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Regulation of the *Staphylococcus aureus* Plasmid pI258 Mercury Resistance Operon

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Experiments involving fusion between the *Staphylococcus aureus* plasmid pI258-encoded *mer* operon and the reporter gene β-lactamase, mutational analysis, and *trans*-complementation studies have shown that the *merR* gene of pI258, which shows DNA sequence similarity with known *merR* genes from other bacteria, regulates the expression of the *mer* operon in vivo. The *merR* gene product is a *trans*-acting protein that activates *mer* operon transcription in the presence of the inducers Hg^{2+} and Cd^{2+}. A glutathione-*S*-transferase–MerR fusion protein specifically bound and protected a 27-nucleotide operator sequence from DNase I digestion. This operator sequence is highly homologous with *mer* operator sequences of other known systems.

Mercury-resistant bacteria from environmental and clinical isolates enzymatically detoxify inorganic mercury salts and sometimes organomercurial compounds (for recent reviews, see references 3, 13, 20, 22, 23, 25, and 26). The mercury resistance (*mer*) genes are often found on plasmids and transposons and are usually clustered in an operon. The highly substrate-specific mercuric ion reductase catalyzes the reduction of Hg^{2+} to chemically inert elemental Hg^0, which is water insoluble and volatilizes because of its high vapor pressure. The organomercurial resistance operon encodes another enzyme, organomercurial lyase, that catalyzes the cleavage of the C-Hg bond from organomercurials such as phenylmercury to yield benzene and Hg^{2+}.

The regulation of *mer* operon expression in gram-negative bacteria has been extensively studied (for reviews, see references 13, 20, 23, and 25). The major regulatory gene, *merR*, is transcribed divergently from the other *mer* genes. The regulatory gene product, MerR, represses transcription of the *mer* operon via the P*merT* promoter in the absence of the inducer Hg^{2+} and activates transcription in the presence of Hg^{2+}. MerR protein also regulates its own synthesis from the P*merR* promoter, which is divergently oriented from P*merT*. A 7-bp dyad sequence is located within the −10 and −35 sequences of P*merT*. The −10 and −35 sequences for P*merT* are separated by 19 bp, 2 bp more than the optimal spacing for efficient transcription by the *Escherichia coli* σ^{70} RNA polymerase. The 19-bp spacing, dyad sequences, and relative position of the dyad sequence with respect to the −10 and −35 sequences are important for induction and repression of the operon (11, 19). It was recently reported that the MerR-Hg^{2+} complex bends DNA in the promoter region and underwinds the spacer region of the promoter, thus facilitating the formation of a transcriptionally active open complex by RNA polymerase (1). We have shown that another regulatory protein, MerD, down-regulates the expression of the *mer* operon and is involved in fine-tuning the operon (14).

The sequence of the chromosomally encoded *mer* operon of the *Bacillus* sp. strain RC607 has been reported elsewhere (28). The MerR protein, which was identified by sequence similarity searches, was overexpressed in *E. coli* (8). The *Bacillus* MerR positively regulated transcription of the *Bacillus mer* operon, but not the Tn*501 mer* operon, in the presence of Hg^{2+}.

The nucleotide sequence of the *mer* operon of the *Staphylococcus aureus* plasmid pI258 was reported earlier (10). The pI258 *mer* operon, located within a 6.4-kb DNA fragment, contains several open reading frames. The mercuric ion reductase and organomercurial lyase genes were initially defined on the basis of searches of amino acid sequence similarity with the corresponding enzymes encoded by other known *mer* operons from gram-negative bacteria. Babich et al. (2) published data from mutational studies confirming the assignment of the mercuric ion reductase and organomercurial lyase genes. The regulatory gene was predicted from comparison of the amino acid sequences of the MerR proteins from *Bacillus* sp. strain RC607 and from gram-negative bacteria with the deduced amino acid sequence of the pI258 *merR* gene product (2, 8, 24). There is also no report of in vivo *trans* regulation of *mer* operon expression in gram-positive bacteria. In the present report, we have defined the regulatory gene of the pI258-encoded *mer* operon by mutation, operon fusion (with a reporter gene), and *trans*-complementation experiments. The regulatory gene product, MerR, activates transcription in the presence of Hg^{2+} or Cd^{2+}. A glutathione-*S*-transferase (GST)-MerR fusion protein bound specifically to a sequence that is highly homologous with the gram-negative bacterial *mer* operator and precedes the *merR* sequence of the *S. aureus mer* operon.

Regulation of *blaZ* expression in *mer* operon-*blaZ* fusion constructs. In order to confirm the regulatory role of the *merR* gene, a promoterless *blaZ* gene encoding the β-lactamase enzyme of *S. aureus* was used as a reporter gene in transcriptional gene fusions. *S. aureus* RN4220 (9) and the β-lactamase transcriptional fusion vector pSA3800 (15) were obtained from R. P. Novick. Plasmid pSK270, which was derived from pE194 after the pUC19 polylinker cloning sequence was added into the *Hin*dIII site, was obtained from S. A. Khan (University of Pittsburgh). Cells were grown in Luria-Bertani medium containing chloramphenicol (5 μg/ml) or erythromycin (10 μg/ml), as necessary. DNA manipula-

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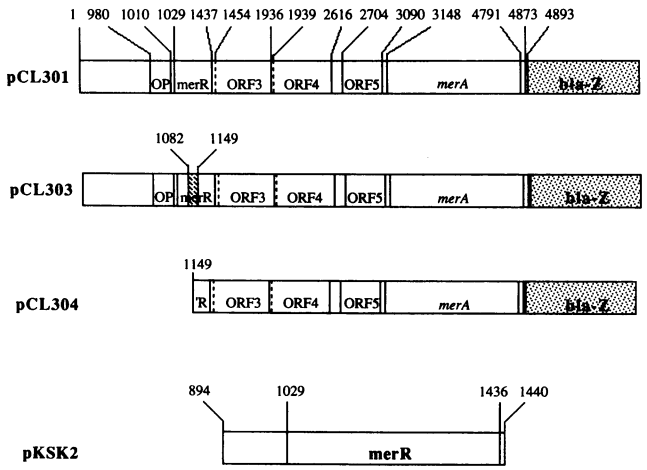

FIG. 1. Construction of plasmids used for measuring in vivo transcription from the *mer* promoter. The DNA sequence and organization of the genes are summarized in a diagram based on data from references 2 and 15 and from the data presented in this report. Numbers on the top represent nucleotide numbers as published in reference 15. OP, operator-promoter region; *merR*, regulatory gene; *merA*, the gene coding for mercuric ion reductase. The 5′ end of open reading frame 4 (ORF4) overlaps with two nucleotides of the 3′ end of ORF3. The boundaries of ORF3 shown by broken lines. pCL301 was derived after a DNA fragment bounded by nucleotides 1 and 4893 (10) was cloned into pSA3800, which contains the pI258-encoded *blaZ* gene. The transcriptional direction of the *blaZ* gene is the same as those of the *mer* genes (left to right). pCL303 is the same as pCL301 except that 68 bp from nucleotides 1082 to 1149 was deleted. The hatched section represents the internal *merR* deletion region. pKSK2 was obtained after a DNA fragment spanning nucleotides 894 to 1440 was cloned into pSK270, which is a derivative of pE194. (Note that pSA3800 and pE194 are compatible plasmids.) Plasmids were cloned into *S. aureus* RN4220 for in vivo experiments. The figure is not drawn to scale.

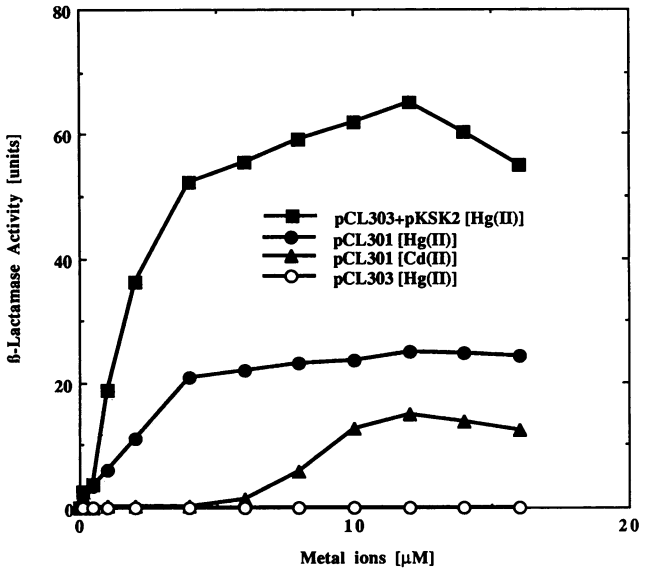

FIG. 2. Inducibility of *mer* operon measured as expression of *blaZ*. Exponentially growing cells were induced with different concentrations of $HgCl_2$ or $CdCl_2$ for 1 h, and β-lactamase activity was assayed by the nitrocefin method (27).

tions were performed as described by Sambrook et al. (21). *S. aureus* was transformed by using protoplasts formed after treatment of cells with lysostaphin, as described previously (16). Transformants were selected on regeneration medium containing either chloramphenicol (5 μg/ml) or erythromycin (10 μg/ml) or both when necessary.

Plasmid pCL301 contains all the genes necessary for Hg^{2+} resistance and *blaZ* fused in the *merB* gene 20 bases after the translational start site of *merB* (Fig. 1). pCL303 is a derivative of pCL301 with an internal deletion of 68 bases from the *merR* gene. *S. aureus* cells harboring plasmid pCL301 were induced to synthesize β-lactamase in the presence of Hg^{2+} or Cd^{2+} (Fig. 2). The maximum level of induction was achieved when cells were induced with 5 μM Hg^{2+} or 12 μM Cd^{2+}. Note that the *mer* determinant does not confer resistance to Cd^{2+}; hence, Cd^{2+} is a gratuitous inducer in this system. Cu^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Pb^{2+} failed to induce β-lactamase synthesis from pCL301. No β-lactamase activity was detected in cells containing pCL301 when they were induced with the organomercurial compound phenylmercuric acetate (PMA). Weiss et al. (29) reported that the *mer* operon was inducible by PMA in *S. aureus* cells harboring pI258 plasmid. In the present study with the β-lactamase gene fusion in the *merB* gene, the pI258 *mer* operon was not inducible by PMA. This finding is in contrast with the observation that the pDU1358-encoded *mer* operon is induced by PMA in *E. coli* even in the absence of *merB*, the gene for organomercurial lyase (17). It was shown with the pDU1358 *mer* operon that after the C-terminal 17 amino acids were deleted from the MerR protein, the *mer* operon was not induced by PMA but was still induced by Hg^{2+} in *E. coli* (17). The Tn*501* and Tn*21* operons were not induced by PMA in *E. coli*. Amino acid sequence comparisons of the MerR proteins from Tn*501*, Tn*21*, and pDU1358 show that the C-terminal nine amino acid residues are not conserved in pDU1358 MerR, implying that these amino acid residues might be necessary for the recognition of PMA as an inducer. Interestingly, these nine amino acid residues are missing from the pI258 MerR. Induction by PMA with the intact pI258 *mer* operon in *S. aureus* might result from the presence of inducing concentrations of Hg^{2+} produced from the catalytic degradation of PMA by the organomercurial lyase enzyme. Cells harboring the *merR* deletion mutant plasmid pCL303 did not synthesize measurable amounts of β-lactamase in the presence and absence of Hg^{2+} and Cd^{2+}. After prolonged incubation (>12 h) of the culture broth with nitrocefin, the substrate for β-lactamase, a light brown color could be seen in the incubation mixture with cells harboring pCL303 but not with cells containing the vector plasmid pSA3800. It is plausible that the *mer* promoter functions very poorly in the absence of *merR* and its activity is not detectable by the β-lactamase assay used for the present study. The *merR* defect in pCL303 was complemented in *trans* by wild-type *merR* from plasmid pKSK2, and the β-lactamase activity was about threefold higher in cells containing pCL303 and pKSK2 than in cells containing pCL301. The higher level of β-lactamase activity may be attributed to the presence of an increased concentration of the *merR* gene product produced from the high-copy-number plasmid pKSK2, a derivative of pE194. No β-lactamase activity was detected in the absence of the inducers and in the presence of the *merR* gene product in *trans*-complementation experiments. From the results described above, we conclude that the product of the *merR* gene is a *trans*-acting

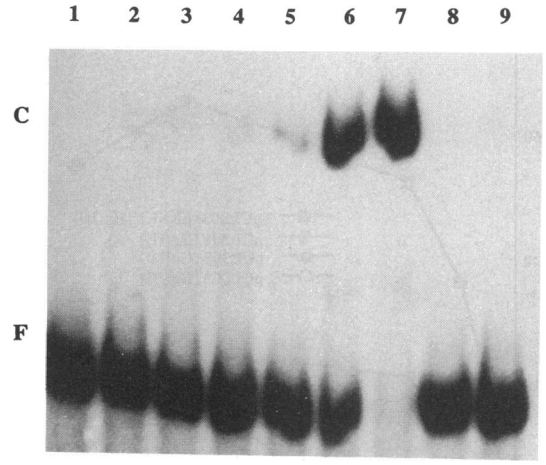

FIG. 3. Electrophoretic mobility shift of a DNA fragment encompassing the *mer* promoter bound with a GST-MerR fusion protein. Four nanograms of a 188-bp ^{32}P-labeled DNA fragment bounded by nucleotides 895 and 1082 (Fig. 1) was used in all lanes. Lanes contain the following amounts of GST-MerR fusion protein (in nanograms): 1, 0.0; 2, 1.3; 3, 2.6; 4, 5.2; 5, 13.0; 6, 26.0; 7, 130.0. Lane 8, same as lane 7, except 320 ng of the 188-bp unlabeled DNA was added to the preformed ^{32}P-labeled DNA-protein complex and incubated at 37°C for 10 min; lane 9, 0.25 μg of added GST protein. F, 188-bp fragment; C, DNA–GST-MerR complex.

factor that activates transcription of the *mer* operon in the presence of specific inducer Hg^{2+} or Cd^{2+}.

Binding of a GST-MerR fusion protein to the *mer* promoter region. Sequence analysis revealed a putative operator sequence upstream from the *merR* coding region. The transcriptional start sites were located at nucleotides 1018 and 1019 by reverse transcriptase mapping (24). *merR* was cloned and overexpressed in *E. coli* by using the pGEX-KG vector and was purified to approximately 99% homogeneity by affinity chromatography (7). The specific binding of the GST-MerR fusion protein with the putative *mer* operator sequence was studied in an electrophoretic mobility shift assay (4, 6). A 188-bp radiolabeled DNA fragment encompassing nucleotides 895 to 1082 (Fig. 1) (10) was used for the binding study. The DNA was end labeled with [α-^{32}P]dCTP (3,000 Ci/mmol). The binding assay conditions were as described in reference 14. The electrophoretic mobility of the DNA was retarded by the fusion protein (Fig. 3, lanes 4 through 7). GST protein alone did not have any effect on the mobility of the radiolabeled *mer* DNA fragment (Fig. 3, lane 9). Prebound fusion protein exchanged with unlabeled *mer* DNA (Fig. 3, lane 8). It can be concluded from these results that the *trans*-acting *merR* gene product is a protein (not an RNA) and that it regulates *mer* operon expression by interacting with an operator sequence.

Mapping of the *mer* operator sequence. In order to map the GST-MerR fusion protein binding sequence, a radiolabeled 496-bp DNA fragment bounded by nucleotides 895 and 1390 (Fig. 1) (10) was subjected to DNase I footprinting analysis (Fig. 4). DNase I protection footprinting was carried out by a modification of the procedure described by Galas and Schmitz (5) and Mukhopadhyay et al. (14). The sequence protected by the fusion protein spans nucleotides 980 to 1006 and is highly homologous with gram-negative bacterial *mer* operator sequences (Tn*501*, Tn*21*, and pDU1358). It should be noted that there is a 1-bp mismatch in the dyad sequences of the pI258 operator and that the dyad sequences extend up

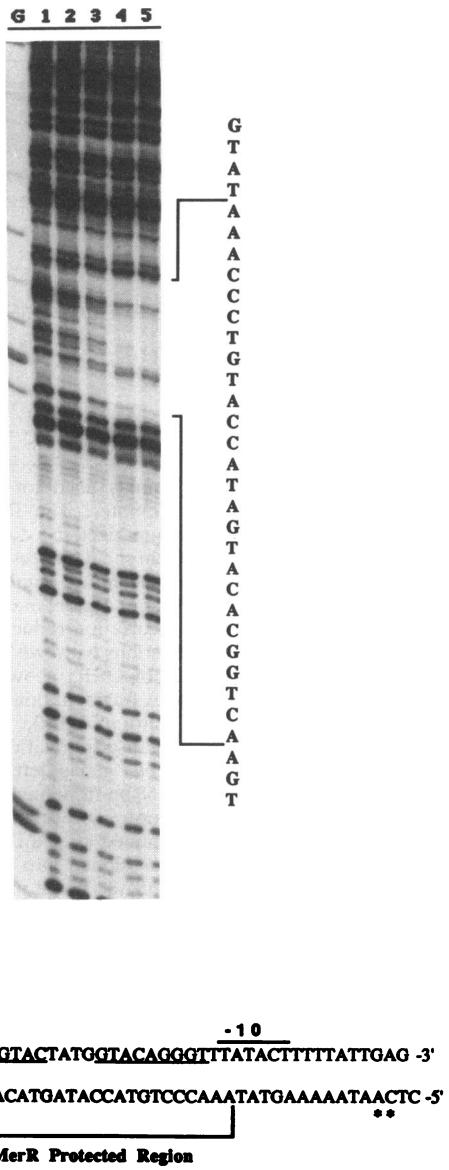

FIG. 4. DNase I protection footprinting of protein DNA complexes (top) and nucleotide sequence of the *mer* operator-promoter region (bottom). Twenty-six nanograms of a ^{32}P-labeled 496-bp DNA bounded by nucleotides 895 and 1390 (Fig. 1) was incubated with the GST-MerR fusion protein prior to digestion with DNase I. Lanes contain the following amounts of fusion protein (in nanograms): 1, 0; 2, 26; 3, 52; 4, 104; 5, 208. G, chemical "G" reaction according to Maxam and Gilbert (12). The protected sequences are shown on the right. At the bottom, the mRNA start sites are identified with asterisks. Consensus −10 and −35 sequences are overlined. Dyad sequences are underlined.

to 9 bp (Fig. 4, bottom), but in Tn*21*, Tn*501*, and pDU1358 *mer* operators the dyad sequences extend up to 7 bp. The *Bacillus* sp. strain RC607 *mer* operator sequence has not been defined directly by footprinting, and the 9-bp dyad sequences in the putative operator region have a 2-bp mismatch (8). It has been demonstrated that the Tn*501* MerR interacts with its operator DNA, altering the local structure of the operator-promoter region and thus making an adjustment for the RNA polymerase to form an open complex and

initiate transcription (1, 18). Studies of the interactions of the MerR proteins from different bacteria with the structurally different but naturally occurring operators and their correlations with the activation of transcription from the *mer* promoters will provide interesting information about the evolution of the *mer* regulatory genes and the corresponding operators.

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