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Electrotransformation of *Thiobacillus ferrooxidans* with Plasmids Containing a *mer* Determinant

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The *mer* operon from a strain of *Thiobacillus ferrooxidans* (C. Inoue, K. Sugawara, and T. Kusano, Mol. Microbiol. 5:2707–2718, 1991) consists of the regulatory gene *merR* and an operator-promoter region followed by *merC* and *merA* structural genes and differs from other known gram-negative *mer* operons. We have constructed four potential shuttle plasmids composed of a *T. ferrooxidans*-borne cryptic plasmid, a pUC18 plasmid, and the above-mentioned *mer* determinant as a selectable marker. Mercury ion-sensitive *T. ferrooxidans* strains were electroporated with constructed plasmids, and one strain, Y4-3 (of 30 independent strains tested), was found to have a transformation efficiency of 120 to 200 mercury-resistant colonies per μg of plasmid DNA. This recipient strain was confirmed to be *T. ferrooxidans* by physiological, morphological, and chemotaxonomical data. The transformants carried a plasmid with no physical rearrangements through 25 passages under no selective pressure. Cell extracts showed mercury ion-dependent NADPH oxidation activity.

Thiobacillus ferrooxidans is a gram-negative, obligately chemolithotrophic bacterium. This microorganism is of interest because of its industrial use in metal leaching from ores and desulfurization of coal. The bacterium has also been used in the treatment of mine drainage runoff, hydrometallurgical flue dust, and hydrogen sulfide gas (38, 42). These uses are based on the Fe(II)-oxidizing activity of this microorganism. A higher Fe(II)-oxidizing activity obtained through genetic manipulation may allow wider application of this organism. Considerable progress in understanding the molecular genetics of the organism (13–16, 22–25, 30, 32, 37) has resulted from cloning genes from *T. ferrooxidans* and studying them in *Escherichia coli*. Genetic transformation and conjugational gene transfer of other members of the same genus such as *Thiobacillus novellus* (6), *Thiobacillus neapolitanus* (21), *Thiobacillus thioparus* (46), and *Thiobacillus thiooxidans* (18) have also been reported. However, introduction of DNA back into *T. ferrooxidans* cells has not been previously successful. DNA transformation opens the way to investigate the function of genes and to understand gene regulation in this microorganism.

To establish such a system, a proper vector, a selectable marker gene(s), and receptive host cells are all required. The most promising vectors are endogenous *Thiobacillus* plasmids. Several groups have reported the occurrence, cloning, and characterization of plasmids from *T. ferrooxidans* (7, 8, 12, 27–29, 36, 44). A selectable marker gene is the second requirement for establishing a gene transfer system. In a previous attempt (31), the arsenic resistance gene originated from an *E. coli* plasmid was used but without success. Considering the acidic habitat (pH about 2) of this bacterium, an inorganic-ion resistance gene is thought to be appropriate because inorganic ions are chemically stable in low pH conditions. Over the past few years, we have cloned and characterized the mercury resistance (*mer*) determinant from a strain (E-15) of *T. ferrooxidans* (14–16, 22, 37). The *T. ferrooxidans mer* system is composed of one *cis* element operator-promoter sequence and three essential genes, *merR* (the regulatory gene), *merC* [whose gene product plays the role of an Hg(II) membrane transporter], and *merA* (encoding the enzyme mercuric reductase) (39). The *mer* determinant has the advantage of having originated from *T. ferrooxidans* and is therefore guaranteed expression in *T. ferrooxidans* cells.

Electrotransformation has been widely used to introduce exogenous DNA into mammalian cells, which are resistant to routine methods (5). This technique has also been used extensively in transforming protoplast cells of various species of plants (10), yeasts (41), and bacteria (2, 4, 9, 43, 45).

In this paper, we report the construction of vectors to shuttle between *T. ferrooxidans* and *E. coli* that have the *mer* determinant found in *T. ferrooxidans* as a marker gene. We also demonstrate electrotransformation of a plasmid into a strain of *T. ferrooxidans*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains of *T. ferrooxidans* were cultured in 9K liquid medium (40) at 30°C with vigorous shaking or on 9K-impregnated colloidal-silica plates. *E. coli* strain DH5α (17) was cultured in Luria broth or on Luria agar. Competent *E. coli* cells were prepared by the method of Hanahan (11). A broad-host-range plasmid, pKT240 (1), was kindly provided by Y. Itoh (National Food Research Institute, Tsukuba, Japan). Plasmid pTM314 (37) contained the 4.6-kb *Sal*I fragment (which includes an operator-promoter region followed by *merC* and *merA* genes) from *T. ferrooxidans* E-15 in the *Sal*I site of pUC18. pTM315 (37) contained the same *Sal*I fragment in reverse orientation on pUC18. pTMR25 (15) has the 1.0-kb *Eco*RI fragment containing the *merR2* gene from *T. ferrooxidans* E-15 on pUC18. Two cryptic *T. ferrooxidans* plasmids (pTNA33 [2.4 kb] and pTSB121 [5.1 kb]) (36) were also used. pTNA33 is a plasmid found in *T. ferrooxidans* MA3-3.

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pTSB121, isolated from the strain *T. ferrooxidans* B-12, is the smallest plasmid of seven.

Construction of shuttle vectors for *T. ferrooxidans* and *E. coli*. Using standard protocols for DNA manipulation (34), we constructed five shuttle vectors: two cryptic *T. ferrooxidans* plasmids, pTNA33 and pTSB121, each cloned into pUC18 at two different restriction enzyme sites as described below, and a fifth construct derived from pKT240. pTNA33 was cloned into the *Bam*HI or *Kpn*I sites of pUC18. The corresponding plasmids, pTA321 and pTA322 (36), were digested with *Sal*I and ligated with the 4.6-kb *Sal*I fragment of pTM314 (37), resulting in the plasmids pTMA641 and pTMA642, respectively. The 1.0-kb *Eco*RI fragment of pTMR25 was then cloned into *Eco*RI sites of pTMA641 and pTMA642, resulting in pTMZ47 (10.7 kb) and pTMZ48 (Fig. 1A; 10.7 kb). pTSB121 was digested with *Pst*I or *Sal*I and cloned into pUC18, and the recombinant was designated pTB311 (7.8 kb) or pTB312 (7.8 kb). Both plasmids were cleaved with *Bam*HI and ligated with the 3.4-kb *Bam*HI fragment of pTM315. The resulting plasmids, pTMB631 (11.2 kb) and pTMB632 (11.2 kb), were digested with *Eco*RI and ligated with the 1.0-kb *Eco*RI fragment of pTMR25, resulting in pTMZ132 (Fig. 1B; 12.2 kb) and pTMZ134 (12.2 kb), respectively. pKT240 was digested with *Eco*RI and *Sph*I, and the longer (12.4-kb) fragment was recovered. The 4.6-kb *Eco*RI-*Sph*I fragment of pTM315 (both cleavage sites were derived from the multicloning sites of pUC18) was ligated with the recovered 12.4-kb fragment. The hybrid plasmid, designated pTM138 (17.0 kb), was cleaved with *Eco*RI and ligated with the 1.0-kb *Eco*RI fragment of pTMR25. The final construct was designated pKMZ51 (Fig. 1C; 18.0 kb).

Electrotransformation procedure. Host strains from our *T. ferrooxidans* strain collection (36) were selected on the basis of $HgCl_2$ sensitivity (MIC of <0.1 μg/ml) and presence of no or at most one plasmid. *T. ferrooxidans* at full-growth phase (20 ml) was inoculated into 400 ml of fresh 9K medium and incubated at 30°C for 16 h with vigorous shaking. The following steps were carried out at 4°C unless otherwise mentioned. The cells were pelleted by centrifugation at 8,000 rpm (Hitachi RPR-20-2 rotor) for 10 min and washed twice with low-pH solution (9K medium-basal salts, 0.16 M $MgSO_4$, pH 1.9) to remove iron precipitates, twice with high-pH solution (25 mM phosphate buffer, 0.3 M sucrose, 10 mM EDTA, pH 8.0), once quickly with electroporation buffer [3 mM piperazine-*N*-*N*'-bis(2-ethanesulfonic acid) (PIPES), 272 mM sucrose, pH 6.4]. Cells (total numbers, about 6×10^9 to 8×10^9) were resuspended in 3.2 ml of electroporation buffer. An aliquot (0.4 ml) was mixed with the vector DNA (5 μg/10 μl) and transferred into a precooled cuvette with an electrode gap of 2 mm. The pulses were generated by using electroporation apparatus (Gene-pulser; Bio-Rad Laboratories) equipped with a pulse controller. After the pulse was delivered, the cells were quickly layered onto 10 ml of a fresh 9K-liquid medium and incubated at 30°C to express the genes of the electroporated plasmids.

Induction of the *mer* gene electroporated in *T. ferrooxidans*. The *mer* system is inducible (39). Therefore, the system needs to be induced by low subtoxic concentrations of mercury. The following two methods were taken to express the gene electroporated before starting selection. In method A, cells were incubated in 9K medium with shaking at 30°C for 3 h, 0.02 μg of $HgCl_2$ per ml was added, and cells were incubated for 16 h at 30°C with shaking. During incubation, the viable cells increased 1.5- to 1.8-fold. In method B, electroporated cells were incubated in 9K medium with shaking at 30°C for 3 h, 0.002 μg of $HgCl_2$ per ml was added, and the cells were incubated statically at 30°C for 40 h. During this static incubation, no increase in cell numbers was observed. After induction by one of these methods, aliquots of the cultured cells were spread over 9K medium-impregnated colloidal-silica plates containing 0.25 μg of $HgCl_2$ per ml and further incubated for 7 to 10 days at 30°C until colonies appeared.

Southern hybridization. Transformants were cultured in 10 ml of 9K liquid medium containing 0.075 μg of $HgCl_2$ per ml. Cells were pelleted and washed as described previously (37). Plasmid fractions were prepared by the slightly modified procedure of Birnboim and Doly (3) (adding twice the lysozyme in solution I) and separated by electrophoresis on 0.7% agarose gels. After gel electrophoresis, DNA was blotted onto membrane filters (Hybond-N; Amersham Co. Ltd.) by a slight modification (15) of the procedure of Reed and Mann (33). Detection of DNA was done by ^{32}P-labeled probe and nick translation. Prehybridization and hybridization were carried out by using the standard protocol (34). Each filter was washed with 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0)–0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min twice and with 0.1× SSC–0.1% SDS at 68°C for 15 min twice and autoradiographed.

Hg^{2+}-dependent NADPH oxidation assay. Original host cells and transformant cells were cultured in 50 ml of 9K medium and induced by adding 0.05 μg of $HgCl_2$ per ml. The cell pellets were collected, washed as described above, and disrupted by sonication. After undisrupted cells were removed, the supernatant fraction was used for measurement of Hg^{2+}-dependent NADPH oxidation. The reaction mixture for assay was the same as described previously (37).

RESULTS

Structures of five potential shuttle vectors. Five potential vectors to shuttle between *T. ferrooxidans* and *E. coli* were constructed. These five vectors mainly differ in the *T. ferrooxidans* plasmid part. In pTMZ47 and pTMZ48 (Fig. 1A), a cryptic plasmid, pTNA33 from *T. ferrooxidans* MA3-3, was used. In pTMZ132 (Fig. 1B) and pTMZ134, another cryptic plasmid, pTSB121 from *T. ferrooxidans* B-12, was used. Dorrington and colleagues (7, 8) reported that the plasmid pTF-FC2, isolated from a strain of *T. ferrooxidans*, has a broad-host-range property and a high degree of nucleotide sequence homology with the IncQ-type plasmid RSF1010. Therefore, broad-host-range plasmid pKT240 (IncQ type) was used as another starting vehicle with plasmid pKMZ51 (Fig. 1C). As a marker gene for selection, the *mer* determinant isolated from *T. ferrooxidans* E-15 was used (15, 37). The regulatory gene *merR* is spaced apart from the *merC* and *merA* genes in this strain of *T. ferrooxidans* (15); therefore, in some constructs (pTMZ47 and pTMZ48), the *merR* gene is separated from the *mer* structural genes.

Introduction of shuttle vectors into host strains. Host strains were selected from our collection on the basis of being possibly plasmid free and $HgCl_2$ sensitive. Host cells were electroporated at a field strength of 12,500 V/cm and a pulse controller resistance of 400 Ω with the five vectors which were prepared from *E. coli* DH5α. A single pulse was delivered in this work. The pulse duration ranged from 3.5 to 4.5 ms, and the viability of the cells after the pulse was delivered was 55 to 67%. Of 30 strains examined, strain Y4-3 gave transformants at an efficiency of 120 to 200 CFU/μg of DNA with four of the five chimeric plasmids (Table 1). Strain

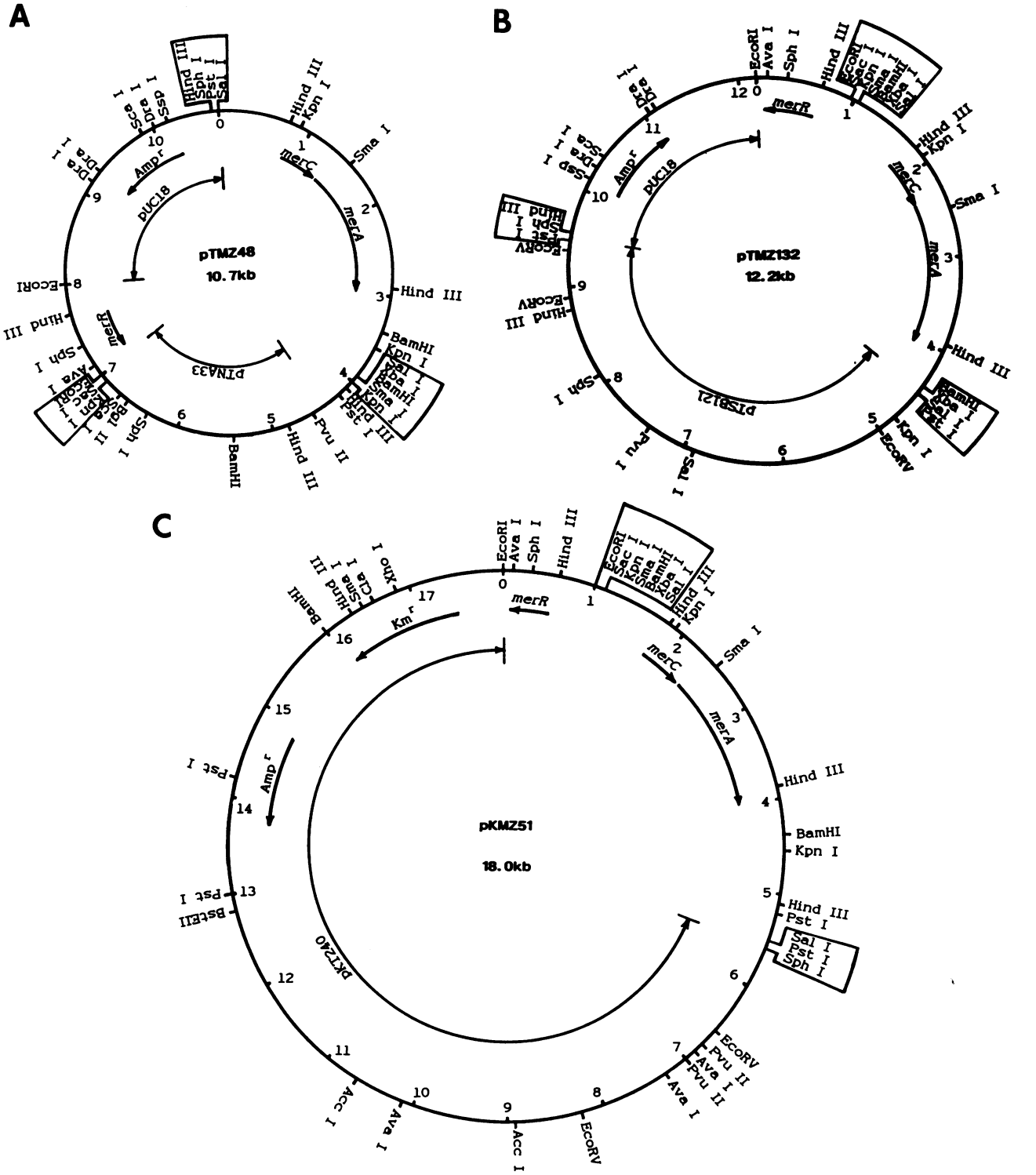

FIG. 1. Structures of vectors pTMZ48 (A), pTMZ132 (B), and pTKZ51 (C) that were used to shuttle between *T. ferrooxidans* and *E. coli*. The construction of these plasmids is described in Materials and Methods. pTNA33 and pTSB121 were cryptic plasmids isolated from *T. ferrooxidans* strains (36), and pKT240 is an IncQ-type broad-host-range plasmid (1). Arrows indicate directions of transcription of the genes. Lines with arrowheads at both ends indicate the regions of the vectors. Boxed restriction enzyme sites are derived from the multicloning site of pUC18.

TABLE 1. Transformation of *T. ferrooxidans* Y4-3 with five plasmids

Vector	Size (kb)	Source of replicon	Cleavage site(s) of *T. ferrooxidans* plasmids and pKT240	No. of transformants/μg of DNA[a]
pTMZ47	10.7	pTNA33	*Bam*HI	0
pTMZ48	10.7	pTNA33	*Kpn*I	168 ± 23
pTMZ132	12.2	pTSB121	*Pst*I	203 ± 14
pTMZ134	12.2	pTSB121	*Sal*I	162 ± 11
pKMZ51	18.0	pKT240	*Sal*I, *Eco*RI	119 ± 16

[a] Efficiency of transformation was calculated as the average number ± standard deviation of transformants obtained from three independent experiments. In each experiment, ca. 0.75×10^8 to 1×10^8 cells were mixed with 5 μg of plasmid DNA and charged with a single pulse as described in Materials and Methods. Efficiency was calculated as follows: apparent colony numbers per microgram of DNA × (numbers of positive clones in Southern analysis/total number of clones analyzed in Southern experiment).

Y4-3 carried no detectable plasmid originally. Among four successful vectors, there was little difference in efficiency. Transformants could not be reproducibly obtained by using plasmid pTMZ47. At any lower field strength (less than 10,000 V/cm), no transformants were obtained in this strain (data not shown). The following experiments used plasmid pTMZ48 unless otherwise mentioned. As seen in Fig. 2, more colonies were obtained on Hg^{2+} selection plates after electroporation with pTMZ48 (Fig. 2B) than with the mock DNA (Fig. 2A) (eliminating the *mer* determinant from the final constructs) or no plasmid (not shown). The efficiency of transformation was not affected by the two alternative induction methods (see Materials and Methods). In this selection system, we always observed background colonies (Fig. 2A), and the details of $HgCl_2$ selection were critical. This is a major drawback of the system. To confirm that the colonies represent real transformants, independent colonies grown on the selection plates were inoculated into 10 ml of fresh 9K medium containing 0.075 μg of $HgCl_2$ per ml and cultured at 30°C with vigorous shaking until stationary phase. The plasmid DNA fractions were prepared from harvested cells. Agarose gel electrophoresis of these plasmid fractions showed that most of the colonies carried a single plasmid which was indistinguishable from the input plasmid with regard to size (data not shown). A single plasmid was

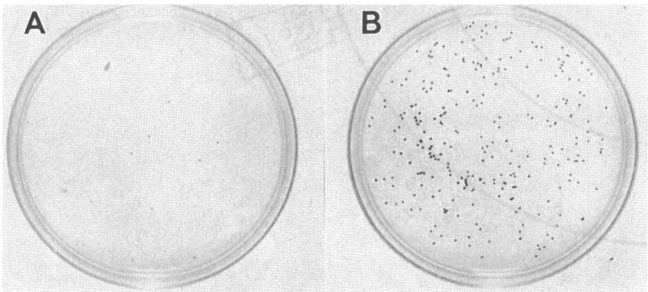

FIG. 2. Mercury ion-resistant colonies obtained by electrotransformation with plasmid pTMZ48. After electroporation and induction with 0.002 μg of $HgCl_2$ per ml for 40 h at 30°C (see Materials and Methods), aliquots (containing ca. 5×10^7 cells each) of cells electroporated with pTNA311 (plasmid without *mer* genes) (A) or pTMZ48 (B) were plated onto 9K medium–colloidal-silica plates containing 0.25 μg of $HgCl_2$ per ml and incubated for 10 days at 30°C.

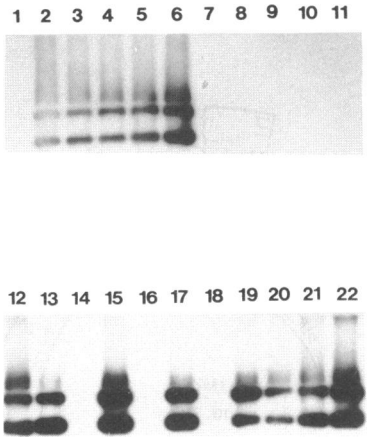

FIG. 3. Southern blot hybridization analysis of the transformant potentially carrying plasmids. The *mer* probe was the ^{32}P-labeled 4.6-kb-*Sal*I fragment of pTM314. Each lane contains DNA prepared from the cells of 10 ml of full-growth culture.

visible in the gels after ethidium bromide staining and therefore may exist as a few to 10 copies in a transformant. Plasmid DNA in the gel was alkaline blotted onto a nylon membrane, and Southern analysis with the 4.6-kb *Sal*I fragment of *mer* plasmid pTM314 was performed (37). Of 22 clones analyzed (Fig. 3), 13 clones (lanes 2 through 6, 12, 13, 15, 17, and 19 through 22) harbored plasmids which hybridized with this *mer* probe. The transformation efficiency given in Table 1 was calculated as follows: apparent colony numbers per microgram of DNA × (numbers of positive clones in Southern analysis [13 in Fig. 5]/number of clones analyzed in Southern experiment [22 in Fig. 3]).

Transformants carry plasmids without apparent rearrangements. Each transformant except false ones carried a single plasmid whose size was identical to that of plasmid pTMZ48. This suggested that no rearrangement occurred in these transformants. Restriction nuclease patterns of plasmids recovered from the transformants and the input plasmid were compared. The two plasmids showed indistinguishable cleavage patterns with five different enzymes (Fig. 4). When the plasmid DNA fraction from the transformant was used, *E. coli* competent cells were transformed and selected in Luria broth containing 50 μg of ampicillin per ml. The plasmid rescued in ampicillin-resistant *E. coli* cells had restriction patterns identical to those of the input DNA (data not shown). These indicate that rearrangements had not occurred in transformants and that the plasmid was shuttling between the two microorganisms. It is interesting that the input plasmid pTMZ48 was prepared from *E. coli* DH5α (*dam*$^+$ *dcm*$^+$). *Mbo*I and *Eco*RII recognize 5′-GATC-3′ and 5′-CC(A/T)GG-3′ sequences, respectively, whereas the methylated respective sequences modified by Dam methylase and Dcm methylase were resistant to *Mbo*I and *Eco*RII, respectively. Therefore, the plasmid prepared from strain DH5α was resistant to *Mbo*I and *Eco*RII (Fig. 4B, lanes 7 and 8). By contrast, the plasmid recovered from the transformant was found to be sensitive to these two enzymes (Fig. 4B, lanes 3 and 4). Chromosomal and plasmid DNAs prepared from *T. ferrooxidans* strains as far as investigated are sensitive to these two enzymes. This strongly supports the conclusion that the electroporated plasmid replicated in transformant cells. To know whether the plasmid prepared from the *dam dcm* double-mutant *E. coli* cells or whether the

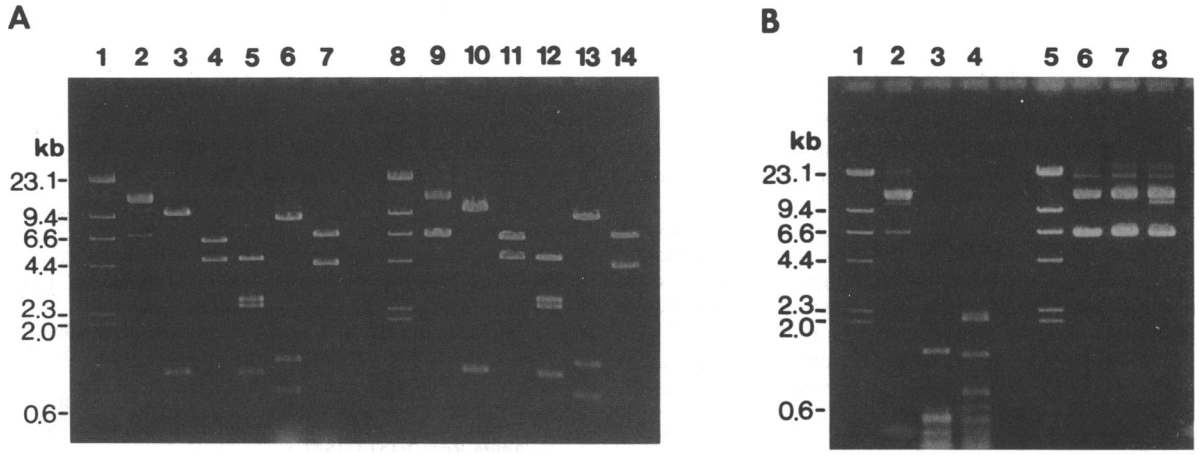

FIG. 4. Restriction enzyme analysis of the input plasmid (before electroporation) and the output plasmid (recovered from one transformant). (A) Lanes 1 and 8, λ phage DNA digested with *Hin*dIII; lanes 2 to 7, plasmid recovered from the transformant; lanes 9 to 14, plasmid pTMZ48 prepared from *E. coli* DH5α; plasmid was incubated with no enzyme (lanes 2 and 9), *Eco*RI (lanes 3 and 10), *Sal*I (lanes 4 and 11), *Kpn*I (lanes 5 and 12), *Bam*HI (lanes 6 and 13), or *Pst*I (lanes 7 and 14). (B) Lanes 1 and 5, λ phage DNA digested with *Hin*dIII; lanes 2 to 4, plasmid recovered from the transformant; lanes 6 to 8, plasmid pTMZ48 prepared from *E. coli* DH5α; lanes 2 and 6, no addition of enzyme; lanes 3 and 7, digested with *Mbo*I; lanes 4 and 8, digested with *Eco*RII.

one prepared from the *T. ferrooxidans* Y4-3 transformant itself affects transformation efficiency, plasmid pTMZ48 was prepared from *E. coli* GM2929 (*dam-13*::Tn*9 dcm-6*) (provided by Barbara J. Bachmann, Yale University) or from the *T. ferrooxidans* transformant. Both plasmids, however, failed to increase transformation efficiency (data not shown). A cell extract prepared from the transformant showed activity of mercury ion-dependent NADPH oxidation (3.4 nmol of NADPH oxidized per mg of protein per min), whereas the extract of the original host cell did not show any such activity. Similar results were obtained with *T. ferrooxidans* transformants of the plasmids pTMZ132, pTMZ134, and pKMZ51 as well as with pTMZ48 (data not shown).

Plasmid stability in transformants. A serial cultivation with or without selective pressure was performed (Fig. 5). With 20-fold growth during one passage, the plasmid was kept at the original level after 10 passages (about 43 generations) (Fig. 5, lane 3) and 25 passages (more than 110 generations) (Fig. 5, lane 4) in the transformant. Furthermore, the plasmid was not segregated out from the transformant even in the absence of selective pressure (Fig. 5, lane 5).

Confirmation of host cell strain Y4-3 as *T. ferrooxidans*. Strain Y4-3 is able to grow not only on ferrous ions but also on inorganic sulfur as energy source. Addition of more than 0.01% (wt/vol) glucose into 9K liquid medium inhibited the growth of strain Y4-3, but less than 0.001% (wt/vol) glucose did not have any effect on its growth. This suggests that heterotrophs did not contaminate the strain. Scanning electron microscopical observation showed that each cell of this strain is a short rod of uniform size (ca. 0.5 by 1 to 2 μm) and not a spiral (20). The cellular fatty acid composition of the strain was as follows: 12:0, 5%; 14:0, 2%; 15:0, trace; 16:0, 11%; 17:0, 1%; 18:0, 3%; 16:1, 15%; 18:1, 60%; 17 cyc, 1%; 19 cyc, 2%; 3-OH-14:0, 6%; 3-OH-16:0, 2%. First two digits, third digit, cyc, and 3-OH indicate the length of the carbon chain, the number of double bonds, and the existence of cyclopropane and 3-hydroxy groups, respectively. A major ubiquinone of this strain has eight isoprene units (Q-8) (data not shown). These chemotaxonomical data also agreed with those of typical strains of *T. ferrooxidans* (19). These lines of evidence led us to the conclusion that strain Y4-3 is *T. ferrooxidans*.

DISCUSSION

The system of transferring plasmid genetic material into *T. ferrooxidans* was demonstrated. Although the efficiency is still low (Table 1), there were no apparent DNA rearrangements in the transformants (Fig. 4A). pKMZ51 is 18.0 kb in

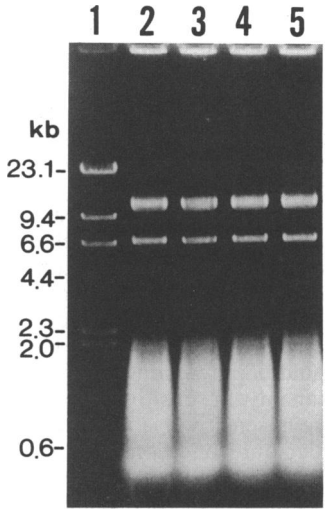

FIG. 5. Stability of the plasmid in the transformant with or without selective pressure. At each passage, 1/20 volume (0.5 ml) of the fully grown culture was transferred to 10 ml of fresh 9K medium with or without 0.075 μg of $HgCl_2$ per ml. The plasmid fraction was prepared from 30 ml of the full-growth culture of the cells in 30-ml scale (three tubes with 10 ml of culture each), and halves of the preparations were analyzed in 0.7% agarose gel. Lane 1, λ phage DNA digested with *Hin*dIII; lane 2, passage 1 with $HgCl_2$; lane 3, after 10 passages with $HgCl_2$; lane 4, after 25 passages with $HgCl_2$; lane 5, after 25 passages, 10 with $HgCl_2$ and 15 without $HgCl_2$.

size and replicated without deletions. pKMZ51 showed that the replication origin of pKT240 functions in *T. ferrooxidans* cells similarly to that of pTF-FC2 (7, 8). The plasmid isolated from the transformant was sensitive to enzymes *Mbo*I and *Eco*RII (Fig. 4B), whereas the input DNA was resistant to these enzymes because of Dam and Dcm methylation in *E. coli* (Fig. 4B). This indicates that plasmid replication occurred in host *T. ferrooxidans* cells.

Only strain Y4-3, confirmed to be *T. ferrooxidans* by several criteria (see Results section), of 30 *T. ferrooxidans* strains allowed successful electrotransformation. Three days after electroporation with pTMZ48, plasmid fractions were prepared from the pulse-delivered cells of 29 other *T. ferrooxidans* strains and rescued to competent *E. coli* cells. The fractions from 2 strains gave ampicillin-resistant transformants, but those from the other 27 strains gave no such transformants. Restriction enzyme and Southern analyses showed that the plasmids recovered from the such Amp^r transformants have lost the *merC* and *merA* genes totally in the cases of both strains (data not shown). The presence of a strong restriction system is probable in most *T. ferrooxidans* strains. In general, it is known that the DNA modified by a microorganism itself is not restricted by that microorganism (26). Plasmid pTMZ48 prepared from the *T. ferrooxidans* transformant failed to increase the rate of transformation. Further work is required to explain why the plasmid failed to increase the efficiency.

The pUC18 derivative containing the *T. ferrooxidans mer* determinant was unable to transform Y4-3 cells. This indicates that transformation is dependent on the *T. ferrooxidans* plasmid replicon. Among the five plasmids, only pTMZ47 was unable to transform strain Y4-3. The *Bam*HI site of pTNA33 (Table 1) is supposed to occur in the region of the replication origin in this plasmid or in the gene which participates in plasmid segregation and/or replication. DNA sequencing of pTNA33 and maxicell labeling of the plasmid-coded protein(s) showed that there is no potential open reading frame around the *Bam*HI site (35). The *ori* (origin of DNA replication) region of the *T. ferrooxidans* cryptic plasmids will be identified and characterized by using this system.

One weakness of the system developed is the marker gene, because the difference in threshold values for $HgCl_2$ between the original host and the transformant is small. Another effective marker gene for selection is required. During the preparation of this paper, Jin et al. (18) reported the conjugational transfer of an IncP plasmid to another acidophilic chemolithotroph, *T. thiooxidans*, from *E. coli* at frequencies of 10^{-5} to 10^{-7} per recipient cell. They used the kanamycin resistance gene as the selective marker. pKMZ51 used in this work also carried the kanamycin resistance gene. So it would be interesting to know whether there is a difference in kanamycin sensitivity between Y4-3 cells (originally kanamycin sensitive) and its transformant carrying pKMZ51. Under our conditions, the kanamycin resistance cassette from pKT240 did not work in *T. ferrooxidans*. To increase the usefulness of this transformation system, further refinements in both the host bacterium and plasmid vector will be necessary.

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