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Cloning and Expression of *Thiobacillus ferrooxidans* Mercury Ion Resistance Genes in *Escherichia coli*

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A search of various domestic isolates of *Thiobacillus ferrooxidans* revealed that some were fairly resistant to mercury ion. A proportion of mercury-resistant clones were able to volatilize mercury, and their corresponding gene was localized not in the plasmid DNA but in chromosomal DNA. This mercury ion resistance gene was cloned in *Escherichia coli*. *E. coli* carrying the recombinant plasmid was able to grow in the presence of more than 40 μg of $HgCl_2$ per ml. Deletion analysis of the recombinant plasmid showed that the entire coding sequence of the mercury ion resistance gene was located within a 2.3-kilobase fragment of the chromosomal DNA from strain E-15. At least two polypeptides (molecular mass, 56 and 16 kDa, respectively) were coded by this fragment.

Thiobacillus ferrooxidans is an acidophilic chemoautotroph that utilizes energy generated by the oxidation of inorganic ferrous ion to ferric ion. This organism has been a focus of interest because of its industrial utility in so-called bacterial leaching. However, the slow growth of this organism has limited its further use. To fully exploit the properties of this bacterium by molecular breeding, an understanding of both its gene structure and its expression is essential. However, knowledge about the genetic background of this organism is scarce. Recently, Rawlings and co-workers (24, 25) reported that the *oriV*, *oriT*, and *mob* functions of plasmids from *T. ferrooxidans* are expressed in *Escherichia coli*. Furthermore, this group (1, 20, 22) has cloned and determined the nucleotide sequences of the glutamine synthetase gene and nitrogenase iron protein gene from *T. ferrooxidans*. The *Thiobacillus glnA* gene is able to complement the *E. coli glnA* strain (1). These findings indicated that genetic information from *T. ferrooxidans* is functional in the heterotroph *E. coli*.

It has been reported that *T. ferrooxidans* is quite resistant to iron, acidity, and some heavy metals (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, etc.) but exceedingly sensitive to uranium, silver, and mercury ions (26). So far, two groups (3, 19) have reported the presence of the enzyme mercuric reductase in mercury ion-resistant *T. ferrooxidans* strains.

In the present study, we showed that (i) the mercury ion resistance gene of *T. ferrooxidans* is present in the chromosomal DNA but not in plasmid DNAs, (ii) it was possible to clone the corresponding gene into *E. coli*, (iii) this gene was expressed in *E. coli*, and (iv) two polypeptides were coded by the minimum essential region.

MATERIALS AND METHODS

Bacterial strains and media. Independent colonies of *T. ferrooxidans* used in this study were isolated from various domestic mining sites. *T. ferrooxidans* was identified taxonomically by comparison with the physiological characteristics listed in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (31). *T. ferrooxidans* was enriched in the 9K medium of Silverman and Lundgren (28) and purified on colloidal silica plates (M. Kawarazaki, personal communication). Each silica plate was composed of 900 ml of colloidal silica no. 30 (Nissan Kagaku Co., Ltd., Tokyo, Japan), 70 ml of 10-fold 9K basal salts, and 30 ml of saturated $FeSO_4 \cdot 7H_2O$ solution. Each solution was separately autoclaved, and then mixed and adjusted to pH 3.6 with sulfuric acid. A 20-ml portion was poured into each petri dish and incubated at 60°C for 16 h. After the incubation, the pH of each silica plate became 2.8. A purified *T. ferrooxidans* clone was grown with vigorous shaking in inorganic 9K medium at 30°C.

MIC of $HgCl_2$. *T. ferrooxidans* clones grown in 9K medium were streaked onto $HgCl_2$-containing colloidal silica plates. For *E. coli* transformants, the MIC was determined by the serial dilution method in LB broth under aerobic conditions. Growth of *E. coli* DH5α or *E. coli* DH5α carrying pUC18 was totally inhibited by a concentration of 5 μg of $HgCl_2$ per ml of broth.

Assay of Hg-dependent NADPH oxidation. Crude extracts of *T. ferrooxidans* isolates were prepared at 4°C as follows. Cells in 150 ml of full-growth culture in the presence of 0.5 μg of $HgCl_2$ per ml were harvested by centrifugation at 8,000 rpm for 20 min (Hitachi RPR20-2 rotor). Harvested cells were washed twice with low-pH solution (9K basal salts containing 0.16 M $MgSO_4$, pH 1.9) to remove insolubles [mainly $Fe(OH)SO_4$], then with high-pH solution (25 mM phosphate buffer, 0.3 M sucrose, 10 mM EDTA, pH 8.0) once, followed by 50 mM phosphate buffer (pH 7.4) once. The cell pellets were resuspended in 1 ml of 50 mM phosphate buffer (pH 7.4) and disrupted with a Branson sonifier (setting 3, 20-s intervals, three times) on ice. Debris were removed by microcentrifugation at 8,000 × *g* for 15 min. Supernatants were used as crude extracts. Assay solutions in a total volume of 100 μl contained 50 mM K_2HPO_4-NaH_2PO_4 (pH 7.4) buffer, 0.5 mM EDTA, 0.2 mM $MgSO_4$, 1 mM β-mercaptoethanol, 0.2 mM NADPH, 0.5 mg of bovine serum albumin per ml, and 0.1 mM $HgCl_2$ with or without cell extract (5 μg of protein). Amounts of protein in extracts were measured by the method of Lowry et al. (14). Mixtures were kept at 37°C, and the absorbance was moni-

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tored at 340 nm, this value representing the maximum absorption of reduced NADP.

Preparation of chromosomal DNA or plasmid DNA from *T. ferrooxidans*. *T. ferrooxidans* cells (250-ml culture) were harvested at the stationary phase and washed with low-pH solution (see above) twice. After spinning, the cell pellets were washed with high-pH solution twice as described above. Cells suspended in 4 ml of 25 mM Tris hydrochloride (pH 8.0)–50 mM glucose–10 mM EDTA containing 10 mg of lysozyme per ml were incubated for 10 min at room temperature, followed by the addition of 0.5 ml of 0.25 M EDTA for 10 min on ice. After complete lysis by the addition of 0.5 ml of 10% sodium dodecyl sulfate (SDS), 50 μl of 20-mg/ml proteinase K was added and the mixture was kept at 37°C for 1 h. The homogenate was deproteinized with an equal volume of phenol-chloroform mixture (1:1, vol/vol) three times. The aqueous phase was recovered and precipitated with ethanol. This chromosomal DNA fraction was further purified by cesium chloride-ethidium bromide ultracentrifugation (Hitachi Hi-MAC, CP120H).

T. ferrooxidans plasmids were prepared by the modified alkaline lysis method of Birnboim and Doly (2) or by the procedure of Kado and Liu (12).

Source of enzymes. Restriction enzymes, the Klenow fragment of DNA polymerase I, exonuclease III, mung bean nuclease, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from Takara Shuzo Co. Ltd., Toyobo Co. Ltd., or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used according to the instructions of the suppliers.

Transformation. *E. coli* DH5α was used as the transformation host. Competent cells were prepared by the procedure of Hanahan (10). Efficiency of transformation was usually 10^7/μg of pUC18 DNA.

Agarose gel electrophoresis. The DNAs were resolved by 0.7% agarose gel electrophoresis in TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8). The gels were strained in 0.2 μg of ethidium bromide (Aldrich Chemical Co., Milwaukee, Wis.) per ml, and the patterns were photographed with a Polaroid MP-4 Land camera with a red filter under UV light (302 nm).

Nick translation. For nick translation (15), the Tn*501* mercury resistance determinant (*mer*) fragment originated from pME285 (11). The *mer* fragment (4.5 kilobases [kb], from the *Ava*I to the *Hin*dIII site of pME285) was blunted with Klenow fragment, tagged with *Bam*HI linker (Takara Shuzo Co.), and then subcloned in pBR322 (termed pM610). The 4.5-kb fragment was cut out from pM610 and recovered by electroelution with a dialysis tube and then nicktranslated; the mixture of 0.1 to 0.5 μg of fragment DNA, 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass., or Amersham Corp., Arlington Heights, Ill.), 1 ng of DNase I per ml, 20 μM each dATP, dTTP, and dGTP, and *E. coli* DNA polymerase I (10 U) was incubated at 20°C for 2 h. Unincorporated [^{32}P]dCTP was eliminated with a Sephadex G-50 minicolumn (1.0 by 10 cm).

Hybridization. Dot and Southern blottings were done with nylon filters (Biodyne A). In the Southern method, DNAs were electroblotted onto the filter. Hybridization (15) was done in the presence of 10% dextran sulfate (30). Each filter was finally washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS at 65°C. Colony hybridizations were performed with Biodyne A nylon membranes (BNNG82) by the protocol of Grunstein and Hogness (9).

Cloning of mercury resistance gene from *T. ferrooxidans* and construction of deletion plasmids. The chromosomal DNA of *T. ferrooxidans* E-15 was partially digested with *Sal*I and separated on a 0.7% agarose preparative gel. The fragments corresponding to the areas hybridized with the Tn*501 mer* probe were recovered by Gene-Clean (Bio 101 Co., Ltd.). Recovered fragments were ligated with *Sal*I-cleaved pUC18 after treatment with calf intestinal alkaline phosphatase. Competent *E. coli* DH5α was transformed with this ligated sample.

Samples of 2 μg of the resulting plasmids, pTM314 and pTM315 (see Results), were digested with *Xba*I and *Sac*I. The linearized fragment was digested with exonuclease III at 60-s intervals for 20 min. Digested mixtures were blunt ended with mung bean nuclease, followed by Klenow fragment to ensure complete blunting. Ligation with T4 DNA ligase was done at 16°C for 16 h. The ligated sample was cleaved with *Xba*I and then transformed in *E. coli* DH5α. About 200 clones each were analyzed. A series of plasmids with deletions of every ca. 300 base pairs were selected. Sixteen deletion plasmids originating from pTM314 were named, in order, pTM401 to pTM416. For pTM315, 15 deletion plasmids were named pTM501 to pTM515.

Detection of bacterial volatilization of mercuric chloride. A qualitative method for the detection of mercury volatilization activity without using ^{203}Hg was recently established by Nakamura and Nakahara (18). In brief, *E. coli* DH5α harboring pTM314 or its derivatives was cultured at 37°C overnight in Luria broth containing 2.5 μg of $HgCl_2$ per ml unless otherwise stated. Each culture was streaked onto a Luria agar plate containing 1 μg of $HgCl_2$ per ml and then incubated at 37°C overnight. Next day, each cell mass was collected with a toothpick and resuspended in reaction mixture in 1 well of a 96-well microdilution plate. The reaction mixture consisted of 1/15 M phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.2 mM magnesium acetate, 5 mM sodium thioglycolate, and 20 to 40 μg of $HgCl_2$ per ml in a total volume of 50 μl. Immediately afterward, an X-ray film (Kodak X-OMAT AR) and acrylic plate were sequentially mounted over each microdilution plate in a dark room, and both ends were fixed with clips. The plate was then put into a dark box and incubated for 60 min at 37°C. After the incubation, the X-ray film was developed. The fogged areas on the film were the result of reduction of the Ag^+ emulsion by mercury vapor.

Maxicell labeling of plasmid-coded polypeptides. For maxicell labeling (27), plasmids pTM314, pTM315, pTM351, pTM352, pTM364, pTM371, and pTM362 were isolated from *E. coli* DH1 *hsdR17* (r_K^- m_K^+) and used to transform *E. coli* CSR603. *E. coli* CSR603 transformants carrying pTM314 or its derivatives were incubated aerobically at 37°C in K medium (M9 medium supplemented with 1% Casamino Acids [Difco Laboratories, Detroit, Mich.] and 0.1 μg of thiamine per ml). After UV irradiation (50 J/m^2) for 45 s, cells were treated with D-cycloserine (150 μg/ml). Then maxicell proteins were labeled with [^{35}S]methionine (1,000 Ci/mmol; Dupont) according to the original protocol (27). Gel electrophoresis was performed by the method of Laemmli (13), and sodium salicylate was used for detection of the ^{35}S-labeled polypeptide (6).

RESULTS

Presence of mercury ion resistance gene in chromosomal DNA of *T. ferrooxidans*. In the process of establishing the present host-vector system, we tested 10 domestic isolates of *T. ferrooxidans* for sensitivity to mercuric chloride

TABLE 1. MIC of $HgCl_2$ for and mercury-dependent NADPH oxidation activity of 10 *T. ferrooxidans* isolates[a]

Strain	MIC of $HgCl_2$ (μg/ml)	Change in A_{340} over 20 min[b]
B-12	0.2	NT[c]
B-19	0.2	NT
E-6	1.0	0.97
E-7	0.75	1.35
E-9	0.3	NT
E-15	0.75	1.02
E-24	1.5	0.12
M4-6	0.2	NT
U4-25	1.0	0.07
Y5-9	0.2	NT

[a] Ten *T. ferrooxidans* isolates grown in 9K medium at 30°C were streaked over colloidal silica 9K plates containing 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, and 1.5 μg of $HgCl_2$ per ml. MICs were determined after 4 to 5 days of incubation at 30°C.

[b] Initial $A_{340} - A_{340}$ after 20 min of incubation at 37°C.

[c] NT, Not tested.

($HgCl_2$). Five strains (B-12, B-19, E-9, Y5-9, and M4-6) were rather sensitive, and the remaining five strains (E-6, E-7, E-15, E-24 and U4-25) were resistant to more than 0.5 μg of $HgCl_2$ per ml (Table 1). The presence of mercuric reductase has been reported in acidophilic *T. ferrooxidans* (3, 19). To clarify the mode of resistance of our strains against mercury, we detected mercury-dependent NADPH oxidation activity. Three clones (E-6, E-7, and E-15) of five oxidized NADPH in a mercury-dependent manner (Table 1). When mercury chloride (20 μg/ml) was added to an assay mixture with the cell extracts from the three clones, A_{340} (λ_{max} of reduced NADP) was decreased significantly. In the absence of mercury chloride, A_{340} was not changed even after 60 min of incubation. Half of the reaction mixture in which $HgCl_2$ had been omitted was removed, mixed with 20 μg of $HgCl_2$ per ml, and incubated at 37°C. The remaining half of the reaction mixture was allowed to continue as before. NADPH oxidation was only observed in the half of the reaction mixture to which $HgCl_2$ had been added (data not shown). The activity required the sulfhydryl group as a cofactor and was stimulated by the addition of EDTA (data not shown). These characteristics were the same as those previously reported (19). The activity was diminished by heat treatment (100°C, 5 min) of cell extracts (data not shown). On the other hand, no significant amount of NADPH oxidation was detected in the other two strains (E-24 and U4-25, Table 1). It was thus apparent that resistance to $HgCl_2$ was due to the mode of enzymatic action in isolates E-6, E-7, and E-15, whereas another mechanism (for example, transport barrier or efflux) might be responsible in strains E-24 and U4-25. No plasmids were found in the three former clones even when plasmids were prepared by the alkaline-SDS method (2) or by the method of Kado and Liu (12) for possibly large plasmids. Therefore, the mercury volatilization gene was possibly localized in the chromosomal DNA.

To verify this, we performed dot-blot hybridization. The chromosomal DNA was prepared from the 10 isolates and hybridized with a ^{32}P-labeled 4.5-kb transposon Tn*501 mer* fragment as a probe. From the consistency of the results in right column of Table 1, again three chromosomal DNAs (E-6, E-7, and E-15) appeared to hybridize with the *mer* probe under stringent washing conditions (0.1× SSC, 65°C) (Fig. 1). In addition, more than 50 plasmids from 29 other *T. ferrooxidans* isolates including E-24 and U4-25 were tested for hybridization with the Tn*501 mer* probe, and no hybridization was observed (data not shown). From the above evidence, it was concluded that at least one group of mercury ion-resistant *T. ferrooxidans* cells volatilized mercury and that the coding gene responsible was located in the chromosomal DNA.

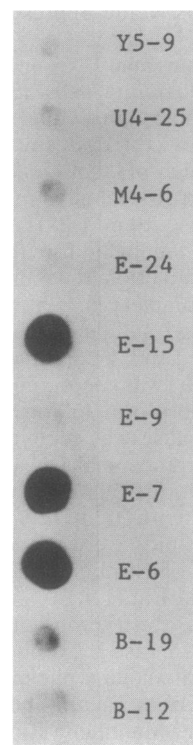

FIG. 1. Dot-blot hybridization of *T. ferrooxidans* chromosomal DNAs with the transposon Tn*501 mer* probe. A 5-μg sample of each chromosomal DNA was blotted onto Biodyne A nylon membrane (Pall Co. Ltd.). The filters were denatured, neutralized, and then baked at 80°C for 1 h. After prehybridization, the ^{32}P-labeled *mer* probe (originating from pME285) was added in the presence of 10% dextran sulfate and incubated at 65°C for 20 h. Filters were washed in 0.1× SSC–0.5% SDS at 65°C. Autoradiographs were taken with an intensifying screen (Du Pont) at −80°C.

Cloning and expression of *T. ferrooxidans* mercury ion resistance gene in *E. coli*. Three chromosomal DNAs, E-6, E-7, and E-15, were digested with several restriction enzymes. The digests were separated on a 0.7% agarose gel as described above and blotted onto nylon membranes. Each filter was hybridized with the Tn*501 mer* fragment. In a double-digestion experiment with *Hin*dIII and *Eco*RI, a 2.1-kb fragment hybridized with the probe in all three cases. In E-6 and E-7, however, an additional 6.5-kb fragment was hybridized (Fig. 2). After *Sal*I digestion, a 4.6-kb fragment in all three clones, in addition to the fragment of greater than 20 kb in E-6 and E-7, was hybridized (Fig. 2). There was thus a possibility that two clones, E-6 and E-7, contained two copies of the mercury ion resistance gene. Therefore, the mercury ion resistance gene was cloned by using chromosomal DNA of the E-15 isolate as a starting source. Direct selection of mercury-resistant transformants on $HgCl_2$-containing agar plates was not successful even after several trials. Therefore, colony hybridization was performed with a radioactive probe. Among ca. 1,000 white colonies, two positive colonies were obtained. The resulting plasmids were termed pTM314 and pTM315, respectively. Both contained

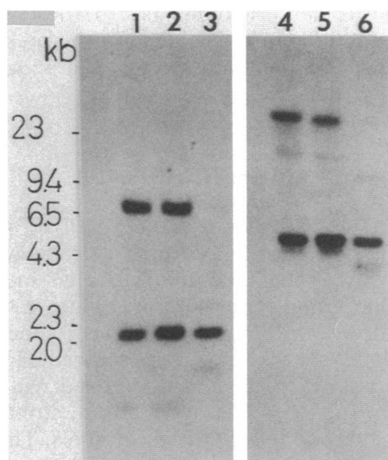

FIG. 2. Southern hybridization of *T. ferrooxidans* chromosomal DNAs with the Tn*501 mer* probe. A 5-μg sample of each chromosomal DNA from strains E-6 (lanes 1 and 4), E-7 (lanes 2 and 5), and E-15 (lanes 3 and 6) was digested with *Hin*dIII and *Eco*RI (lanes 1 to 3) or *Sal*I (lanes 4 to 6). Hybridization was done as described in the legend to Fig. 2.

exactly the same insert fragment (4.6 kb), although it was positioned in a reverse orientation relative to the vector. *E. coli* DH5α harboring either of these plasmids was able to grow in Luria broth containing more than 40 μg of $HgCl_2$ per ml and could also volatilize mercury (see below). This result suggested that expression of the *T. ferrooxidans* mercury ion resistance gene in *E. coli* took place from a *T. ferrooxidans* promoter(s).

Localization of *T. ferrooxidans mer* gene. Inserts (4.6 kb) of plasmids pTM314 and pTM315 each had one *Bam*HI, one *Pst*I, and one *Sma*I cleavage site and three *Hin*dIII sites. By partial or double digestion of these inserts, relative cutting sites were determined (Fig. 3). Several deletion plasmids were constructed to determine the minimum functional region. Resistance to $HgCl_2$ was fully retained with pTM352 and pTM351, significantly lost with pTM364 and pTM371, and totally lost with the other plasmids.

A qualitative method for detecting mercury volatilization activity without the use of ^{203}Hg has been developed by Nakamura and Nakahara (18). Using this method, we determined mercury volatilization activity. Activity was detected in *E. coli* transformed with the following plasmids: pTM314, pTM315, pTM352, pTM351, pTM364, and pTM371 (Fig. 3). The reason for the partial loss of mercury resistance with

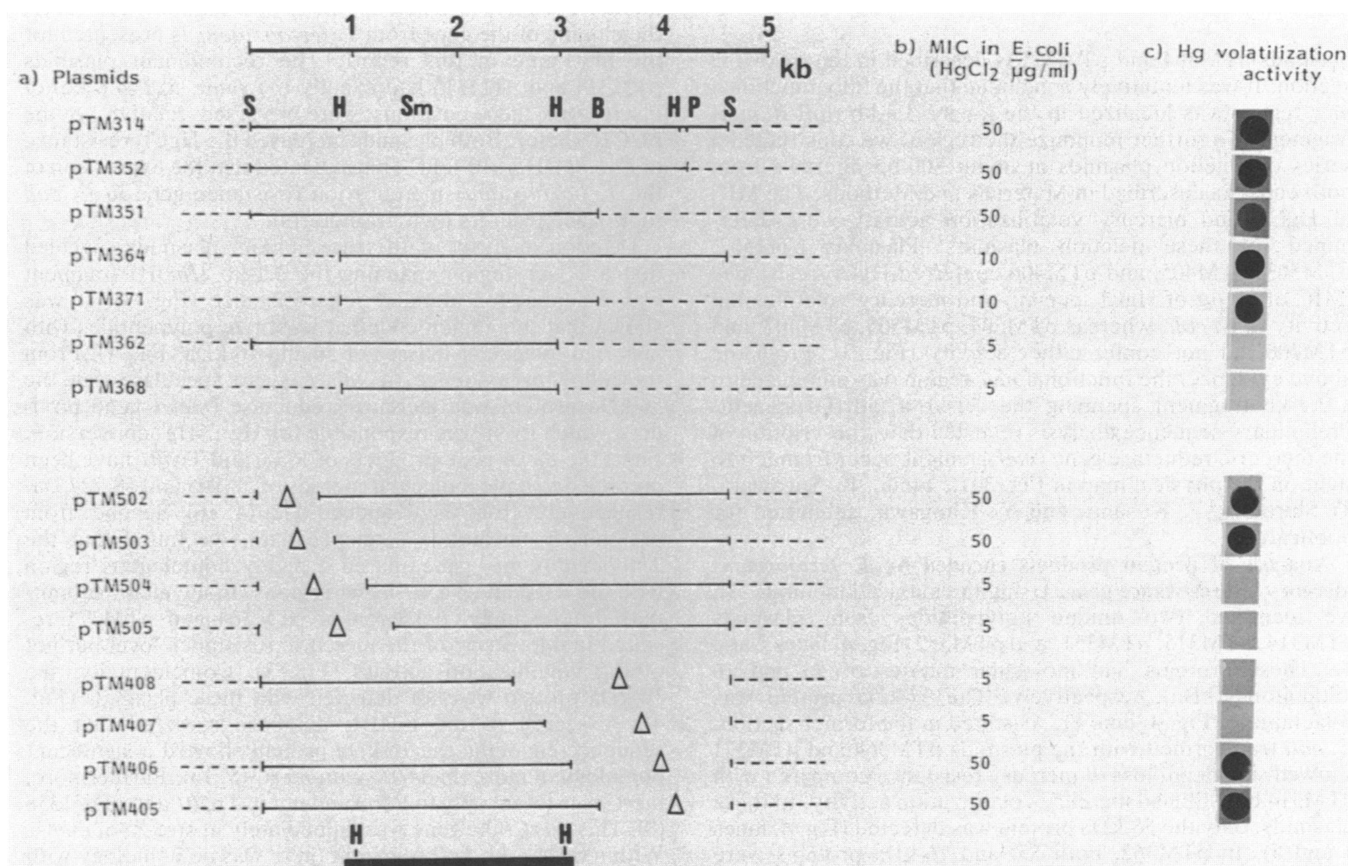

FIG. 3. Restriction endonuclease map and biological activity of pTM314 and its deletion derivative plasmids. Deletion derivatives were obtained in two ways. In the top half of the figure, the internal restriction sites were utilized and subcloned into the pUC18 polylinker site. In the lower half of the figure, exonuclease III was used. Plasmids pTM405-8 and pTM502-5 were deletion products of pTM314 and pTM315, respectively (for details, see Materials and Methods). *E. coli* DH5α cells carrying pTM314 and other deletion derivatives were assayed for mercury ion resistance and mercury volatilization activity. The dashed line indicates plasmid pUC18. The thick line indicates the region essential for the expression of the mercury ion resistance phenotype. S, *Sal*I; H, *Hin*dIII; Sm, *Sma*I; B, *Bam*HI; P, *Pst*I.

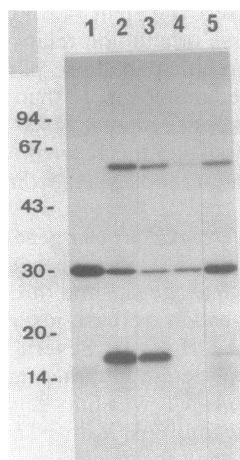

FIG. 4. Identification of *T. ferrooxidans* mercury ion resistance gene-encoded polypeptides. *E. coli* CSR603 harboring the recombinant plasmid was UV irradiated and labeled with [^{35}S]methionine. Labeled cells were collected, lysed, and analyzed on SDS-polyacrylamide (10 to 15% gradient) gels. The gels were prepared for fluorography as described in the text and exposed for 20 h at −80°C. Samples were prepared from maxicells carrying the following plasmids: lane 1, pUC18; lane 2, pTM315; lane 3, pTM351; lane 4, pTM364; lane 5, pTM371. The numbers in the left margin of the figure indicate molecular mass markers in kilodaltons.

plasmids pTM364 and pTM371 is described in the following section. It was tentatively concluded that the fully functional *mer* region was localized in the longer 3.4-kb *Sal*I-*Bam*HI fragment. To further minimize the region, we constructed a series of deletion plasmids at about 300-bp intervals from both ends, as described in Materials and Methods. The MIC of $HgCl_2$ and mercury volatilization activity were determined for these deletion plasmids. Plasmids pTM502, pTM503, pTM405, and pTM406 conferred $HgCl_2$ resistance (MIC of 50 μg of $HgCl_2$ per ml) and mercury volatilization activity to *E. coli*, whereas pTM504, pTM505, pTM407, and pTM408 did not confer either activity (Fig. 3). From the above evidence, the functional *mer* region was minimized to a 2.3-kb fragment spanning the 2.1-kb *Hin*dIII fragment. Preliminary sequence analysis revealed that transcription of the mercuric reductase gene (*merA*) might occur from left to right on the physical map in Fig. 3 (C. Inoue, K. Sugawara, T. Shiratori, T. Kusano, and Y. Kitagawa, submitted for publication).

Analysis of protein products encoded by *T. ferrooxidans* mercury ion resistance gene. Using the maxicell method (27), we identified two unique polypeptides from plasmids pTM314, pTM315, pTM351, and pTM352 (Fig. 4, lanes 2 and 3). These proteins had molecular masses of 56 and 16 kilodaltons (kDa), respectively. The 32-kDa protein was β-lactamase (Fig. 4, lane 1). As stated in the former section, *E. coli* transformed with the plasmids pTM364 and pTM371 showed significant loss of mercury resistance compared with pTM314 but still had mercury volatilization activity. In these plasmids, only the 56-kDa protein was detected (Fig. 4, lanes 4 and 5). In pTM362, both 55- and 16-kDa proteins were detected (data not shown). The 55-kDa protein seemed to be a truncated product of the 56-kDa protein. Plasmid pTM362 was unable to confer to *E. coli* resistance or hypersensitivity (17) to mercury, i.e., the MIC was 5 μg of $HgCl_2$ per ml, the same as that for *E. coli* carrying pUC18. The above evidence suggested that (i) the 56-kDa protein responsible for mercury volatilization was mercuric reductase, (ii) the 16-kDa protein strengthened the mercury resistance in the presence of the 56-kDa protein, and (iii) both the 56- and 16-kDa proteins were necessary for full resistance activity.

DISCUSSION

Among our 10 isolates of *T. ferrooxidans*, 5 strains were resistant to $HgCl_2$ (Table 1). These five strains were divided into at least two groups according to their mechanism of resistance. One group, consisting of clones E-6, E-7, and E-15, was endowed with mercury ion resistance through an enzymatic mode, since this group showed Hg-dependent NADPH oxidation activity and hybridized with the Tn*501* *mer* fragment (Table 1). The other group, strains E-24 and U4-25, did not show either of these activities. Therefore, the latter two strains might have Hg resistance owing to a different mode, probably a transport barrier. In the first group, the corresponding gene was localized in the chromosomal DNA, not in the plasmid DNA (Fig. 1). The mercury ion resistance system is mainly found on plasmids and transposons in other bacteria (7, 25, 26) with a few exceptions (32, 33). The origin of the *T. ferrooxidans* mercury ion resistance genes is indeed interesting, but at present we do not have any information to warrant further discussion.

Two groups (3, 19) have already reported the presence of mercuric reductase in acidophilic *T. ferrooxidans*. However, the cloning of the gene from *T. ferrooxidans* is presented for the first time in this report. The recombinant plasmids pTM314 and pTM315 had exactly the same size (4.6 kb) of insert, but their positions were reversed relative to the pUC18 vector. Both plasmids increased the $HgCl_2$ resistance of *E. coli* DH5α 10-fold. This indicated that the expression of the *T. ferrooxidans* mercury ion resistance gene in *E. coli* took place from its own promoter(s).

Deletion analysis of the recombinant plasmids revealed that a 2.3-kb region spanning the 2.1-kb *Hin*dIII fragment was essential for mercury ion resistance (Fig. 3). It was shown that this region coded at least two polypeptides with apparent molecular masses of 56 and 16 kDa (Fig. 4). From the following evidence, it was easy to speculate that the 56-kDa protein was mercuric reductase (*merA* gene product), which itself was responsible for Hg^{2+}-Hg^0 conversion. First, the *merA* gene products of R100 and Tn*501* have been predicted to have molecular masses of 58,905 and 58,727 Da, respectively, from the sequence data (4, 16). Second, from preliminary nucleotide sequence data, we found that the *Thiobacillus mer* gene shared a highly homologous region with the R100 and Tn*501* *merA* regions (Inoue et al., submitted). Interestingly, deletions of pTM364 and pTM371 resulted in a lowering of the mercuric resistance level but not of the volatilization activity (Fig. 3). Coincidentally, the 16-kDa protein was not detected with these plasmids (Fig. 4). A search of the EMBL database revealed that the sequence encoding the 16-kDa protein showed a significant homology of more than 60% with *merC* (5, 16). Furthermore, *merC* was found only in R100 and not in Tn*501* and pDU1358 (8). This *merC*-like gene was immediately upstream of *merA*. Within the 4.6-kb *Sal*I fragment there was no homology with *merR*, *merT*, or *merP* (7, 26, 29). Details will be described elsewhere (T. Kusano, C. Inoue, K. Sugawara, and Y. Kitagawa, submitted for publication). In *T. ferrooxidans* E-6, E-7, and E-15, the mercury resistance genes appeared to be constitutive (data not shown), similar to that found by Olson et al. (19) and suggestive of the absence of *merR*.

Furthermore, plasmid pTM362, which lacked the sequence corresponding to the carboxy-terminal region of mercuric reductase, did not confer mercury hypersensitivity (17) to the host cells (see Results), indicative of the absence of *merT*. Thus, the gene composition of the *mer* region of this acidophilic bacterium was quite unique when compared with those of other gram-negative bacteria. Further work is under way to determine the functions of the 16-kDa protein.

As far as we know, several genes (for example, the glutamine synthetase gene, *ori* fragment, *recA*-like fragment, and the present gene) from *T. ferrooxidans* are functional in *E. coli* (1, 21, 24). However, no evidence that a foreign gene(s) is expressed in the acidophilic *T. ferrooxidans* has been obtained. Rawlings et al. (23) constructed recombinant plasmids for *T. ferrooxidans* which contained an arsenic resistance gene originating from the *E. coli* IncN plasmid R46. Transformation of their plasmids in *T. ferrooxidans* has not yet been successful. We have also tried to establish the conditions for foreign gene introduction into *T. ferrooxidans* through examination of chloramphenicol acetyltransferase activity with pACYC184, but we have never detected it. It is still doubtful whether a foreign bacterial gene(s) would be functional in this bacterium. From this viewpoint, the native mercury ion resistance gene shows guaranteed expression in *T. ferrooxidans*. We have also recently cloned the *T. ferrooxidans* plasmids and constructed potential shuttle vectors for this bacterium, which has its own mercury ion resistance genes to be used as a selection marker (T. Kusano, manuscript in preparation).

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