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Nucleotide Sequence of a Chromosomal Mercury Resistance Determinant from a *Bacillus* sp. with Broad-Spectrum Mercury Resistance

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A 13.5-kilobase *Hin*dIII fragment, bearing an intact mercury resistance (*mer*) operon, was isolated from chromosomal DNA of broad-spectrum mercury-resistant *Bacillus* sp. strain RC607 by using as a probe a clone containing the mercury reductase (*merA*) gene. The new clone, pYW33, expressed broad-spectrum mercury resistance both in *Escherichia coli* and in *Bacillus subtilis*, but only in *B. subtilis* was the mercuric reductase activity inducible. Sequencing of a 1.8-kilobase mercury hypersensitivity-producing fragment revealed four open reading frames (ORFs). ORF1 may code for a regulatory protein (MerR). ORF2 and ORF4 were associated with cellular transport function and the hypersensitivity phenotype. DNA fragments encompassing the *merA* and the *merB* genes were sequenced. The predicted *Bacillus* sp. strain RC607 MerA (mercuric reductase) and MerB (organomercurial lyase) were similar to those predicted from *Staphylococcus aureus* plasmid pI258 (67 and 73% amino acid identities, respectively); however, only 40% of the amino acid residues of RC607 MerA were identical to those of the mercuric reductase from gram-negative bacteria. A 69-kilodalton polypeptide was isolated and identified as the *merA* gene product by examination of its amino-terminal sequence.

Continuing environmental heavy-metal pollution has favored the proliferation of microorganisms carrying metal resistance determinants (2, 11, 18, 37). Resistance to mercury encoded by bacterial plasmids and transposons in several gram-negative bacteria (11, 34, 37) and in one gram-positive organism (16, 43) has been investigated.

We previously described a gram-positive, aerobic sporeformer, *Bacillus* sp. strain RC607 (18, 42), whose mercury resistance determinants were located chromosomally and which showed a broad-spectrum (43) mercury resistance phenotype, i.e., growth both in the presence of such organomercurials as phenylmercuric acetate (due to the activity of the enzyme organomercurial lyase) and in the presence of inorganic mercury salts (with ability to convert Hg^{2+} to Hg^0 via the enzyme mercuric reductase). Plasmid pYW40, containing a 6.2-kilobase (kb) fragment of RC607 chromosomal DNA, was transferred to *Escherichia coli*, in which it produced mercuric reductase constitutively (42). Mercury resistance, however, was produced only in the presence of a second plasmid, pYW22, which encoded a functional mercury transport system (42); i.e., pYW40 did not contain the intact RC607 *mer* operon. Thus, both transport function and mercuric reductase activity are required for expression of microbial mercury resistance (17, 42).

The bacterial transposons Tn*21* and Tn*501*, of gram-negative origin and conferring narrow-spectrum resistance, possess similar *mer* operons. Essential gene products encoded include MerR, a metalloregulatory DNA-binding protein; MerT, a transport protein; MerP, a periplasmic Hg^{2+}-binding protein; and MerA, the mercuric reductase enzyme (22, 26, 34, 37). The *merD* gene, of unknown function, is found in both Tn*501* and Tn*21* (8), whereas an additional gene, *merC*, is found only in Tn*21* (22).

Of the *mer* genes, *merA* has been studied in greatest detail. This NADPH-dependent enzyme functions by reduction of flavin adenine dinucleotide followed by reduction of the Hg^{2+} substrate bound to the cysteine residues of the protein (12, 29). The amino acid sequences of the Tn*21* and Tn*501* MerA share 85% identity (23) but have only 40% identity with the amino acid sequence of the polypeptide predicted from the *merA* region of pI258 (16). However, when only the active-site positions and the regions believed responsible for NADPH and flavin adenine dinucleotide contacts were considered, there was more than 90% conservation (16).

Nucleotide analyses of two *merB* regions are available (14, 16). Plasmid pDU1358, isolated from gram-negative *Serratia marcescens*, encodes a predicted polypeptide of 212 amino acids, whereas plasmid pI258, isolated from gram-positive *Staphylococcus aureus*, encodes a predicted organomercurial lyase of 216 amino acids. The overall amino acid identity of these two MerB proteins is 40%. In both plasmids, the *merB* gene immediately follows the *merA* gene. In contrast, *merA* and *merB* of *Bacillus* sp. strain RC607 are separated by 2.5 kb (42), and mapping experiments carried out with the *Serratia* plasmid R831b revealed a *merB* gene 13.5 kb distant from other functional *mer* genes (25).

We now report the isolation of a larger clone, pYW33, containing the intact *Bacillus mer* operon and expressing

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TABLE 1. Plasmids and bacterial strains used and mercuric chloride and PMA resistance

Strain or plasmid	Mercury resistance[a]		Reference or source[b]
	$HgCl_2$	PMA	
Strain			
Bacillus sp. strain RC607	r	r	18
B. subtilis			
1A40	s	s	BGSC
1A40(pYW65)	r	r	
E. coli			
JM83	s	s	44
JM83(pYW22)	ss	NT	42
JM83(pYW40)	s	s	42
JM83(pYW22,pYW40)	r	r	42
JM83(pYW33)	r	r	
JM83(pYW54)	ss	NT	
JM83(pYW54B)	ss	NT	
JM83(pYW40, pYW54B)	r	NT	
JM83(pYW54BB)	s	NT	
JM83(pYW40, pYW54BB)	s	NT	
JM83(pYW54E)	s	NT	
JM83(pYW40, pYW54E)	s	NT	
JM83(pDB7)	r	s	3
Plasmid			
pUC9			40
pMK3			36

[a] Assessed both by the plate and disk methods (see Materials and Methods) as resistant (r), sensitive (s), or hypersensitive (ss). NT, Not tested.

[b] Where no reference or source is indicated, the strain was constructed in this study. BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus.

mercury resistance both in *E. coli* and in *Bacillus subtilis*. We have analyzed the nucleotide sequence of the *Bacillus* mercury resistance determinant and some characteristics of the isolated MerA protein.

MATERIALS AND METHODS

Strains and culture conditions. Strains and plasmids used in this study are listed in Table 1. *E. coli* carrying pDB7 (*mer* operon of plasmid R100) was provided by A. O. Summers. All strains were cultured in LB broth or on LB agar at 37°C. When necessary, the medium was supplemented with ampicillin (50 to 100 μg/ml), tetracycline (10 to 25 μg/ml), chloramphenicol (5 μg/ml), or kanamycin (7 μg/ml).

Resistance of bacterial strains to $HgCl_2$ and PMA. Resistant cells were selected on LB plates containing 25 to 125 μM $HgCl_2$ or 15 to 20 μM phenylmercuric acetate (PMA). Sensitivity of isolated strains was tested by either of two methods. (i) In the disk method, LB agar (18 ml per plate) was overlaid with 3 ml of soft LB agar (0.75% agar) containing 5×10^7 cells per ml. A 6-mm-diameter disk, bearing 125 nmol of $HgCl_2$ or 50 nmol of PMA, was placed on the surface, and the zone of inhibition (less the 6-mm disk diameter) was measured after incubation at 37°C for 16 h. Strains with a zone of inhibition of 18 to 20 mm were hypersensitive or supersensitive (Hg^{ss}), sensitive (Hg^s) strains had a zone of inhibition of 10 to 15 mm, and resistant (Hg^r) strains had an inhibition zone of <7 mm. (ii) Resistance criteria by the plate method were determined as described by Wang et al. (42) and were based on inhibition of growth on LB plates in which various concentrations of $HgCl_2$ or PMA had been incorporated.

Plasmids and chromosomal DNA isolation. For large-scale plasmid DNA preparation, the method of Tanaka et al. (39) was used. Small-scale plasmid DNA extractions were carried out as described by Birnboim and Doly (4). Chromosomal DNA was prepared by the method of Marmur (20).

Transformation. *B. subtilis* and *E. coli* were transformed after having been rendered competent for uptake of DNA (5, 19). Transformants were selected on LB agar containing antibiotics, $HgCl_2$, or both.

Labeling DNA and Southern DNA-DNA hybridization analysis. Plasmid DNA was labeled in vitro (28) with [α-^{32}P]dCTP. Southern blot transfers (35) were carried out with Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, Calif.). Hybridization was carried out at 45°C for 16 to 20 h in a solution (4× SSC [3.5% NaCl plus 1.76% sodium citrate], 50% formamide) containing ca. 10^6 cpm of probe DNA per ml.

Mercury volatilization. Cells were grown with shaking in tryptone broth (Difco Laboratories, Detroit, Mich.) (tryptone, 8 g/liter; NaCl, 5 g/liter) at 37°C to a Klett (red filter) reading of 60 (2×10^7 to 4×10^7 cells per ml) and then grown for an additional 60 min in the presence or absence of inducing concentrations of $HgCl_2$ (*B. subtilis* and *E. coli*, 10 μM; *Bacillus* sp. strain RC607, 25 μM). The cells were harvested by centrifugation, washed once with 25 mM Tris hydrochloride (pH 7.2), and then used in mercury volatilization assays (32, 43) with 5 μM $^{203}Hg(NO_3)_2$.

DNA sequencing. The dideoxy method of Sanger et al. (31), adapted for plasmid DNA by Chen and Seeburg (9), was followed except that either DNA polymerase I (Klenow fragment) or modified bacteriophage T7 DNA polymerase (Sequenase) (38) was used. Fragments were cloned into plasmid pUC9. Sequencing deletions were generated by digestion with appropriate restriction enzymes and nuclease *Bal*31. Synthetic oligonucleotides were used as primers. The cloned DNA was sequenced in both directions.

Protein purification, amino-terminal sequence analysis, and enzyme assays. Mercuric reductase was purified both from wild-type *Bacillus* sp. strain RC607 and from *E. coli* JM83(pYW40) grown in LB broth. RC607 cells were induced by the addition of 2.5 μM $HgCl_2$ at a cell density of 95 Klett units. After 20 min at 37°C, a second sample of $HgCl_2$ was added to 5.0 μM total. JM83(pYW40) produced enzyme constitutively. Both RC607 and JM83(pYW40) cells were harvested at ca. 140 Klett units and were stored as pellets at −70°C. Cells were suspended in purification buffer (20 mM sodium phosphate [pH 7.4], 0.5 mM EDTA, 0.1% 2-mercaptoethanol) and passed twice through a French pressure cell, and cellular debris was separated by centrifugation for 30 min at 27,000 × *g*. A 40 to 60% $(NH_4)_2SO_4$ precipitate containing >90% of the mercuric reductase activity was desalted on a Bio-Rad P6-DG desalting column and loaded onto a RedA Dye Matrex (Amicon Corp., Lexington, Mass.) column. After the preparation was washed with purification buffer, the enzyme was eluted with either 1 mM $NADP^+$ or 100 μM NADPH.

Approximately 250 μg of mercuric reductase purified from RC607 was exhaustively reduced and carboxymethylated as described by Allen (1), using iodoacetic acid recrystallized from chloroform. The carboxymethylated protein was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane as described by Matsudaira (21). Edman degradation and phenylthiohydantoin derivative analysis was done by using an Applied Biosystems model 470A automated sequencer equipped with a 1020A online PTH analyzer. Protein electrophoresis, electroblotting, and N-terminal sequence analysis were performed by D. Andrews and

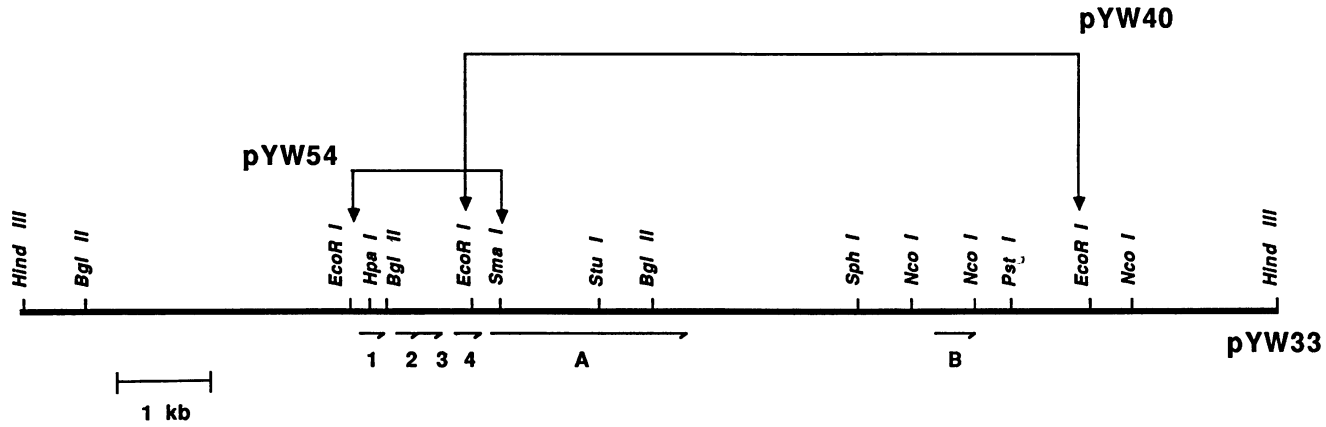

FIG. 1. Restriction map of fragments cloned from *Bacillus* sp. strain RC607 chromosomal DNA and inserted into plasmid pUC9. Plasmid pYW33 contains the intact *mer* operon. A and B represent the *merA* (mercuric reductase) and *merB* (organomercurial lyase) genes, respectively. ORFs 1 to 4 are located as indicated. The *Pst*I site of pYW33 was absent in pYW40.

W. Lane at the Harvard Microanalysis Facility, Cambridge, Mass.

Enzyme activity during purification was monitored by following NADPH oxidation in the presence of 100 μM $HgCl_2$ and 2-mercaptoethanol, i.e., $Hg(SR)_2$ (12, 42). Kinetic parameters for $Hg(SR)_2$ reduction by purified enzyme were determined at 25°C in 50 mM potassium phosphate (pH 7.0)–100 μM NADPH–2 mM 2-mercaptoethanol. Protein concentrations were determined by the procedure of Bradford (6), using bovine serum albumin as a standard. Units of enzyme activity were defined as the amount of enzyme catalyzing the Hg^{2+}-dependent oxidation of 1.0 μmol of NADPH per min (12).

For proteolysis studies, 100 μg of purified mercuric reductase was treated with either *N*-α-*p*-tolyl-L-lysine chloromethyl ketone-treated α-chymotrypsin or tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (1%, wt/wt in 100 μl of purification buffer at ambient temperature.

Chemicals. Antibiotics and chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes and enzymes involved in nucleic acid metabolism were purchased from New England BioLabs, Inc. (Beverly, Mass.) and used as directed. [α-^{32}P]dCTP, [α-^{35}S]dATP, and $^{203}Hg(NO_3)_2$ were purchased from Dupont, NEN Research Products (Boston, Mass.). DNA sequencing kits and protein molecular weight standards were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and United States Biochemical Corporation (Cleveland, Ohio). Synthetic oligonucleotide primers were prepared in a Cyclone DNA synthesizer (Biosearch, Inc., San Rafael, Calif.).

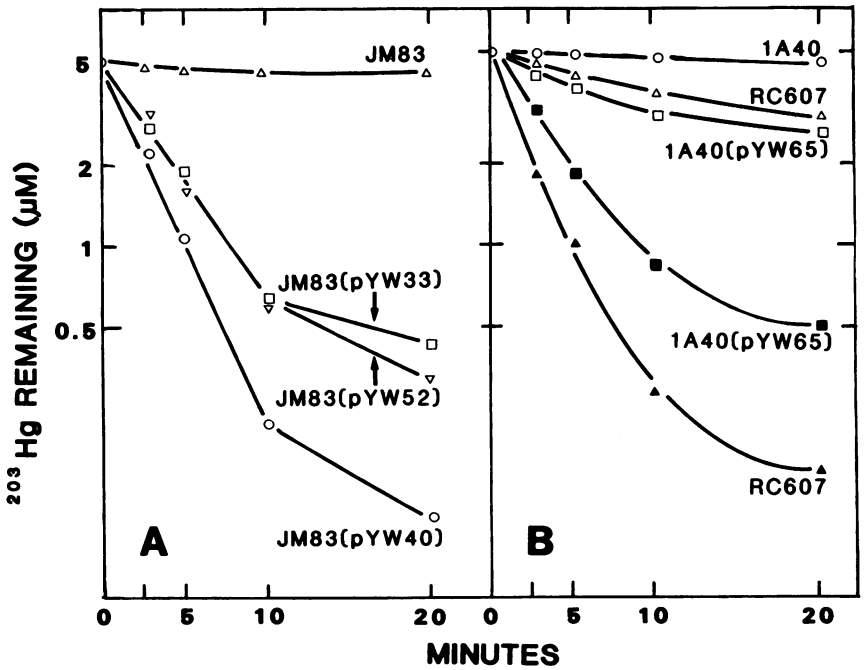

FIG. 2. Volatilization of mercury by intact cells of *E. coli* JM83 (A) and of *Bacillus* sp. strain RC607 and *B. subtilis* 1A40 (B). Open symbols, Cells without induction; closed symbols, cells induced with subtoxic concentrations of Hg^{2+}. Mercury-volatilizing *E. coli* strains produced mercuric reductase constitutively.

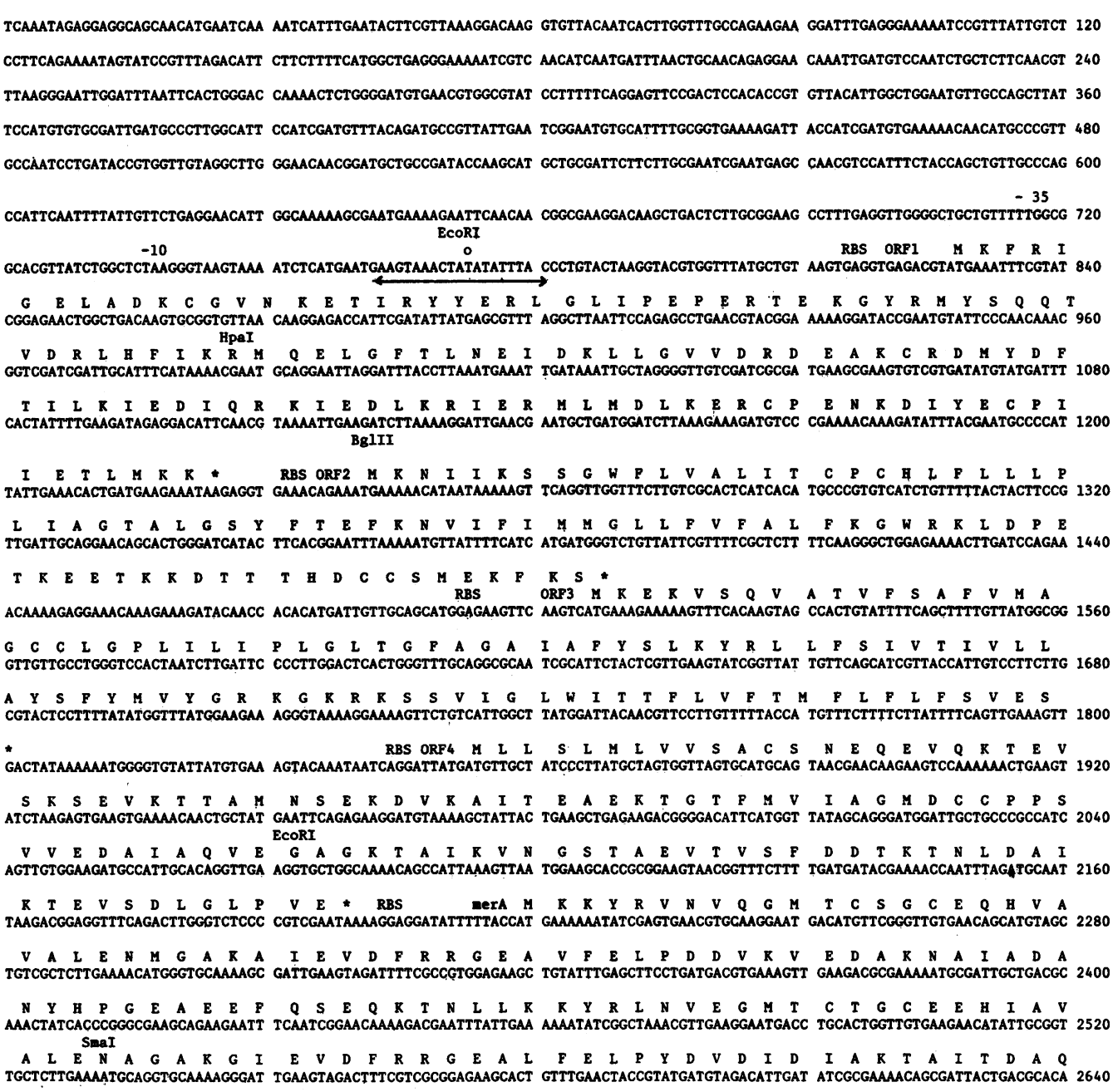

FIG. 3. Nucleotide sequence of cloned RC607 DNA including a short segment upstream of pYW54, pYW54 (containing ORF1 to ORF4), and the *merA* gene. Both strands were sequenced. Symbols: ↔, potential promoter-operator site with dyad symmetry upstream of ORF1; *, stop codon. A putative transcriptional terminator after *merA* is underlined; potential ribosome-binding sites (RBS) are marked. The primary structure of each predicted gene product is given by a one-letter amino acid code.

Treated trypsin and α-chymotrypsin were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

RESULTS

Evidence that plasmid pYW33 codes for mercury resistance. A *Hin*dIII-cut *Bacillus* sp. strain RC607 chromosomal DNA fragment containing the intact *mer* operon was isolated by using an [α-^{32}P]dCTP-labeled (28) *Eco*RI-excised fragment from pYW40 (Fig. 1; 42) as a probe in the Southern (35) hybridization procedure. The recombinant plasmid formed by inserting the cloned fragment into *Hin*dIII-digested pUC9 was used to transform *E. coli* JM83. Colonies resistant to ampicillin and to 37.5 μM $HgCl_2$ were selected and transferred to LB plates containing 125 μM $HgCl_2$. The plasmid isolated from such a mercury-resistant colony, pYW33 (Fig. 1), contained a 13.5-kb insert which transformed JM83 to broad-spectrum resistance (Table 1) and produced mercuric reductase activity constitutively (Fig. 2A).

To determine the expression of the cloned fragment in *B. subtilis*, plasmid pYW33 was digested with *Hin*dIII and ligated to the *Hin*dIII-cut vector pMK3, and the mixture was

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  Y  Q  P  G  E  A  E  E  I  Q   V  Q  S  E  K  R  T  D  V  S   L  N  D  E  G  N  Y  D  Y  D   Y  I  I  I  G  S  G  G  A  A
ATATCAACCGGGCGAAGCAGAAGAAATACA AGTGCAATCGGAAAAAAGGACAGATGTAAG TTTAAATGATGAAGGTAACTATGATTATGAT TACATCATCATCGGTTCTGGTGGAGCTGC 2760

  F  S  S  A  I  E  A  V  A  L   N  A  K  V  A  M  I  E  R  G   T  V  G  G  T  C  V  N  V  G   C  V  P  S  K  T  L  L  R  A
CTTTTCATCTGCCATTGAAGCCGTTGCTTT GAACGCAAAAGTGGCTATGATTGAGCGTGG AACGGTGGGTGGAACTTGCGTTAATGTCGGA TGCGTTCCTTCTAAGACCTTATTAAGAGC 2880

  G  E  I  N  H  L  A  K  N  N   P  F  V  G  L  H  T  S  A  S   N  V  D  L  A  P  L  V  K  Q   K  N  D  L  V  T  E  M  R  N
AGGGGAAATCAATCATCTAGCAAAAAATAA TCCATTTGTGGGATTACACACTTCGGCTTC AAATGTTGATTTAGCGCCATTAGTAAAACAA AAGAATGATTTAGTAACCGAGATGCGAAA 3000

  E  K  Y  V  N  L  I  D  D  Y   G  F  E  L  I  K  G  E  S  K   F  V  N  E  N  T  V  E  V  N   G  N  Q  I  T  A  K  R  F  L
TGAAAAATATGTGAATTTAATTGATGATTA TGGTTTTGAATTAATAAAAGGTGAATCAAA ATTCGTAAATGAAAATACAGTTGAAGTAAAT GGCAATCAAATCACAGCCAAAAGATTTTT 3120

  I  A  T  G  A  S  S  T  A  P   N  I  P  G  L  D  E  V  D  Y   L  T  S  T  S  L  L  E  L  K   K  V  P  N  R  L  T  V  I  G
AATAGCTACAGGTGCTTCTTCAACTGCACC TAATATTCCCGGATTAGATGAAGTAGATTA TTTAACAAGCACTAGCTTATTGGAATTAAAG AAGGTTCCAAATCGTCTTACCGTAATTGG 3240

  S  G  Y  I  G  M  E  L  G  Q   L  F  H  N  L  G  S  E  V  T   L  I  Q  R  S  E  R  L  L  K   E  Y  D  P  E  I  S  E  A  I
TTCAGGATATATCGGCATGGAATTAGGACA ACTATTTCATAACCTCGGGTCAGAAGTCAC TTTGATTCAAAGAAGCGAGCGTCTATTAAA AGAATACGATCCTGAAATTTCAGAAGCCAT 3360

  T  K  A  L  T  E  Q  G  I  N   L  V  T  G  A  T  Y  E  R  V   E  Q  D  G  D  I  K  K  V  H   V  E  I  N  G  K  K  R  I  I
TACTAAGGCCTTAACAGAACAGGGAATTAA TTTAGTAACAGGTGCAACCTATGAACGAGT TGAGCAAGATGGAGACATTAAAAAAGTTCA TGTTGAGATAAATGGTAAAAAGCGAATTAT 3480

  E  A  E  Q  L  L  I  A  T  G   R  K  P  I  Q  T  S  L  N  L   H  A  A  G  V  E  V  G  S  R   G  E  I  V  I  D  D  Y  L  K
TGAAGCAGAACAATTGCTAATTGCCACTGG AAGAAAACCAATACAGACATCATTAAACTT ACATGCAGCAGGCGTTGAAGTTGGTTCCCG TGGTGAAATTGTCATTGATGATTATCTTAA 3600

  T  T  N  S  R  I  Y  S  A  G   D  V  T  P  G  P  Q  F  V  Y   V  A  A  Y  E  G  G  L  A  A   R  N  A  I  G  G  L  N  Q  K
AACGACCAATTCCCGAATTTATTCAGCTGG AGATGTCACTCCCGGTCCCCAATTTGTTTA TGTAGCTGCTTATGAAGGTGGACTTGCTGC TCGTAATGCAATCGGAGGACTAAATCAAAA 3720

  V  N  L  E  V  V  P  G  V  T   F  T  S  P  S  I  A  T  V  G   L  T  E  Q  Q  A  K  E  K  G   Y  E  V  K  T  S  V  L  P  L
GGTCAATTTAGAAGTGGTTCCAGGCGTTAC GTTTACTTCTCCATCGATTGCAACGGTTGG TTTAACGGAGCAACAGGCAAAAGAAAAAGG ATATGAAGTGAAAACATCGGTATTGCCGTT 3840

  D  A  V  P  R  A  L  V  N  R   E  T  T  G  V  F  K  L  V  A   D  A  K  T  L  K  V  L  G  A   H  V  V  A  E  N  A  G  D  V
GGATGCTGTTCCAAGAGCGCTCGTTAATCG GGAAACAACAGGTGTTTTCAAATTAGTGGC AGACGCGAAAACATTGAAAGTGTTAGGGGC GCATGTAGTGGCAGAAAACGCAGGAGACGT 3960

  I  Y  A  A  T  L  A  V  K  F   G  L  T  V  G  D  L  R  E  T   M  A  P  Y  L  T  M  A  E  G   L  K  L  A  V  L  T  F  D  K
AATTTATGCAGCAACATTAGCTGTGAAATT CGGTTTAACTGTTGGAGATCTGAGAGAAAC GATGGCTCCATATCTAACAATGGCAGAAGG ATTGAAGCTGGCTGTCCTAACTTTTGATAA 4080
                                BglII
  D  V  S  K  L  S  C  C  A  G   *                        o
AGATGTTTCGAAATTATCTTGCTGTGCTGG ATAAGTTAGAAAAAAGATTCCTGATTATTG CTCAGGAATCTTTTTTCTGTTTTATGAATA AGGTTTTCCGTACTTTGCCAAATTAACTTT 4200

AAACTGTTTAAGGAAATGAAATATTTATAT AGCATATTTGTTTTACACGGTTTATTATGT CAACAATCTGTGCACTTTGGTTAAGTCTTC TTGCTATTGAAATCTATAAAACTTACTTAA 4320

TAAAAAGGATTGAGTTTCTCTACAAATTAT CGAGGAATGACTAATGGTGGCAGAGGAATA AATCCCTTTTTAACAGCCATTGAGTCAGTG GCTAGGTGCAACGATTTGCACGATGATAAT 4440

TACGAATAAATGCATTCACTTACGTCTGCT CGATGGCTCGAAATTGCCCCAGTATTCCAA AGTCCATAAATCCCTGTACGGCGAAATAAT GTCCAATGAGTATCTTCTGGTCATCTATAA 4560

ACAGGCGTTTGAATATCTCCTAGTTCCTTA ACTGTTTTTGATTCCGAAATAGCATTATAT GGAGCTGGAACAGTGTAACCAAGACGAATT AAAATTTGATATAACTCTGTTAGATTGGGT 4680

TCGCATACATCCCGACGATAGATTTCCATA AGTTGTTTTAATTGAGGATTCTTTAAAATC TGAATGTATAAATTACATTACCCCAAGCAA ACCCATTCGCGAAGGTAGCCTTGGGTTACC 4800

ATATAATACTCGCTAGTATCAAGGAAACGG TTTTCATGTCGAGTTTATTATAGAAATGTT GCAATAACACCATTT 4875
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transformed into *B. subtilis* 1A40. Transformants were selected on kanamycin plates and tested on plates containing 125 μM $HgCl_2$. Plasmid pYW65, the 13.5-kb cloned fragment inserted into pMK3, transformed competent cells of *B. subtilis* to broad-spectrum mercury resistance (Table 1). Mercuric reductase activity of *B. subtilis* 1A40(pYW65) was inducible; mercury was volatilized more rapidly when the cells had been induced with subtoxic concentrations of mercury (Fig. 2B).

Location, isolation, and nucleotide sequence of the *merA* gene. Plasmid pYW40 digested with *Eco*RI and *Sph*I, followed by self-ligation, was transformed into JM83. Ap^r transformants were tested for mercuric reductase activity. Cells carrying the 3.8-kb *Eco*RI-*Sph*I fragment still produced enzyme activity. JM83 cells carrying pYW40, which had been digested with *Bgl*II, failed to synthesize mercuric reductase activity. The 3.8-kb fragment was shortened by digestion with *Bal*31, and the DNA fragments obtained were inserted into the *Sma*I site of pUC9 by blunt-end ligation.

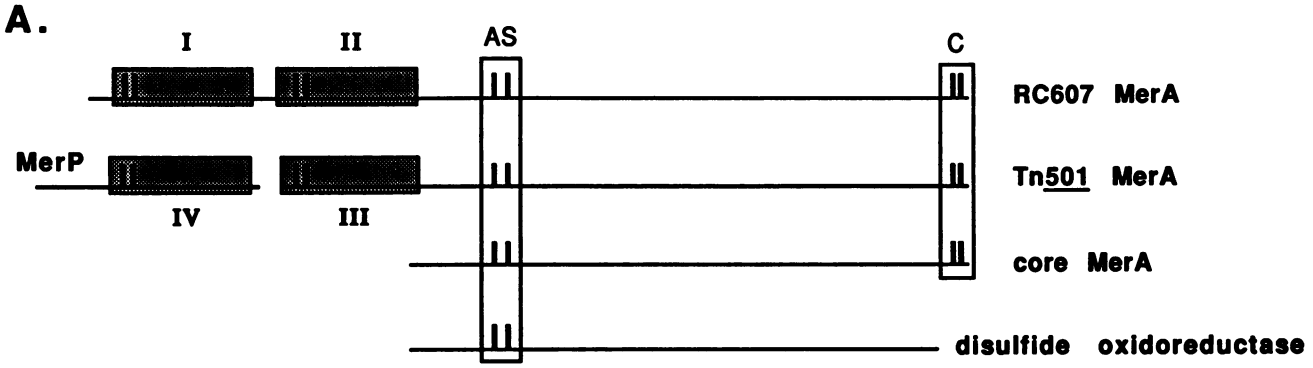
The shortest DNA fragment still capable of producing mercuric reductase activity in *E. coli* (pYW52, Fig. 2A) measured 2.1 kb and included the intact *merA* gene. DNA sequencing of this fragment revealed a potential ribosome-binding site (30) followed by an ATG start codon and encompassed an open reading frame (ORF) of 1896 base pairs (positions 2218 to 4114, Fig. 3). After the stop codon there was a potential transcriptional terminator (30), a 17-base-pair perfect inverted repeat (underlined in Fig. 3).

Comparison of the predicted *Bacillus* MerA polypeptide sequence with the three other known MerA sequences (Fig. 4), using the multiple-alignment program of Feng and Doolittle (10), showed that the MerA sequence from *S. aureus* plasmid pI258 was closest to the new *Bacillus* sequence, with 67% identical amino acids as aligned. The *Bacillus* MerA sequence was significantly less homologous (40 to 41% matches) with the MerA polypeptides of Tn*21* and Tn*501*, which are from gram-negative bacterial sources.

The predicted size (69 kilodaltons) of the *Bacillus* MerA protein (Fig. 4) was significantly greater than those of the putative mercuric reductase of pI258 (16) and the mercuric reductase enzymes isolated from gram-negative bacteria (3, 23). The increased length of the *Bacillus merA* DNA sequence (Fig. 3) encoded 79 additional amino acid residues at the beginning of the gene. These initial 79 amino acids appear to have resulted from a duplication. The alignment of amino acid residues 1 to 80 with residues 81 to 161 (Fig. 5B) showed 73% identity between the two sets.

Characterization of purified *Bacillus* MerA. Given the

FIG. 4. Alignment (gap penalty = 25) of amino acid sequences predicted from *Bacillus* sp. strain RC607 *merA* with sequences from pI258, Tn*21*, and Tn*501*. The multiple-protein-alignment program of Feng and Doolittle (10) was used.



B.

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I    1 MKKYRVNVQGMTCSGCEQHVAVALE-NMGAKAIEVDFRRGEAVFEL-PDDVKVEDAKNAIADANYHPGEAESF--QSEQKTNLL- 80
II  81 -KKYRLNVEGMTCTGCEEHIAVALE-NAGAKGIEVDFRRGEALFEL-PYDVDIDIAKTAITDAQYQPGEAEEIQVQSEKRTDVS- 161
III  1   MTHLKITGMTCDSCAAHVKEALEKVPGVQSALVSYPKGTAQLAIVPG-TSPDALTAAVAGLGYKATLADAPLADNRVGLL- 79
IV  24   -VTLSVPGMTCSACPITVKKAISEVEGVSKVDVTFETRQAVVTFDDAKTSVQKLTKATADAGYPSSVKQ 91
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C. Met-Lys-Lys-Tyr-Arg-Val-Asn-Val-Gln-Gly-Met-Thr-Cys-Ser-Gly-Cys-Glu-Gln-His-Val-

FIG. 5. Comparison of the N-terminal domains of *Bacillus* MerA (I and II), Tn*501* MerA (III), and Tn*501* MerP (IV). (A) Schematic of primary sequences showing cysteine pairs (‖) and regions of N-terminal-domain homology (▨). AS and C denote the active-site redox-active and C-terminal cysteine pairs, respectively. (B) Sequence comparison of N-terminal domains of *Bacillus* sp. strain RC607, Tn*501* MerA, and Tn*501* MerP. Symbols: :, amino acid residues which are identical in more than one sequence; ., conservative replacement, defined (15) by the groupings (I,L,M,V), (H,K,R), (D,E,N,Q), (A,G), (F,Y,W), (S,T), (C), and (P). (C) N-terminal sequence of *Bacillus* MerA as determined by protein sequencing. Residues not identical between the N-terminal regions are underscored.

novelty of the N-terminal-domain duplication indicated by the DNA sequence of *merA* (Fig. 3) and the translated polypeptide sequence (Fig. 4 and 5), the protein was isolated and sequenced. Analysis of the *Bacillus* MerA protein confirmed the expected sequence (Fig. 5C). Thus, the (amino acid) sequencing data coupled with the high molecular weight of the protein (Fig. 6) established that the *Bacillus* mercuric reductase contained both N-terminal domains.

There were several differences between RC607 MerA and Tn*501* MerA. The *Bacillus* MerA did not bind to the OrangeA (Amicon) Affinity Matrex used to purify Tn*501* MerA but bound to RedA instead, implying that the two proteins differ in the nicotinamide-binding-site region. Unlike the gram-negative, single N-terminal-domain proteins, the *Bacillus* MerA did not appear sensitive to N-terminal-domain proteolysis. The *Bacillus* MerA stored at 4°C for more than 2 months showed little diminution in molecular weight, whereas similarly stored Tn*501* protein became increasingly shortened (i.e., clipped), eventually resulting in removal of the first 85 amino acids, without affecting catalytic activity (13). The *Bacillus* MerA could be partially proteolyzed by incubation with trypsin or α-chymotrypsin, with production of sodium dodecyl sulfate-polyacrylamide gel electrophoresis bands consistent with sequential removal of the two domains (Fig. 6).

The catalytic properties of purified *Bacillus* MerA were examined. The enzyme had a pH optimum of 7.0 and K_m and V_{max} for $HgCl_2$ (in 2 mM β-mercaptoethanol, 25°C) of 15 μM and 19 U/mg, respectively, kinetic parameters which are not

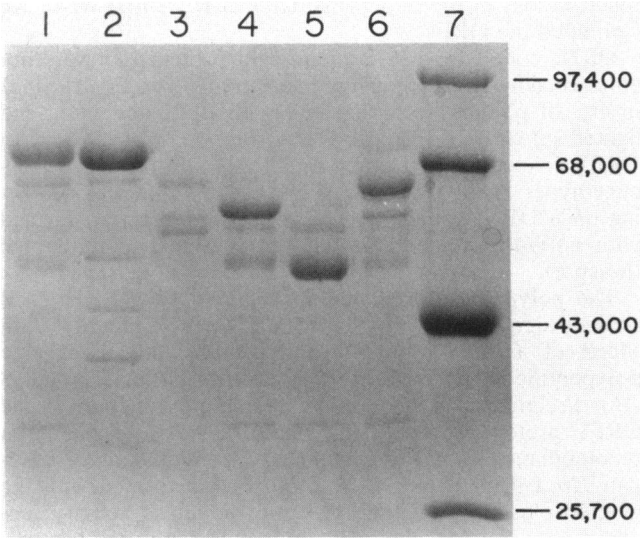

FIG. 6. Polyacrylamide gel electrophoresis of purified and partially proteolyzed MerA. Lanes: 1, purified *Bacillus* MerA protein from *Bacillus* RC607; 2, purified *Bacillus* MerA from *E. coli* JM83(pYW40); 3, purified Tn*501* MerA showing unclipped (upper band) and clipped (lower bands) protein; 4 to 6, *Bacillus* MerA from JM83(pYW40) after treatment with trypsin for 10 min (lane 4), after treatment with trypsin for 2 h (lane 5), and after treatment with α-chymotrypsin for 2 h (lane 6); 7, molecular weight standards (α-chymotryspinogen, 25,700; ovalbumin, 43,000; bovine serum albumin, 68,000; and phosphorylase *b*, 97,400).

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GCTCTCTCTTTTGGAAACGA ATTGAAAAAATTTTTATCAT AGGGTAGAGCCCATGAAGTT TCAAAAATCAGACAAGCTAT GAAAAGGATGCTTTTATCGG TCAATTAGTCCAACAAATGG 120

                                                        RBS    merB M K T  E I Q E I V T  R L D Q Q S
AGTTTTTAGGTAATGAAAAG TAATATCGAAAATAATAATA GGAACAAAAAATAACAAAGG AGGAAAAAGACATGAAAACT GAAATTCAGGAAATCGTAAC CCGACTTGACCAACAGTCGA 240

N K G E G G E  S M K W L F R  P L L Q M L  A G G E S V T  I E D M A T T  T G K P V E
ACAAGGGAGAGGGAGGAGAA TCCATGAAGTGGCTGTTTCG CCCGTTGTTACAAATGCTAG CAGGGGGGGAGTCTGTCACC ATTGAAGATATGGCGACAAC GACCGGAAAACCTGTTGAAG 360

E V K K V L Q  S L P S V E I  D E Q G R V  V G L G L T L  I P T P H H F  T V D G K Q
AGGTGAAGAAAGTTCTCCAG TCTCTGCCAAGCGTGGAAAT CGACGAACAAGGCCGTGTCG TTGGTTTAGGTCTTACCCTT ATCCCTACCCCTCACCACTT TACGGTTGATGGGAAGCAAC 480

L Y A W C A L  D T L I F P A  L I G R S V  N I E S P C H  S T G E P I R  L N V E P D
TATATGCCTGGTGCGCCCTT GACACACTTATATTTCCAGC ACTCATCGGTCGTTCGGTCA ACATCGAGTCACCCTGTCAC AGCACCGGAGAACCTATACG GTTGAATGTGGAACCGGACC 600

H I V S V E P  S T A V V S I  V T P D D M  S S I R T A F  C N E V H F F  S S P N A A
ACATTGTAAGCGTGGAACCT TCCACTGCCGTTGTCTCAAT CGTGACGCCAGATGATATGT CCTCGATTCGTACGGCATTC TGCAACGAAGTCCATTTCTT CAGCTCACCGAATGCAGCCG 720

E D W L D Q H  P G G K V L S  V E D A F E  L G R L M G T  R Y E E S R P  A N G S C C
AAGACTGGCTTGACCAACAT CCAGGGGGGAAAGTGTTATC TGTAGAAGATGCCTTTGAAC TGGGTCGCCTAATGGGGACG CGTTACGAGGAATCTAGACC TGCCAATGGGTCATGCTGTC 840
                                                                                    XbaI
H I *
ACATTTAGTCGCAAGTAACA CGATACAGAAGGAGCACAGG TCTGAAGATGAACCAGGCAG CTCAGGACAGGTTCCTCTTT ATACCTGATTCTTATATCTC TGACCGCTAACTACGCGAAG 960

TATGCAAAGGTGGTAAAACC TACAAAAATACCCGGTATTT CTGTACTAAGGAGCCGATCC GTATATGCCATGGTACCGGA 1040
                                                                  NcoI
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FIG. 7. Nucleotide and derived amino acid sequence of the *merB* gene of *Bacillus* sp. strain RC607.

significantly different from those previously determined at 37°C for Tn*501* mercuric reductase (12 μM and 13 U/mg; 12).

Nucleotide sequence and transport function of a region involved in hypersensitivity. Cells carrying a *mer* operon with intact transport genes but without functional *merA* become mercury hypersensitive (24). The location of the *Bacillus merA* gene having been determined (Fig. 1), vector pUC9 was used to subclone a 1.8-kb *Eco*RI-*Sma*I fragment upstream of *merA*. The recombinant plasmid, pYW54, was transformed into *E. coli*. Transformants had increased sensitivity to Hg^{2+} (Table 1). Sequencing of the *Bacillus* DNA of pYW54 (positions 649 to 2410, Fig. 3) revealed four ORFs capable of coding for four small polypeptides. Since deletion of a gene responsible for encoding transport protein would result in loss of the hypersensitivity of *E. coli*(pYW54), we examined the ORFs.

ORF1 coded for a 132-amino-acid protein that was not concerned in determining hypersensitivity. The *Eco*RI-*Sma*I insert (of pYW54) was digested with *Bgl*II and blunt-end ligated into pUC9. The new construction, pYW54B (with ORF1 deleted), transformed into JM83 still produced mercury-hypersensitive cells (Table 1). In addition, cells carrying pYW54B became mercury resistant when transformed with pYW40, which indicated the presence of functional transport.

The polypeptide predicted from *Bacillus* ORF1 (Fig. 3) showed significant homology (37% amino acid identity) with MerR of Tn*501* (8) and strong identity (58%) with the polypeptide predicted from ORF2 of *S. aureus* plasmid pI258 (16). Preliminary support for a regulatory function of the ORF1 protein product was obtained by constructing a recombinant plasmid in which ORF1 contained a 252-base-pair *Hpa*I-*Bgl*II deletion. *B. subtilis*, transformed with the deleted plasmid, had low mercuric reductase activity; enzyme activity was no longer inducible (data not shown). Although Laddaga et al. (16) considered ORF1 of pI258 as the probable regulatory gene, *Bacillus* ORF1 and pI258 ORF2 may well be responsible for the regulation of these gram-positive *mer* operons.

The *Eco*RI-*Sma*I fragment was digested with *Bgl*II, followed by digestion with *Bal*31. Ligated to pUC9, the new construct, pYW54BB, lacked ORF1 and ORF2 but contained ORF3 and ORF4. ORF2 could code for a 90-amino-acid polypeptide, with a Cys-Pro-Cys and a Cys-Cys sequence (Fig. 3). ORF2 may play a role in mercury transport and in producing cellular hypersensitivity. Cells carrying pYW54BB showed normal mercury sensitivity (Table 1), i.e., loss of hypersensitivity, and did not yield mercury-resistant transformants when transformed with pYW40 (Table 1). Comparison of the predicted amino acids of *Bacillus* ORF2 with those of MerT of Tn*501* (22) showed 30% identity. There was some similarity (20% identity) between the *Bacillus* ORF2 putative polypeptide and the protein predicted from ORF5 of pI258 (16).

ORF3, a sequence which coded for a 99-amino-acid polypeptide, showed a potential signal sequence (41) followed by a Cys-Cys pair (Fig. 3).

ORF4 coded for a 115-amino-acid polypeptide featuring an initial short run of hydrophobic amino acids meeting the theoretical criterion for a signal sequence (27). Plasmid pYW54 was digested with *Eco*RI and transformed into JM83 after self-ligation. Cells carrying the new construction, pYW54E, with ORF4 deleted, had normal mercury sensitivity (Table 1), i.e., were not hypersensitive; when cells were transformed with pYW40, no mercury-resistant transformants appeared. Thus, it is suggested that ORFs 2 and 4 play a role in mercury transport.

Location and nucleotide sequence of *merB*. Cells carrying both the helper plasmid, pYW22, and pYW40 were resistant to both inorganic mercury and PMA (Table 1). *Nco*I digestion as well as double digestion of pYW40 with *Eco*RI and *Sph*I (Fig. 1) resulted in loss of PMA resistance. A portion of the 2.4-kb *Eco*RI-*Sph*I fragment was sequenced and revealed a 657-base-pair ORF capable of encoding a MerB protein of 218 amino acids (Fig. 7) related to those described by Griffin et al. (14) and by Laddaga et al. (16). Alignment with the predicted amino acids coded by the *merB* region of plasmid pI258 revealed an overall identity of 73% (158 of 216 shared amino acids), whereas comparison with the predicted MerB encoded by plasmid pDU1358 (of gram-negative origin) showed less identity, only 39% (Fig. 8).

DISCUSSION

The mercury resistance determinants cloned from chromosomal DNA of *Bacillus* sp. strain RC607 showed both similarities with and differences from cloned and sequenced mercury resistance genes obtained from plasmids of gram-negative microorganisms and from *S. aureus*.

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          **        *              * *** ** * *    *    * * * **   * * * *
Bacillus  MKTEIQEIVTRLDQQSNKGEGGESMKWLFRPLLQMLAGGESVTIEDMATTTGKPVEEVKKVLQSLPSVEIDEQG  74
PI258     MK NISEFSAQLDQTFDQGEAV SMEWLFRPLLKMLAEGDPVPVEDIAAETGKPVEEVKQVLQTLPSVELDEQG  72
pDU1358   MK LAPYILERLT SVNRTNGTAD  LLVPLLRELAKGRPVSRTTLAGILDWPAERVAAVLEQATSTEYDKDG  69

             * **** *  * *  ********** *******    * *  **    * * *     ***    *
Bacillus  RVVGLGLTLIPTPHHFTVDGKQLYAWCALDTLIFPALIGRSVNIESPCHSTGEPIRLNVEPDHIVSVEPSTAVV 148
PI258     RVVGYGLTLFPTPHRFEVDGKQLYAWCALDTLMFPALIGRTVHIASPCHGTGKSVRLTVEPDRVVSVEPSTAVV 146
pDU1358   NIIGYGLTLRETSYVFEIDDRRLYAWCALDTLIFPALIGRTARVSSHCAATGAPVSLTVSPSEIQAVEPAGMAV 143

          * * *     *  **  **** *  * **  *         *  ** **
Bacillus  SIVTPDDMSSIRTAFCNEVHFFSSPNAAEDWLDQHP   GGKVLSVEDAFELGRLMGTRYEESRPANGSCCHI  218
PI258     SIVTPDEMASVRSAFCNDVHFFSSPSAAQDWLNQHP   ESSVLPVEDAFELGRHLGARYEESGPTNGSCCNI  216
pDU1358   SLVLPQEAADVRQSFCCHVHFFASVPTAEDWASKHQGLEGLAIVSVHEAFGLGQEFNRHLLQTMSSRTP      212
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FIG. 8. Alignment (gap penalty = 7) of the predicted organomercurial lyase (MerB) sequences of *Bacillus* sp. strain RC607 with those reported for pI258 (16) and pDU1358 (14).

The predicted *Bacillus* MerB (organomercurial lyase) showed a high degree of identity (73%) with MerB of *S. aureus* pI258. The *Staphylococcus merB* sequence was originally identified by similarity of the gene product (40% amino acid identity) to a MerB from the gram-negative *Serratia* plasmid pDU1358 (14, 16). In both pI258 and pDU1358 the *merA* and *merB* genes are contiguous, but the *Bacillus merB* is located 2.5 kb downstream from *merA*, following a strong termination signal, and thus may be regulated independently of the rest of the *mer* operon.

When considering the aligned mercuric reductase sequences which are now available (Fig. 4), it is apparent that the conserved residues are not evenly spaced through the sequences. Laddaga et al. (16) showed that the active-site peptide and the amino acids putatively involved in flavin adenine dinucleotide and NADPH binding are highly conserved among the pI258, Tn*501*, and Tn*21* MerA sequences. With the addition of the RC607 sequence, it is now evident that the N- and C-terminal regions are also homologous. Most striking is the similarity in the C-terminal region of all four MerA sequences: 22 of 28 amino acids are completely conserved (Fig. 4). This conservation is consistent with recent mutagenesis studies by M. Moore and C. Walsh (unpublished data) showing that the pair of cysteines at positions 558 and 559 (Fig. 4) of Tn*501* MerA are essential for enzyme activity. In addition, S. Miller et al. (personal communication) have shown these residues to interact with the redox-active disulfide (33) and have proposed a role for them in catalysis.

The three previously published *merA* sequences encode an 80- to 85-amino-acid N-terminal domain which includes a pair of cysteine residues near the N terminus. The sequence around this cysteine pair is highly conserved in all four MerAs, with the consensus being Gly-Met-Thr-Cys-X-X-Cys-X-X-His-Val-X-X-Ala-Leu-Glu (Fig. 4). The *Bacillus merA* sequence encodes a total of 631 amino acids, compared with 561 for Tn*501*, 564 for Tn*21*, and 547 for pI258, the additional RC607 residues resulting apparently from a duplication of the N-terminal domain (Fig. 5B).

The N-terminal domain of Tn*501* MerA is very susceptible to proteolysis, and such proteolysis does not affect the in vitro catalytic properties of the enzyme (13). Because of its similarity to the *merP* gene product (Fig. 5A and B), a role for the N-terminal domain in Hg^{2+} transport was proposed (7, 34, 37), namely, that the two cysteines near the N-terminus of MerA transiently bind Hg^{2+} ions while transferring Hg^{2+} from MerT to the active site of MerA. However, J. Altenbuchner (personal communication) has recently sequenced a *merA* gene from *Streptomyces lividans* which codes for only 474 amino acid residues and lacks an N-terminal domain. In addition, the essentiality of the N-terminal cysteines of Tn*501* MerA remains unclear, since Moore and Walsh (unpublished data) have demonstrated, by mutagenesis, that resistance is retained even in the absence of this cysteine pair.

Whereas the *merA* and *merB* genes from gram-negative and gram-positive bacteria possess a high degree of similarity, the transport genes show only limited identity. Since the *mer* operon from *S. aureus* pI258 was not expressed in *E. coli* (16), it was considered that transport proteins from gram-positive sources might not function in a gram-negative cell. The finding that the intact RC607 *mer* operon of pYW33 expressed mercury resistance in *E. coli* indicated that the *Bacillus* proteins are capable of functioning in a gram-negative cell. Consistent with this, a degree of sequence similarity exists between the gram-negative MerT proteins and those encoded by pI258 ORF5 (16) and *Bacillus* ORF2. While this fact suggests that RC607 ORF2 may encode a transmembrane protein, the precise functions of the ORF3 and ORF4 gene products are less clear. In view of its potential signal sequence, it is possible that ORF4 protein functions extracellularly. However, further analysis of all three gene products, as well as that of ORF1 (the putative regulatory protein), will be required to achieve a more complete understanding of this gram-positive system.

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