JOURNAL OF BACTERIOLOGY, Jan. 2010, p. 608–611 Vol. 192, No. 2
0021-9193/10/$12.00 doi:10.1128/JB.01022-09

The Zinc-Responsive Regulator Zur Controls Expression of the Coelibactin Gene Cluster in *Streptomyces coelicolor*▽†

Dimitris Kallifidas,[1] Ben Pascoe,[1] Gillian A. Owen,[1] Claire M. Strain-Damerell,[1] Hee-Jeon Hong,[2] and Mark S. B. Paget[1]*

Department of Chemistry and Biochemistry, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, United Kingdom,[1] and Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom[2]

Received 2 August 2009/Accepted 2 November 2009

Streptomyces coelicolor **mutants lacking the zinc-responsive Zur repressor are conditionally defective in sporulation, presumably due to the overexpression of one or more Zur target genes. Gene disruption analyses revealed that deregulation of previously known Zur targets was not responsible for the sporulation phenotype. We used microarrays to identify further Zur targets and discovered that Zur controls a cluster of genes predicted to direct synthesis of an uncharacterized siderophore-related non-ribosomally encoded peptide designated coelibactin. Disruption of a key coelibactin biosynthetic gene suppressed the Zur sporulation phenotype, suggesting that deregulation of coelibactin synthesis inhibits sporulation.**

Zinc is an essential element in all organisms, generally acting as a metalloenzyme cofactor or to stabilize protein folds. Several uptake systems have been characterized for bacteria, including the widely distributed high-affinity ZnuABC ABC transporter (16). In addition, ribosome remodeling has emerged as a novel zinc homeostasis mechanism in recent years (1, 12, 15). This involves the production of zinc-free ribosomal proteins (R proteins) to replace up to seven different zinc-binding R proteins. Zinc is usually coordinated by cysteine residues in the zinc R proteins, and these are therefore termed C^+ proteins, whereas the alternative R proteins that lack cysteines are termed C^- proteins (11). The genes that encode ZnuABC systems and alternative C^- R proteins are usually controlled by the zinc-responsive transcriptional repressor Zur (4, 15, 16). In the antibiotic-producing actinomycete *Streptomyces coelicolor*, Zur negatively regulates seven C^- R proteins, including five located in a single "RP^{C-}" cluster, as well the *znuACB* operon (13, 18). Interestingly, when grown as a confluent lawn on solid agar, the *S. coelicolor* Δ*zur*::*apr* mutant S121 (13) displayed a white phenotype, as opposed to the normal gray appearance that derives from mature spore pigment (Fig. 1A). Conversely, single dispersed colonies fully develop the gray spore pigment (data not shown). The sporulation defect was also seen on zinc-depleted minimal media and on MS agar containing excess zinc (100 μM) (data not shown). Scanning electron microscopy revealed poorly septated aerial hyphae in white confluent areas of growth, whereas normal spore chains were detected in gray colonies (data not shown). Therefore, the deletion of *zur* conditionally inhibits sporulation, probably through the overexpression of one or more target genes.

Overexpression of the ZnuABC system or C^- R proteins does not underlie the ΔZur phenotype. We constructed a series of double deletion mutants to test whether the deregulation of the *znuACB* operon or the C^- R proteins was responsible for the ΔZur phenotype. A standard PCR targeting and λ-Red-mediated recombination approach was used to construct mutant alleles in cosmid-borne genes, which were then transferred to *S. coelicolor* by conjugation and homologous recombination (5, 6). First, an ΔRP^{C-}::*apr* allele was constructed in cosmid StE9 (17). The DNA encoding *rpsR2* (SCO3425) to SCO3431 (chromosomally equivalent to nucleotide [nt] 3789250 to nt 3792451) was replaced with an apramycin (*apr*) resistance cassette from pIJ773 (5). The FLP recombinase recognition target (FRT) sequences that flank the *apr* cassette were exploited to recombine out the cassette using *Escherichia coli* EL250 (*araC*-P_{BAD}*flpe* [9]). Following this, the *apr* cassette from pIJ773 was reintroduced into the cosmid, this time replacing the *amp* gene, and the markerless ΔRP^{C-} allele was introduced into *S. coelicolor* M145 by conjugation from the donor strain ET12567(pUZ8002) (14). Following the isolation of single-crossover recombinants (Apr^r), putative double-crossover mutants (Apr^s) were isolated and then verified by Southern analysis (data not shown). The resulting strain, S153 (ΔRP^{C-}), had no obvious phenotype even when grown under zinc depletion, confirming that the five C^- R proteins encoded by this cluster are nonessential. A Δ*zur*::*apr* allele (13) was recombined into the resulting strain to generate S154 (ΔRP^{C-} Δ*zur*::*apr*). S154 retained the white ΔZur phenotype (Fig. 1A), indicating that overexpression of RP^{C-} cluster genes is not the underlying cause of the morphological defect. Similarly, a mutant allele that replaced the entire *znuACB-zur* cluster (nt 2704007 to nt 2707218) with the *apr* cassette from pIJ773 in cosmid StC121 was constructed (17). The Δ*znuACB-zur*::*apr* mutant, designated S151, also maintained the white ΔZur phenotype (Fig. 1A). The Δ*znuACB-zur*::*apr* mutant grew poorly on defined minimal media lacking added zinc but formed normal-sized colonies when supplemented with 5 to 25 μM $ZnCl_2$. This aspect of the phenotype was similar to that of the Δ*znuA* mutant S150, which was constructed in a similar manner to

* Corresponding author. Mailing address: Department of Chemistry and Biochemistry, School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, United Kingdom. Phone: (44) 1273 877764. Fax: (44) 1273 678433. E-mail: M.Paget@sussex.ac.uk.

† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 13 November 2009.

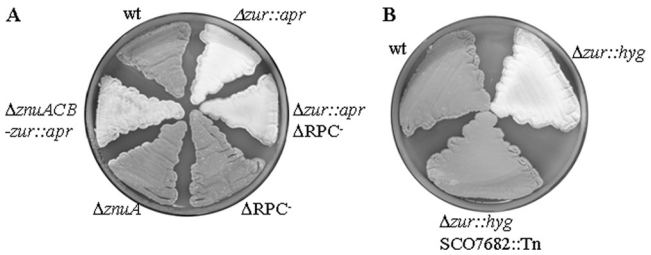

FIG. 1. Attempts to suppress the white ΔZur phenotype by disruption or deletion of Zur target genes. Strains were grown on mannitol-soya flour agar (8) for 5 days. (A) M145 (wt); S121 (Δ*zur*::*apr*); S154 (ΔRP^{C-} Δ*zur*::*apr*); S153 (ΔRP^{C-}); S150 (Δ*znuA*); S151 (Δ*znuACB-zur*::*apr*). (B) M145 (wt); M1016 (Δ*zur*::*hyg*); M904 (Δ*zur*::*hyg* SCO7682::Tn).

S153, in which the entire *znuA* coding sequence was deleted (chromosomally equivalent to nt 2704007 to nt 2704988), and confirmed that the ZnuABC system plays a key role in zinc uptake in *S. coelicolor*.

Zur controls expression of the coelibactin cluster and an additional putative metal uptake system. In order to identify additional candidate genes that might underlie the ΔZur sporulation defect, we investigated the Zur regulon by using a microarray approach. Triplicate cultures of M145 (wild type [wt]) and S121(Δ*zur*::*apr*) were grown to mid-log phase (optical density at 450 nm [OD_{450}] of ~0.8) in NMMP liquid medium (8), which includes 3.5 μM added $ZnCl_2$. Cultures were treated with RNAprotect (Qiagen) to prevent further RNA synthesis and degradation, and then RNA was isolated as described in the supplemental material. M145 or S121 total RNA was labeled with Cy5-dCTP or Cy3-dCTP, respectively, and then fluorescent cDNAs were cohybridized to whole-genome-PCR-spotted microarray slides (University of Surrey Microarray Group) and scanned using an Axon Genepix 4000B microarray scanner. Following filtering procedures to remove bad spots and poorly expressed genes, we identified 52 genes that were >2-fold upregulated in S121 compared to levels for M145 (*P* value of <0.05) (see Table S1 in the supplemental material). We screened these genes and potentially cotranscribed genes for upstream Zur operator sequences by using the consensus ATGnnnnTCnTTTT (where n is any nucleotide) (13), and along with previously identified Zur targets, we identified two new gene clusters that might be controlled by Zur (Fig. 2; Table 1). The SCO472-77 cluster encodes a likely ABC metal uptake system that might encode an alternative route for zinc acquisition. Indeed, SCO0473 is homologous to ZnuA (42% similarity), SCO0475 is homologous to ZnuB (46% similarity), and SCO0476 is homologous to ZnuC (50% similarity). SCO0473 and SCO475 are also related to Rv2059 and Rv2060, respectively, which were shown to be controlled by Zur in *Mycobacterium tuberculosis* (10). The SCO7676-92 cluster is predicted to be involved in the production of an uncharacterized non-ribosomally encoded peptide with siderophore characteristics (2). The 17-gene cluster is arranged as at least three transcriptional units, with transcription initiating upstream of genes encoding ferredoxin (SCO7676), an adenylate-forming enzyme (SCO7681), and a nonribosomal peptide synthetase (SCO7682) (Fig. 2A). Although only four genes in this cluster exhibited >2-fold-increased levels of expression, most of the remaining genes were significantly overexpressed in the Δ*zur* mutant, suggesting that the whole cluster is controlled by Zur. To confirm that Zur binds to the SCO0474/75 and SCO7681/82 intergenic regions, as well as upstream from SCO7676, we performed electromobility shift assays using ~200-bp PCR products that included the putative Zur binding sites and purified Zur (Fig. 2B), as described previously (13). In each case, Zur bound specifically to the probes, exhibiting a concentration-dependent laddering effect, as previously observed at other Zur targets (13).

Quantitative real-time PCR (Q-PCR) was used to confirm

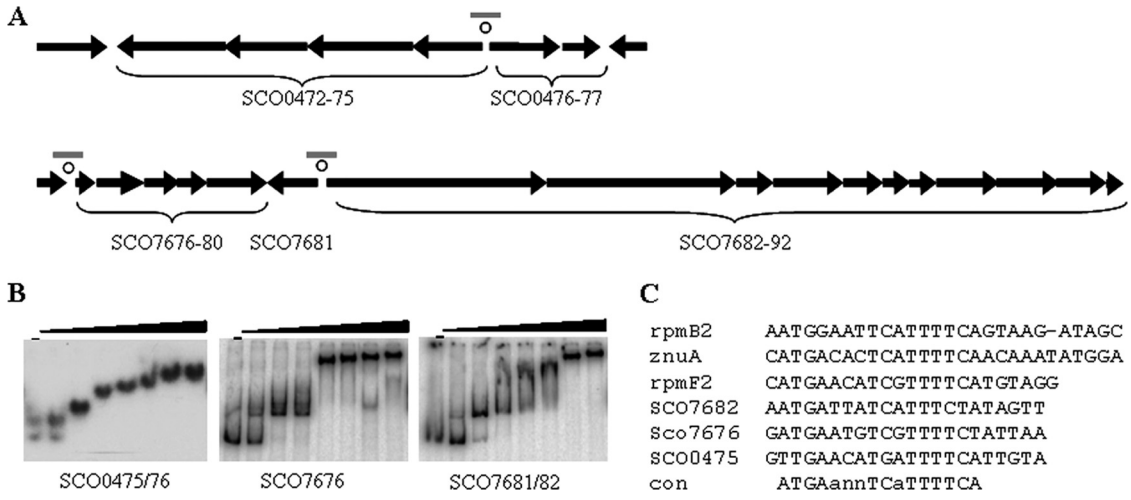

FIG. 2. (A) Organization of the Zur-controlled SCO0472-77 and SCO7676-92 gene clusters. Zur binding sites are marked with an open circle, and the electrophoretic mobility shift assay (EMSA) probes (see the supplemental material) used to test Zur binding are illustrated by a gray line. (B) Electrophoretic mobility shift assays using DNA fragments containing the SCO4075/76 intergenic region, the SCO7676 promoter region, and the SCO7681/82 intergenic region. Assays contained in 10 μl <1 nM [γ-^{32}P]ATP-labeled probe, 1 μg herring sperm DNA, and either no added Zur (−) or increasing concentrations of Zur as follows (in μM): 0.003, 0.034, 0.085, 0.175, 0.340, 0.51, and 0.85. (C) Alignment of Zur binding sites in *S. coelicolor*, indicating a derived consensus. Nucleotide positions in lowercase indicate conservation in >50% but <80% of binding sites.

TABLE 1. Genes induced in S121 (Δ*zur*::*apr*) compared to the parental strain M145 that contain a Zur binding site upstream from the first gene of the operon

Gene identifier[a]	Gene designation and/or function	Fold change
SCO0472	Putative secreted protein	11.5
SCO0473	Putative solute-binding lipoprotein	4.6
SCO0475*	ABC transporter protein, integral membrane subunit	8.7
SCO0476*	ABC transporter protein, ATP-binding subunit	4.2
SCO2505*	*znuA*; putative ABC transporter metal-binding lipoprotein	42.1
SCO2506	*znuC*; putative metal transport ABC transporter	2.3
SCO2507	*znuB*; putative metal transport ABC transporter	1.8
SCO3426	YciC homologue; zinc metallochaperone	3.7
SCO3428*	*rpmG2*; putative 50S ribosomal protein L33	7.1
SCO3429*	*rpmB2*; putative 50S ribosomal protein L28	7.4
SCO3430	*rpsN2*; putative 30S ribosomal protein S14	4.2
SCO3431	Possible membrane protein	2.5
SCO7676*	Putative ferredoxin	5.3
SCO7677	Putative secreted solute-binding protein	5.0
SCO7678	Putative metal transport integral membrane protein	1.4
SCO7679	Putative transport system integral membrane protein	1.6
SCO7684	Conserved hypothetical protein	1.7
SCO7685	Conserved hypothetical protein	1.4
SCO7686	Putative cytochrome P450	2.0
SCO7687	Putative thioesterase	2.2
SCO7688	Conserved hypothetical protein	1.7
SCO7691	Putative lyase	1.5

[a] An asterisk (*) indicates that a Zur binding site is located immediately upstream from this gene. Genes that are likely to be cotranscribed with the listed genes but do not pass our threshold for the GeneSpring Student *t* test ($P < 0.05$) are not included. Detailed descriptions of the microarray experiment can be found in the submission at ArrayExpress (accession number E-MAXD-56).

that genes in these clusters were modulated by zinc. *S. coelicolor* M600 (8) was grown in NMMP medium lacking the addition of minor-elements solution to mid-exponential phase and then treated with 25 μM $ZnSO_4$. RNA was prepared as described previously (7) from mycelium sampled immediately before and 30 min after zinc addition. Following this, the culture was treated with the nonspecific chelator EDTA (2 mM), and RNA was sampled 30 min later. Each gene tested was negatively regulated by zinc in a similar manner to *znuA* (SCO2505) (Fig. 3). Although SCO7681 and SCO7682 did not appear to be induced significantly in the microarray experiment, each was zinc responsive. This apparent discrepancy suggests an additional regulatory mechanism; indeed, it was recently discovered that the pleiotropic regulator AbsC negatively controls SCO7681 and SCO7682 by binding to a site that overlaps the Zur binding site (A. Hesketh and M. J. Bibb, personal communication).

Disruption of the coelibactin cluster suppresses the ΔZur phenotype. We hypothesized that the overexpression of the coelibactin cluster might be the underlying cause of the white ΔZur phenotype. To test this, a Δ*zur*::*hyg* allele was constructed by replacing the *zur* coding sequence with a hygromycin resistance cassette in cosmid StC121 (A. Hesketh and M. J. Bibb, personal communication). The resulting strain, M1016, has a ΔZur phenotype identical to that of the Δ*zur*::*apr* mutant S121. This allele was also introduced into M904, an M145 derivative in which SCO7682 was disrupted 279 nt into the coding region by Tn*5062*. M904 displays a wild-type sporulation phenotype and grows normally on media deprived of added zinc (data not shown; A. Hesketh and M. Bibb, personal communication; 3). Strikingly, the Δ*zur*::*hyg* SCO7682::Tn double mutant also sporulated normally (Fig. 1B), suggesting that the morphological phenotype detected in Δ*zur* mutants is largely caused by the overexpression of the coelibactin cluster.

Concluding remarks. The morphological defect observed in Δ*zur* mutants appears to be due to the overexpression of a cluster of genes predicted to direct the production of coelibactin. Coelibactin has not been isolated or characterized in any way, and yet bioinformatic approaches suggest that it is a siderophore-related compound. By definition, siderophores bind extracellular iron in order to increase bioavailability of this metal. However, our data suggest that coelibactin might be a zinc-chelating compound and, importantly, reveal approaches to overexpress the biosynthetic genes enabling its isolation and characterization. It is unclear why coelibactin overexpression should impede the sporulation process.

Microarray data accession number. The microarray data described in this paper can be accessed at ArrrayExpress (accession number E-MAXD-56).

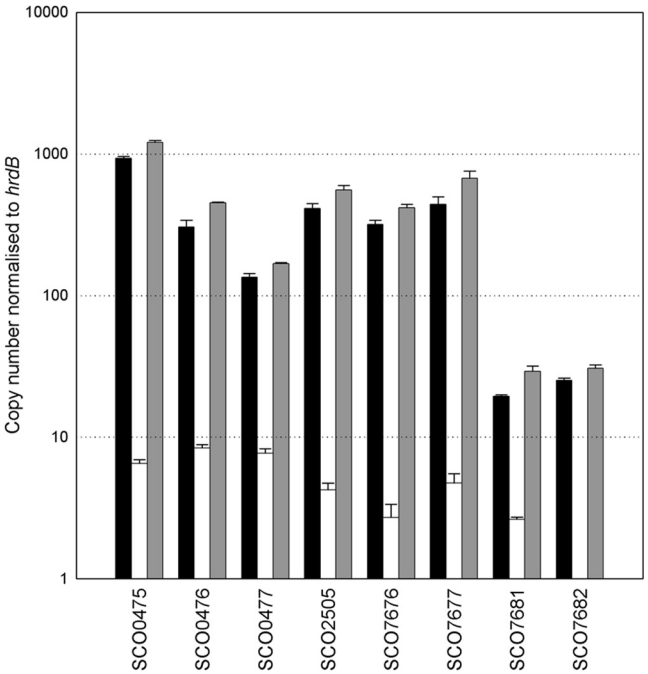

FIG. 3. Expression of Zur target genes relative to that of the principal sigma factor gene *hrdB*, as determined by Q-PCR of total RNA isolated from cultures grown in NMMP minus minor-elements solution before (black bars) and 30 min after (white bars) the addition of 25 μM $ZnSO_4$, followed by the subsequent addition of EDTA for 30 min (gray bars). Q-PCR was carried out as described previously (7), using primers listed in Table S2 in the supplemental material. Standard deviations are indicated by the error bars.

We thank Colin Smith and the other members of the University of Surrey Microarray group for help and advice, Mervyn Bibb and Andrew Hesketh for communication of unpublished data and supply of strains, and Greg Challis for useful discussions. We acknowledge the assistance of Kassimatis Dimitrios and David Randall in the construction and analysis of mutants.

The work was supported by BBSRC grant BBC5038541 and a studentship to B.P. and by a University of Sussex studentship to G.A.O.

REFERENCES

1. **Akanuma, G., H. Nanamiya, Y. Natori, N. Nomura, and F. Kawamura.** 2006. Liberation of zinc-containing L31 (RpmE) from ribosomes by its paralogous gene product, YtiA, in *Bacillus subtilis*. J. Bacteriol. **188:**2715–2720.
2. **Bentley, S. D., K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood.** 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature **417:**141–147.
3. **Bishop, A., S. Fielding, P. Dyson, and P. Herron.** 2004. Systematic insertional mutagenesis of a streptomycete genome: a link between osmoadaptation and antibiotic production. Genome Res. **14:**893–900.
4. **Gaballa, A., and J. D. Helmann.** 1998. Identification of a zinc-specific metalloregulatory protein, Zur, controlling zinc transport operons in *Bacillus subtilis*. J. Bacteriol. **180:**5815–5821.
5. **Gust, B., G. L. Challis, K. Fowler, T. Kieser, and K. F. Chater.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. U. S. A. **100:**1541–1546.
6. **Gust, B., G. Chandra, D. Jakimowicz, T. Yuqing, C. J. Bruton, and K. F. Chater.** 2004. Lambda red-mediated genetic manipulation of antibiotic-producing *Streptomyces*. Adv. Appl. Microbiol. **54:**107–128.
7. **Hesketh, A., W. J. Chen, J. Ryding, S. Chang, and M. Bibb.** 2007. The global role of ppGpp synthesis in morphological differentiation and antibiotic production in *Streptomyces coelicolor* A3(2). Genome Biol. **8:**R161.
8. **Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood.** 2000. Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, United Kingdom.
9. **Lee, E. C., D. Yu, J. Martinez de Velasco, L. Tessarollo, D. A. Swing, D. L. Court, N. A. Jenkins, and N. G. Copeland.** 2001. A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics **73:**56–65.
10. **Maciag, A., E. Dainese, G. M. Rodriguez, A. Milano, R. Provvedi, M. R. Pasca, I. Smith, G. Palu, G. Riccardi, and R. Manganelli.** 2007. Global analysis of the Mycobacterium tuberculosis Zur (FurB) regulon. J. Bacteriol. **189:**730–740.
11. **Makarova, K. S., V. A. Ponomarev, and E. V. Koonin.** 2001. Two C or not two C: recurrent disruption of Zn-ribbons, gene duplication, lineage-specific gene loss, and horizontal gene transfer in evolution of bacterial ribosomal proteins. Genome Biol. **2:**RESEARCH 0033.
12. **Nanamiya, H., G. Akanuma, Y. Natori, R. Murayama, S. Kosono, T. Kudo, K. Kobayashi, N. Ogasawara, S. M. Park, K. Ochi, and F. Kawamura.** 2004. Zinc is a key factor in controlling alternation of two types of L31 protein in the *Bacillus subtilis* ribosome. Mol. Microbiol. **52:**273–283.
13. **Owen, G. A., B. Pascoe, D. Kallifidas, and M. S. Paget.** 2007. Zinc-responsive regulation of alternative ribosomal protein genes in *Streptomyces coelicolor* involves *zur* and σ^R. J. Bacteriol. **189:**4078–4086.
14. **Paget, M. S., L. Chamberlin, A. Atrih, S. J. Foster, and M. J. Buttner.** 1999. Evidence that the extracytoplasmic function sigma factor σ^E is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J. Bacteriol. **181:**204–211.
15. **Panina, E. M., A. A. Mironov, and M. S. Gelfand.** 2003. Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc. Natl. Acad. Sci. U. S. A. **100:**9912–9917.
16. **Patzer, S. I., and K. Hantke.** 1998. The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol. Microbiol. **28:**1199–1210.
17. **Redenbach, M., H. M. Kieser, D. Denapaite, A. Eichner, J. Cullum, H. Kinashi, and D. A. Hopwood.** 1996. A set of ordered cosmids and a detailed genetic and physical map for the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. Mol. Microbiol. **21:**77–96.
18. **Shin, J.-H., S.-Y. Oh, S.-J. Kim, and J.-H. Roe.** 2007. The zinc-responsive regulator Zur controls a zinc uptake system and some ribosomal proteins in *Streptomyces coelicolor* A3(2). J. Bacteriol. **189:**4070–4077.