloproteins (52). However, analogous storage systems in bacteria appear to be rare. SmtA from *Synechococcus* strain PCC 7942 is a zinc metallothionein that is required for resistance to toxic levels of zinc and is induced by zinc (22). Other metallothionein-like proteins in some bacteria have been detected (6), although their biological function(s) remains uncharacterized. The role, if any, of the $C^{+/-}$ group of proteins in providing a system of zinc homeostasis requires further analysis.

We demonstrated here that the promoters *rpmG2p*, *rpmB2p*, and *rpmF2p* are controlled by Zur in a zinc-dependent manner. Zur is one of four members of the Fur family of metalloregulators in *S. coelicolor.* The other characterized members are FurS and CatR, which are thought to be redox-responsive regulators that control the expression of antioxidant genes, and Nur, which controls nickel uptake (1, 19, 33). The regulation of several C^- R proteins by Zur in *S. coelicolor* concurs with results from studies of *B. subtilis* and *M. tuberculosis* (2, 29). In addition, Zur homologues have been predicted to regulate C^- R-protein genes in several other bacteria, including *E. coli* and *Yersinia pestis* (42), emphasizing that the control of paralogous C^- proteins by Zur is widespread in bacteria.

Unexpectedly, we discovered that Zur was not involved in the zinc-dependent regulation of the *rpmG3-rpmJ2* operon and that instead σ^R plays a key role. Although the mechanism behind the zinc responsiveness of σ^R is not yet clear, the known control mechanism of σ^R provides one possibility. σ^R is controlled posttranslationally by RsrA, a member of the ZAS (zinc binding anti-sigma) family of anti-sigma factors (37). Although zinc-free RsrA can bind to σ^R, its affinity may be decreased and all zinc ligands are essential for RsrA activity in vivo (3, 26, 37, 53). Therefore, under conditions of zinc deprivation, RsrA may accumulate in a zinc-free state, with reduced affinity for σ^R, allowing σ^R to interact with RNA polymerase and activate members of the σ^R regulon. The σ^R-RsrA system is the major controller of enzymes involved in thiol-disulfide metabolism, including the thioredoxin system, which maintains cellular thiols in their normal reduced state. The increased expression of such enzymes during zinc deprivation may be beneficial because the very juxtaposition of cysteine zinc ligands may increase the likelihood of intramolecular disulfide bond formation when zinc is absent. Thus, the increased expression of enzymes that can catalyze thiol-disulfide exchange may play an important role in the reassembly of zinc metalloproteins during zinc depletion. There is accumulating evidence

for a link between zinc homeostasis and the oxidative stress response in bacteria. For example, in *B. subtilis*, the deletion of the low-affinity zinc importer ZosA leads to increased sensitivity to the thiol oxidant diamide and hydrogen peroxide (14). Interestingly, rather than being controlled by a zinc-responsive regulator, ZosA is regulated by PerR, a redox-responsive repressor.

Although the control of *rpmG3-rpmJ2* by σ^R may be on account of the ability of the σ^R-RsrA system to sense zinc, it is also possible that the production of $L33^{C-}$ and $L36^{C-}$ provides a selective advantage during disulfide stress. The C^+ versions of these proteins may be susceptible to oxidation, and the production of alternative redox-insensitive forms may compensate for this susceptibility. Such an arrangement is reminiscent of the induction of alternative superoxide-resistant forms of fumarase and aconitase in *E. coli* (17, 27, 51). Further studies are needed to investigate whether $L33^{C+}$ and $L36^{C+}$, and possibly other C^+ R proteins, are sensitive to disulfide stress.

Finally, the results of this study are in close agreement with those of an accompanying study by Shin et al. (47) into the function of *S. coelicolor* Zur. These investigators also noted that Zur negatively regulates *znuA* and C^- R-protein gene expression and additionally showed that Zur positively regulates its own expression. It was also found that σ^R activity was induced by chelating agents, further supporting the possible role of the σ^R-RsrA system as a novel sensor of zinc deficiency.

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