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The *mer* operon of the acidophilic bacterium *Thiobacillus* T3.2 diverges from its *Thiobacillus ferrooxidans* counterpart

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Abstract The chromosomal mercury resistance *(mer)* region of the acidophilic bacterium *Thiobacillus* T3.2 was cloned, characterized, and compared to reported homologous sequences. The *Thiobacillus* T3.2 *mer* resistance system is organized as an operon that transcribes into a polycistronic mRNA encoding the Hg^{2+} ion transport MerT and MerP proteins and the mercuric reductase MerA. In contrast to the *Thiobacillus ferrooxidans mer* determinant, no *merC* gene was detected. Transcription of structural genes is regulated by the product of the regulatory *merR* gene. On the basis of sequence data and expression experiments in *E. coli*, both *merTPA* and *merR* transcription units could be located close to each other and in different strands, with their promoters (P_{TPA} and P_R, respectively) overlapping the putative MerR binding site in the intergenic operator/promoter (O/P) region. Amino acid sequences of *mer* gene products were compared to their homologs. Some sequence features, such as the number and position of cysteine residues, are unique for the Mer proteins of this bacterium. Similarities (−10 and −35 boxes are 19 bp apart in both P_R and P_{TPA} promoters) and differences (inverted repeats in the *Thiobacillus* T3.2 MerR-binding site are 2 bp shorter than in *Thiobacillus ferrooxidans*) exist between the O/P intergenic regions of both *Thiobacilli*. In vivo experiments showed inducible expression of mercury resistance in *E. coli* cells transformed with the entire *Thiobacillus* T3.2 *mer* genetic determinant (structural plus regulatory genes), and little or no expression in clones containing only the structural *merT*, *merP*, and *merA* genes.

Key words *mer* operon · Mercury resistance · *Thiobacillus* T3.2 · Acidophilic bacteria

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Introduction

Mercury ion (Hg^{2+}) and organomercurial compounds are widespread in natural environments (Foster 1987). They are highly toxic because of their strong affinity for thiol groups in proteins, and bacterial cells have evolved resistance mechanisms to avoid the toxic effects of those agents (Misra 1992a,b). The core activity of the system is the cytoplasmic mercuric reductase (MerA), which catalyzes the NADPH-dependent reduction of Hg^{2+} ions to mercury (Hg^0), which is volatile and thus less toxic to the cell. The resistance mechanism against Hg^{2+} ions (narrow-spectrum resistance) is completed by a mercury transport system, made up of one (MerC), two (MerT and MerP), or three (MerT, MerP, and MerC) proteins, which deliver mercury ions inside the cell cytoplasm where they are reduced to $Hg^{0.}$ The organomercurial (broad-spectrum) resistance system is mediated by the organomercurial lyase (MerB), which cleaves the organo group from the Hg^{2+} ion.

The genetic determinant of mercury resistance, the *mer* operon, consists of one, two, or three genes (*merT, merP, merC*) encoding the transport proteins and the *merA* gene, which encodes the mercuric reductase (Summers 1986; Silver and Walderhaug 1992; Hamlett et al. 1992). In some operons an additional gene, *merB*, which codes for an organomercurial lyase, is also present (Griffin et al. 1987; Laddaga et al. 1987; Wang et al. 1989; Sedlmeier and Altenbuchner 1992; Reniero et al. 1995). The expression of the *mer* operon is controlled by the product of the regulatory *merR* gene (Lund and Brown 1989; Summers 1992). Some other genes (*merD*, with some sequence similarity to *merR* and thought to be involved in the fine tuning regulation) and ORFs are located next to the structural genes in some *mer* operons (Brown et al. 1986; Mukhopadhyay et al. 1991).

Among gram-negative bacteria, the *mer* operon is most often located in plasmids [$pKLH_2$, from *Acinetobacter*

calcoaceticus (Kholodii et al. 1993a); pMER327, from *Pseudomonas fluorescens* (Hobman et al. 1994); pMERPH, from *Pseudomonas putrefaciens* (Osborn et al. 1996); pDU1358, from *Serratia marcescens* (Griffin et al. 1987); pPB, from *Pseudomonas stutzeri* (Reniero et al. 1995)] and transposons [Tn*21*, from *Shigella flexneri* (Nakaya et al. 1960); Tn*501*, from *Pseudomonas aeruginosa* (Stanisich et al. 1977); Tn*5053*, from *Xanthomonas* spp. (Kholodii et al. 1993b)]. The only mercury resistance genetic determinant reported to date among acidophilic bacteria is that borne on the chromosome of *Thiobacillus ferrooxidans*. It displays some unique features: (i) its Hg^{2+} transport system has only one protein component, MerC, and (ii) its regulatory protein is encoded by two copies of the *merR* gene, which are located more than 6 kb from the structural *merC-merA* genes (Inoue et al. 1989, 1990, 1991; Kusano et al. 1990; Rawlings and Kusano 1994). A third *Thiobacillus ferrooxidans merR* gene has been found in the plasmid pTF-FC2 (Clennel et al. 1995).

The objective of this work was the study of the *mer* genetic determinant of another acidophilic bacteria, *Thiobacillus* T3.2, isolated from an uranium ore pile (Silóniz et al. 1993). This strain contains a mercury resistance operon found in its chromosome, similar to *Thiobacillus ferrooxidans*, although its gene organization is different.

Materials and methods

Bacterial strains and plasmids

Thiobacillus T3.2 was isolated from a uranium mineral heap in Ciudad Rodrigo (Spain) and grown in mineral medium supplemented with 0.5% sulfur as described (Silóniz et al. 1993). *Thiobacillus ferrooxidans* strain ATCC 13,598 was obtained from the American Type Culture Collection (Rockville, MD, USA) and used as a reference strain. *Escherichia coli* strain MC1061 was used in the DNA cloning experiments and *E. coli* strain DH5a in manipulation and in vivo expression experiments. The plasmids used in this study are listed in Table 1. Plasmid pTM314, containing the *merA-merC* genes of *Thiobacillus ferrooxidans*, was kindly provided by T. Shiratori.

DNA and RNA isolation, manipulation, cloning, and analysis

Total DNA from *Thiobacillus* spp. cultures was prepared as reported (Shiratory et al. 1989) and analyzed by pulsed field gel electrophoresis in a Pharmacia (Uppsala, Sweden) LKB hexagonal apparatus. Cellular DNA preparation in agarose plugs was made following the instructions of the manufacturer. Plasmid preparations, DNA manipulations, conventional electrophoresis, and cloning were performed following standard (Sambrook et al. 1989) or supplier-recommended procedures.

Total RNA was isolated from *E. coli* cultures following previously reported methods (Aiba et al. 1981) and analyzed in 1.2% agarose, 6.2% formaldehyde gels (Sambrook et al. 1989). Primer extension experiments were performed with the avian myeloblastosis virus kit (Promega, Madison, WI, USA) and using two 5′-^{32}P-labeled oligonucleotides as primers: 17-mer, 5′-GGCGGCCAGCCCACCAG-3′ (primer TPA), and 17-mer, 5′CCCCGGCTACCTTGGCG-3′ (primer R) (Sambrook et al. 1989). The extended products were ana-

Table 1. Plasmids and constructions

Plasmid	Construction	Reference/source
pUC19	Ap^r	Yanisch-Perron et al. 1985
pALTER1	Tc^r	Promega
pALTPA	Tc^r; pALTER with a 2.376 kb *Hin*dII/*Hin*dIII fragment from pT32.1	This study
pALR	Tc^r; pALTER with a 0.62 kb *Hpa*I/*Eco*RV fragment from pT32.1	This study
pRS550	Ap^r, Km^r	Simons et al. 1987
pRS551	Ap^r, Km^r	Simons et al. 1987
pPRTPA	Ap^r, Km^r; pRS550 with the structural genes promotor (*mer* T, *mer* P, and *mer* A) from *Thiobacillus* T.3.2	This study
pPRR	Ap^r, Km^r; pRS551 with the regulatory gene promoter (*mer* R) from *Thiobacillus* T.3.2	This study
pTM314	Ap^r, Hg^r; structural genes from the *mer* operon of *Thiobacillus ferrooxidans*	Shiratory et al. 1989
pT32.1	Ap^r, Hg^r; pUC19 with a 5.7 kb *Hin*dIII fragment from *Thiobacillus* T3.2 genomic DNA	This study
pTPA	Ap^r, Hg^r; pUC19 with a 2.8 kb *Alu*I/*Hin*dIII fragment from pT32.1	This study

Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Hg^r, mercury ion resistance

lyzed on 8% polyacrylamide sequencing gels together with the DNA sequencing ladder (Sambrook et al. 1989).

Labeling, hybridization, and detection

Nonradioactive DNA labeling was carried out with the Boehringer (Mannheim, Germany) chemiluminescent digoxygenin (DIG) kit. Preparation of radioactive probes with random hexanucleotides was made as reported (Feinberg and Volgestein 1983). 5′-Oligonucleotide radioactive labeling was carried out with the T4 phage polynucleotide kinase (Epicentre Technologies, Madison, WI, USA). Colony, DNA, and RNA blotting, hybridization and detection followed standard methods (Sambrook et al. 1989) or procedures recommended by the supplier.

In vitro gene expression

In vitro transcription experiments were carried out with ϕ10 promoter containing vectors (Table 1) and T7 RNA polymerase (Promega). In vitro translation was made using the Promega "*E. coli* S30 Extract System" following the manufacturer's instructions. Proteins were analyzed by SDS-PAGE (Schägger and Gebhard 1987). β-Galactosidase assays (Miller 1972) on *E. coli* clones containing different plasmids were used to measure the strength of promoters.

Growth curves

Induction of cultures was carried out by inoculating 7.5 μM $HgCl_2$ LB liquid medium and growing overnight at 37°C. Overnight either induced or noninduced cultures were diluted in fresh LB medium to 0.03–0.05 OD units at 600 nm and grown at different concentrations of $HgCl_2$ (0–100 μM). Growth rate of these cultures was spectrophotometrically measured at 600 nm.

DNA sequencing and sequence analysis

Automatic sequencing of cloned DNA was performed on both strands, using a Pharmacia Sequencer. Alternatively, the method of Sanger et al. (1977) was followed, using the T7 polymerase sequencing kit (Pharmacia) and forward and reverse universal synthetic (Boehringer) or homemade (Gene Assembler, Pharmacia) primers. The EMBL data bank accession number for the sequence reported in this paper is Y11706. Searching of sequence homologies was carried out with the FASTA program (Pearson and Lipman 1988) and EMBL database. Studies and comparisons of *mer* nucleotide and amino acid sequences were carried out using the GCG package (Devereux at al. 1984) and Lasergene programs (Dnastar, Madison, WI, USA).

Results and discussion

Identification, cloning, and analysis of *Thiobacillus* T3.2 *mer* region

Cellular DNA from a *Thiobacillus* T3.2 culture was analyzed by pulsed field electrophoresis, blotted, and probed with a *Thiobacillus ferrooxidans* DNA *mer* fragment containing the *merA-merC* genes. The *Thiobacillus* T3.2 chromosomal band gave a strong positive signal (result not shown). The *Thiobacillus* T3.2 *mer* region was isolated from a total DNA library made up in pUC19 vector (see Table 1) and MC1061 *E. coli*. After colony blotting and hybridization with the same *T. ferrooxidans mer* probe, a positive clone with a plasmid (pT32.1, Table 1) carrying a 5.7 kb *Hind*III fragment was isolated and mapped (Fig. 1). Of the *Thiobacillus* T3.2 *mer* region, 4 kb was sequenced (EMBL Nucleotide Sequence Database accession number Y11706) and the results were analyzed using the GCG programs. Four ORFs with sequences very similar to those described for bacterial *mer* genes and proteins were detected (Fig. 1). One of them (ORF 1, 426 bp long) was located in opposite sense to the other three. ORF 2 (348 bp) and ORF 3 (297 bp) were in the same reading frame; ORF 4 (1674 bp) was in a different reading frame and overlapped ORF 3 by 8 nucleotides. On the basis of the homology to reported *mer* sequences, these ORFs were assigned to *merR*, *merT*, *merP*, and *merA* genes, respectively. The intergenic region between *merR* and *merT* is presumed to contain the operator/promoter (O/P) sequences.

In vitro transcription and translation of *Thiobacillus* T3.2 *mer* genetic determinant in *E. coli* systems

Transcription products of an induced culture of *E. coli* cells transformed with the plasmid pT32.1 (carrying *merR*, *merT*, *merP*, and *merA* genes; Table 1) were analyzed by northern blotting and hybridization with either of two different probes: one made from a sequence encompassing most of *merT* and *merP* sequences and the other from the 5′-region of *merA*. Both probes detected a transcript of identical size (result not shown), which supports an operon-like organization for these three genes. As an approach to the definition of the start points of both *merTPA* and *merR* transcripts, primer extension experiments on total RNA isolated from the same induced *E. coli* culture, using TPA and R primers, respectively (described in Methods), were carried out (Fig. 1). Both sites were located in the O/P intergenic region, being both *merTPA* (structural) and *mer R* (regulatory) genes transcribed in opposite senses (Fig. 1). When the TPA primer extension was performed using RNA from a noninduced *E. coli* culture transformed with the same *mer* pT32.1 plasmid only, negative results (not shown) were obtained, which confirmed that the *Thiobacillus* T3.2 *mer* system is inducible in *E. coli* cells. Putative −10 and −35 boxes have been located in both overlapping P_{TPA} and P_R promoters. To search whether these sequences are active as promoters in *E. coli* cells and to compare their relative

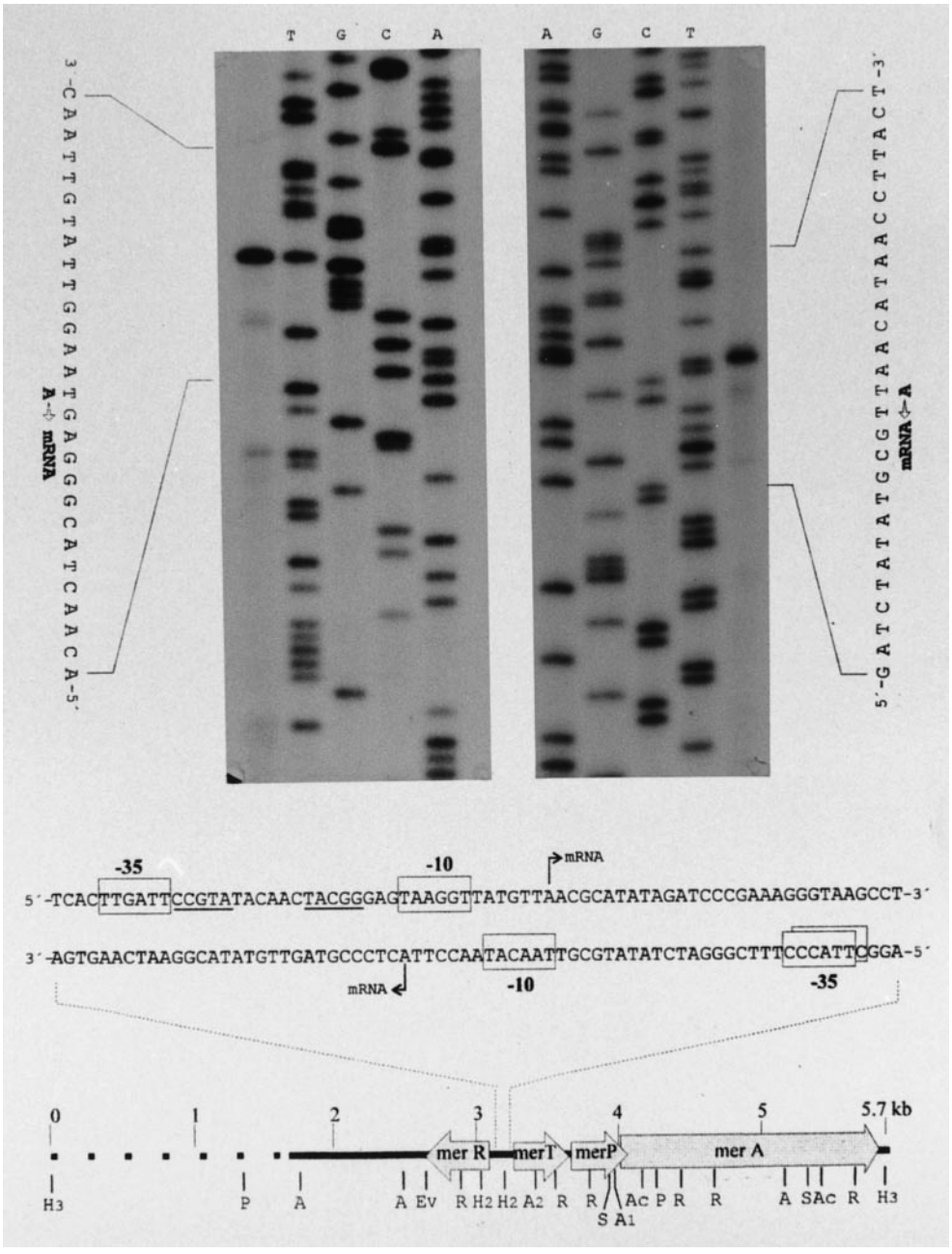

Fig. 1. *Bottom*: Restriction map of the *Thiobacillus* T3.2 chromosomal DNA 5.7 kb *Hin*dIII fragment containing the *mer* region. Enzymes: A, *Acs*I; Ac, *Acc*I; A1, *Ava*I; A2, *Ava*II; EV, *Eco*RV; H2, *Hin*dII; H3, *Hin*dIII; P, *Pst*I; R, *Rsa*I; S, *Sma*I. The sequenced 4 kb segment (*solid bar*) and the four detected ORFs are marked. *Center*: Sequence of the intergenic O/P region: initiation sites (*arrows*), −10 and −35 boxes of both P_R and P_{TPA} promoters, and the 5 pb inverted repeats (*underlined*) are shown. *Top*: Primer extension experiments on total RNA isolated from an induced culture of *E. coli* containing plasmid pT32.1, using R primer (*left*) and TPA primer (*right*)

strength in these cells, the whole intergenic region was cloned, in both senses, in front of the *lacZYA* construction of vectors pRS550 and pRS551 to obtain plasmids pPRTPA and pPRR, respectively (Table 1). The β-galactosidase activity was assayed in cultures of *E. coli* cells with each plasmid (Table 2). This activity was present in both cultures, which confirms that this intergenic sequence contains both promoters; when *lac* transcription was directed by the P_R promoter, the analysis gave a value more than ten times higher than that obtained with the P_{TPA} promoter.

The proteins coded by different sequences of the *Thiobacillus* T3.2 *mer* region were produced in vitro using the pALTER-1 cloning vector (Table 1) and the *E. coli* S30 translation extract. The in vitro expression of the structural *merTPA* genes (in pALTPA plasmid; Table 1) produced three polypeptides of 11, 13, and more than 40 kDa (Fig. 2), which could be provisionally assigned on the basis of their sizes to *MerP*, *MerT*, and *MerA* proteins, respectively. An additional band of 8 kDa was also observed; its occurrence is perhap the result of some post-translation processing acting on one of the other three polypeptides (see following). The in vitro expression of the regulatory *merR* (in pALR plasmid; Table 1) yielded a 16 kDa polypeptide (Fig. 2), in very good accordance with the gene size. To confirm

Table 2. Promotor activity of P_{TPA} and P_R in *E. coli* cells

Plasmid	β-Galactosidase activity (Miller units)
pPRTPA (pRS550 with P_{TPA} in front of *lacZ*)	1154
pRS550 (no insert)	21
pPRR (pRS551 with P_R in front of *lacZ*)	12301
pRS551 (no insert)	13

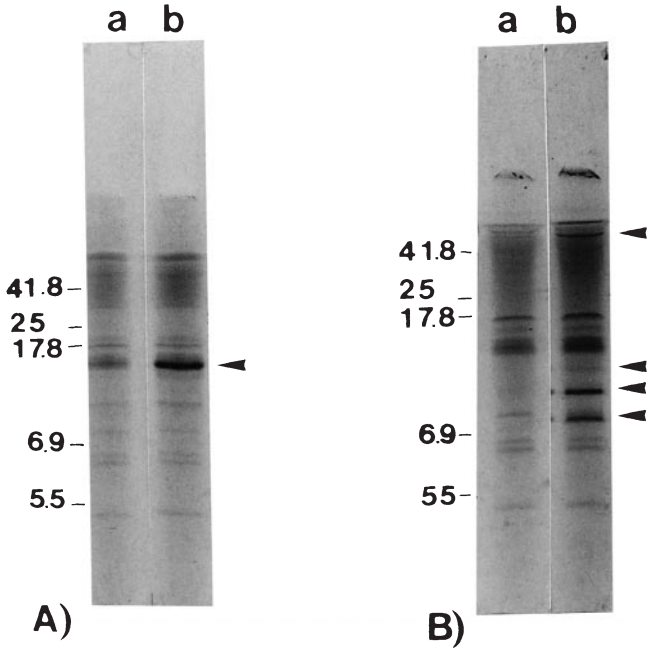

Fig. 2A,B. SDS-PAGE of in vitro synthesized *Thiobacillus* T3.2 Mer proteins from (**A**, *lane b*) *merR* gene cloned in pALR plasmid and (**B**, *lane b*) *merTPA* genes cloned in pALTPA plasmid. Lanes *a* designated are the background proteins. Mer polypeptides are marked with *arrows*. Size marks are in kDa

the origin of all these proteins, several truncations were made on the original sequences cloned in pALTPA and pALR. In vitro expression of the polypeptides encoded by these truncated constructions confirmed previous assignations (results not shown). The 8kDa band only appeared when *merP* was intact but not when the TPA region was truncated either in *merT* or in *merP*, which supports the view of a primary 11kDa MerP polypeptide that is processed to yield a 8kDa mature MerP, as previously reported for other homologous proteins (Sahlman and Jonsson 1992). All these experiments were carried out in *E. coli* cells or in extracts in which the *Thiobacillus* T3.2 *mer* operon may work in a different way than in *Thiobacillus* cells; although only quantitative differences may be expected, results have to be taken cautiously.

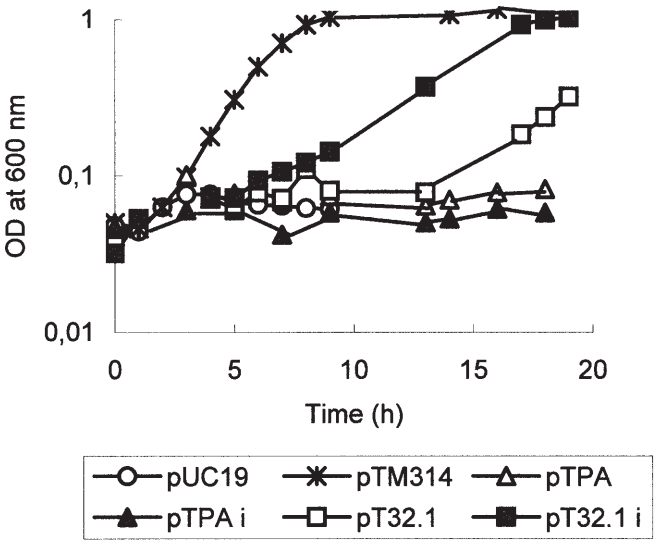

Fig. 3. Growth curves of cultures of *E. coli* clones growing in 20μM Hg^{2+} and containing various plasmids (see Table 1). *i*, induced cultures

In vivo expression of *Thiobacillus* T3.2 *mer* system in *E. coli*

Functional assays have been carried out with several *E. coli* clones containing plasmids with different *mer* constructions (Table 1), namely pT32.1 (with the whole *mer* region), pTPA (with only the structural genes), pTM314 (with the *T. ferrooxidans mer* region), and pUC19 used as a negative control. Growth curves of these cultures, both induced and noninduced, were made at different mercury concentrations. All the cultures grew at both 0 and 5μM Hg^{2+} (results not shown); pTM314 was the only active culture at 40μM Hg^{2+} or higher (not shown); at 20μM Hg^{2+}, differences among cultures were apparent (Fig. 3). From these curves several facts can be inferred: first, the *Thiobacillus* T3.2 *mer* region is a Mer^R determinant, which confers inducible Hg resistance to *E. coli* cells; second, the presence of *merR* is needed both to increase the resistance to higher than basal levels and to show the ability to be induced by Hg^{2+}, which suggests an inducible activator role for this protein. Mercury resistance of pTM314 is constitutively expressed in *E. coli* (Fig. 3) (Inoue et al. 1990); the very different behavior of the pTPA clone (with *merT*, *merP*, and *merA* genes) can be explained on the basis of either the promoter of the structural genes, the different Hg^{2+} transport system (MerC in *T. ferrooxidans* and MerT and MerP in *Thiobacillus* T3.2), or the core activity of the reductase, MerA protein.

The *Thiobacillus* T3.2 *mer* operon

MerT

The three transmembrane regions occurring in all the reported MerT proteins from gram-negative bacteria are also

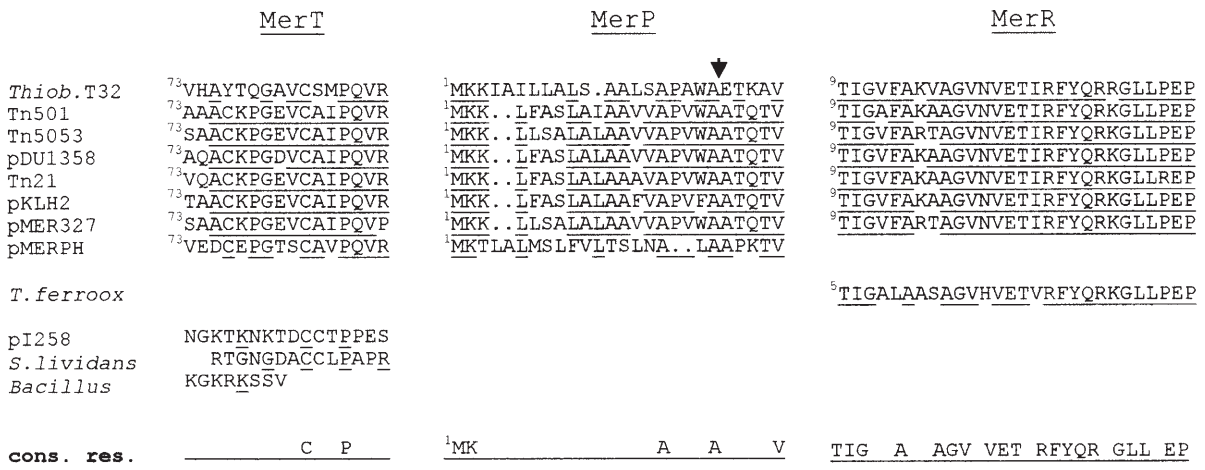

Fig. 4. Homologous animo acid sequences in some regions of MerT, MerP, and MerR proteins from several *mer* systems. Amino acid residues that are conserved in the Tn*501*/Tn*21* group are *underlined*; those which are strictly conserved in all known Mer proteins are shown at the *bottom*. The processing cut site of MerP is marked. Data bank access number or reference of sequences are as follows. **MerT**: Tn*21*, #P04336; $pKLH_2$, translated protein from L04303; pMERPH, #Z49196; Tn*5053*, translated protein from L40585; pMER327, translated protein from X73112; Tn*501*, #P04140; *Thiobacillus* T3.2 (this study); pDU1358, #P13112; pI258, Laddaga et al. (1987); *Bacillus* RC607, Wang et al. (1989); *Streptomyces lividans*, #P30345. **MerP**: Tn*21*, #P04129; $pKLH_2$, translated protein from L04303; pMERPH, #Z49196; Tn*5053*, translated protein from L40585; pMER327, translated protein from X73112; Tn*501*, #P04131; *Thiobacillus* T3.2 (this study); pDU1358, #P13113. **MerR**: Tn*21*, #P07044; $pKLH_2$, translated protein from Z33482; Tn*5053*, translated protein from L03729; pMER327, translated protein from X73112; Tn*501*, #P06688; *Thiobacillus* T3.2 (this study); pDU1358, #P13111; *Thiobacillus ferrooxidans*, translated protein from X57326; pTF-FC2, Clennel et al. (1995)

present in *Thiobacillus* T3.2 MerT. The strictly conserved Cys residue pair, located in the first, N-terminal transmembrane region (^{24}Cys and ^{25}Cys in Tn*21* MerT) and involved in Hg^{2+} binding and transport (Morby et al. 1995), is also present in *Thiobacillus* T3.2 MerT protein. However, the second Cys pair (^{76}Cys and ^{82}Cys in Tn*21* MerT), which occurs between the second and the third transmembrane regions of all the gram-negative bacterial system MerT proteins reported to date, is not so conserved: *Thiobacillus* T3.2 MerT is unique in lacking the first Cys residue of this pair, which is substituted by a Tyr, although it conserves the second Cys (Fig. 4). Both Cys residues seem to be located in the cytoplasm, but its actual role is not completely understood. Directed mutagenesis experiment results suggest this Cys pair to be involved in Hg^{2+} transport across the cytoplasmic membrane, although in a nonessential way because its absence does not cancel ion transport into the cell (Morby et al. 1995). Interestingly, gram-positive bacteria also contain a Cys pair in this region although in different positions: the first one appears in the homologous position of the second Cys residue (^{82}Cys) of Tn*21* MerT protein, which makes this Cys another strictly conserved residue; the second Cys of this pair occurs in the adjacent position, where all the gram-negative MerT proteins contain a conserved ^{83}Ala, with the noteworthy exception of *Thiobacillus* T3.2 MerT, which contains a Ser (Fig. 4). The case of *Thiobacillus* T3.2 MerT is interesting in having a gram-negative wall and a unique pattern of Cys residues.

MerP

Thiobacillus T3.2 MerP is 98 amino acid residues (10.8 kDa) before processing. It is longer than homologous proteins because of the presence of six additional C-terminal residues (GERDHG). It contains the two Cys residues strictly conserved among all the MerP homologous proteins. However, in the N-terminal region some interesting differences are noticed (Fig. 4): *Thiobacillus* T3.2 MerP contains two residues (^{4}Ile and ^{5}Ala) that are absent in all the other reported MerP proteins except in pMERPH MerP (where they are ^{4}Leu and ^{5}Ala); also, the *Thiobacillus* T3.2 MerP protein lacks the ^{10}Leu residue of MerP of Tn*21;* other potentially significant changes are the serines that substitute for hydrophobic highly conserved residues (^{9}Ala, ^{14}Val) and the high polar Glu and Lys, which substitute for well-conserved ^{20}Ala and ^{22}Gln, respectively. All the reported MerP proteins contain a 19 amino-acid N-terminal leader sequence that acts as a signal peptide and is removed during posttranslation processing (Renu et al. 1994). The above-mentioned sequence differences are found in this region (Fig. 4): three changes in the middle part, which clearly decrease the hydrophobicity of this area, and some other changes in the extremely conserved recognition-scission sequence nearby, which could be involved in a peculiar processing signal.

MerA

The molecular weight (58.7 kDa) and length (558 amino acid residues) of MerA of *Thiobacillus* T3.2 are very similar to those of most MerA proteins from gram-negative bacteria (550–560 amino acid residues). All three Cys pairs that occur in all known bacterial mercuric reductases and which are essential in the reduction mechanism (Moore and Walsh 1989; Silver and Walderhaug 1992) are also present in *Thiobacillus* T3.2 MerA. In this protein there are another

five additional Cys residues: two of them are conserved with respect to Tn*21* and Tn*501* MerA proteins, another two are conserved with respect to the pMERPH reductase, and the fifth Cys residue occurs only in *Thiobacillus* T3.2 MerA. The sequences of the Hg^{2+} binding site, the putative FAD binding site, and the C-terminal region are highly homologous to reported MerA sequences. The active center (TIGGTCVNVGCVPSK) is identical to all gram-negative bacterial systems except that of *T. ferrooxidans*, which shows a change (I instead of the second V) (Inoue et al. 1989).

MerR

The regulatory MerR protein of all the *mer* systems reported to date contain three Cys residues, which are involved in the binding of Hg^{2+} ions and in the transcription activation mechanism through interaction with the RNA polymerase (Misra 1992a); these three Cys residues are present in MerR of *Thiobacillus* T3.2. However, in this protein there are another three Cys residues: one of them, located in position 114, also exists in many other homologous MerR proteins (not in those reported from *T. ferrooxidans*); the other two Cys residues, located in the C-terminal region (..KN**C**AE**C**PGD-OH), are unique for *Thiobacillus* T3.2 MerR. The putative RNA polymerase-binding domain (Summers 1992) is very well conserved in *Thiobacillus* T3.2 MerR. Furthermore, the "helix-turn-helix" motif that appears in the N-terminal region of these proteins has also been detected in this new MerR protein, which only diverges in two conservative changes in this region with respect to the wide subgroup that includes Tn*21* and Tn*501* MerR proteins (Silver and Walderhaug 1992). Interestingly, it shows more differences with respect to *T. ferrooxidans* MerR proteins: namely, seven, not all conservative changes (Fig. 4). Given that this domain seems to be a DNA binding site, these changes may reflect significant differences between the binding pattern of both MerR proteins. Finally, the C-terminal amino acid sequence of *Thiobacillus* T3.2 MerR does not contain the organomercurial sensing region that is found in MerRs whose *mer* operons include the *merB* gene, and also in those of Tn*5053* and pMER419, whose *mer* operons lack *merB*.

Operator/promoter region

The operator/promoter region is located in the intercistronic region between the structural *TPA* genes and the regulatory *R* gene (see Fig. 1). The −10 and −35 boxes of the promoter of the structural genes (P_{TPA}) are 19 bp apart, in accordance with most of the reported gram-negative *mer* operons. The distance between these boxes could be important in the mechanism of induction/repression of the *mer* system (Parkhill and Brown 1990), and a model has been proposed to explain this fact (Ansari et al. 1995). In the same intergenic region, 5 bp inverted repeats have been found that overlap P_{TPA} and are thought to be the binding site of the MerR regulatory protein, in a quite similar way to other *mer* operons. Also in this region but in the opposite sense there is the promoter of *merR* (P_R) where two putative −35 boxes, 19 and 20 bp from the −10 box, respectively, can be identified. In the P_R of the *mer* system of Tn*501* and Tn*21*, two −35 boxes, 15 and 19 bp far from the −10 box, respectively, have been found (Summers 1992), and it has been proposed that it is the first one that is functionally active in vivo. No −35 box has been found so close to the −10 module in the *Thiobacillus* T3.2 P_R.

In any event, this promoter displays a higher activity (×10) than P_{TPA} as shown by the in vitro experiments; this is in accordance to previously reported data from the Tn*21* *mer* operon (Summers 1992) and supports the model of a moderate, autoregulated expression of *merR*, based on a fairly good promoter and an inhibitory role of MerR protein, and a low but strongly inducible expression of the structural genes, which results from a poor promoter and a regulatory activator role of MerR protein. The results of northern and primer extension analysis, where *TPA* mRNA is only detected in induced pT32.1-containing *E. coli* cultures, reinforces this interpretation. All these structural features underline the similarities between the regulation system of *Thiobacillus* T3.2 *mer* determinant and those of the core group of gram-negative bacteria. On the other hand, there are differences with the *T. ferrooxidans mer* system: the inverted repeats of *Thiobacillus* T3.2 P_{TPA} are 2 bp shorter than those found in the *T. ferrooxidans* promoter. This difference, put together with those detected between the N-terminal sequences of both MerR proteins, point to a possibly different interaction pattern in the regulation mechanism of the *mer* system in both *Thiobacilli*.

Evolutionary relationships

On the basis of the encoded transport proteins, three groups can be made from the *mer* systems reported to date: one group, represented by the Tn*21* determinant, which codes for all three proteins (MerT, MerP, and MerC); a second group that only code for MerT and MerP proteins, in which Tn*501* is included; and a third, more simple operon, which only codes for MerC protein, whose unique reported representative is the *T. ferrooxidans* Hg^R determinant. The *mer* system of *Thiobacillus* T3.2 has to be located in the same subgroup as the Tn*501* *mer* operon. Recently, the diversity of bacterial *mer* operons has been reviewed and a hypothesis of *mer* operon evolution has been presented (Osborn et al. 1997). The absence of a *merB* gene locates the *Thiobacillus* T3.2 *mer* operon among the narrow-spectrum resistance systems. Furthermore, no organomercurial sensing structure is present in the C-terminal amino acid sequence of the T3.2 MerR, which reinforces a recent divergent evolution of Tn*501* and *Thiobacillus* T3.2 *mer* determinants. Although no transposon-related sequences have been detected to date in close proximity to the T3.2 *mer* region, the possibility of horizontal transfer cannot be excluded. However, some structural details of the transport MerT and MerP proteins may reflect a somewhat divergent

path followed by the *Thiobacillus* T3.2 *mer* system in its adaptation to the acidic media in which this bacterium lives. Differences with respect to the *Thiobacillus ferrooxidans mer* operon, namely the transport protein system and the physical separation of *merA* and *merR*, should be underlined. These two bacterial species share a very acidic habitat and a growth mineral (sulfide) substrate; however, their mercury resistance genetic systems are very different, which poses intriguing and unsolved questions about their origin and evolution.

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