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Construction and Characterization of a *recA* Mutant of *Thiobacillus ferrooxidans* by Marker Exchange Mutagenesis

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To construct *Thiobacillus ferrooxidans* mutants by marker exchange mutagenesis, a genetic transfer system is required. The transfer of broad-host-range plasmids belonging to the incompatibility groups IncQ (pKT240 and pJRD215), IncP (pJB3Km1), and IncW (pUFR034) from *Escherichia coli* to two private *T. ferrooxidans* strains (BRGM1 and Tf-49) and to two collection strains (ATCC 33020 and ATCC 19859) by conjugation was analyzed. To knock out the *T. ferrooxidans recA* gene, a mobilizable suicide plasmid carrying the ATCC 33020 *recA* gene disrupted by a kanamycin resistance gene was transferred from *E. coli* to *T. ferrooxidans* ATCC 33020 by conjugation under the best conditions determined. The two kanamycin-resistant clones, which have retained the kanamycin-resistant phenotype after growth for several generations in nonselective medium, were shown to have the kanamycin resistance gene inserted within the *recA* gene, indicating that the *rec*A::Ω-Km mutated allele was transferred from the suicide plasmid to the chromosome by homologous recombination. These mutants exhibited a slightly reduced growth rate and an increased sensitivity to UV and γ irradiation compared to the wild-type strain. However, the *T. ferrooxidans recA* mutants are less sensitive to these physical DNA-damaging agents than the *recA* mutants described in other bacterial species, suggesting that RecA plays a minor role in DNA repair in *T. ferrooxidans*.

Thiobacillus ferrooxidans is an acidophilic chemolithoautotrophic bacterium that obtains its energy from oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) or of reduced sulfur compounds to sulfuric acid. Its widespread application in mineral leaching and metal remediation has made it an attractive microorganism to study. Considerable progress has been made in studying the biochemistry and molecular biology of *T. ferrooxidans* in recent years (27, 33). However, the absence of genetic tools has impaired the understanding of the physiology of this microorganism. The study of mutants in which a protein of interest is no longer synthesized can help to establish its function, but there have been no reports of the construction of *T. ferrooxidans* mutants. The construction by marker exchange mutagenesis of null mutants would be possible if a reliable genetic transfer system between *Escherichia coli* and *T. ferrooxidans* were available.

Introduction of plasmids into *T. ferrooxidans* by electrotransformation (17) and conjugation (23) has been reported. The plasmids electroporated by Kusano et al. (17) consisted of the *T. ferrooxidans mer* operon, determining resistance to mercury ions, cloned either into the broad-host-range plasmid pKT240 (IncQ group) or into a cryptic *T. ferrooxidans* natural plasmid carrying the pUC18 vector. Of the 30 independent *T. ferrooxidans* private strains tested, only one (Y4-3) gave transformants. The efficiency of electrotransformation was low (120 to 200 mercury-resistant colonies per μg of plasmid DNA). On the other hand, Peng et al. (23) reported the genetic transfer of broad-host-range IncP plasmids (RP4, R68.45, RP1::Tn*501*, and pUB307) by conjugation and the mobilization of a broad-host-range IncQ plasmid (pJRD215) with the aid of an RP4 plasmid to seven *T. ferrooxidans* private strains. Kanamycin resistance was used as the selection marker. The physiological states of both the donor and the recipient, and the mating time, have been shown to be important. The apparent transfer frequency of the large self-transmissible IncP plasmids was 10^{-5} to 10^{-7}, depending on the plasmid, and the apparent mobilization frequency of the IncQ pJRD215 plasmid was about 10^{-5}.

The RecA protein plays an essential role in homologous genetic recombination, DNA repair, induction of the SOS response, and initiation of stable DNA replication (29). The RecA protein is thought to be ubiquitous in eubacteria and is among the most conserved proteins across bacterial organisms (15). The *recA* gene from the *T. ferrooxidans* ATCC 33020 strain has been cloned (14, 25), sequenced (26), and expressed in *E. coli* (14, 25, 26). Both recombinase activity and SOS response were restored in an *E. coli recA* mutant by the *T. ferrooxidans recA* gene (25, 26), showing that RecA has similar activities in *T. ferrooxidans* and *E. coli*.

In this paper, the influence of different factors on the transfer frequency of IncQ plasmids from *E. coli* to *T. ferrooxidans* ATCC 33020 has been analyzed. This study was extended to three other *T. ferrooxidans* strains (ATCC 19859, BRGM1, and Tf-49) and to IncP and IncW plasmids. The feasibility of a marker exchange mutagenesis program has been tested in the ATCC 33020 strain with the construction of a *recA* mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. The *E. coli* growth medium was Luria-Bertani medium (21) except for conjugation experiments (see below). The composition of the *T. ferrooxidans* 9K liquid medium has been reported previously (5). The 2:2 and DOP solid media are described in references 24 and 19, respectively.

Conjugation. Initial conjugation experiments were performed according to the method of Peng et al. (23). The conjugation experiments were optimized as presented in Results. The modified mating procedure was as follows. The *E. coli* donor strains were grown at 37°C until late exponential growth phase in 2:2 basal

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TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
T. ferrooxidans		
ATCC 33020	Isolated from a uranium mine in Japan	ATCC[a]
ATCC 19859	Isolated from acid copper leaching water in Canada	ATCC
Tf-49	Isolated from a coal mine in Yunqin (China)	24
BRGM1	Isolated twice on DOP medium from the BRGM strain	8
E. coli		
HB101	Δ(*gpt-proA*)62 *leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcr-mrr*) *rpsL20 xyl-5 mtl-1 recA13*	
MC1061	*araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL hsdR2*(r_K^- m_K^+) *mcrA mcrB1*	
MOS Blue	*endA1 hsdR17*(r_K^- m_K^+) *supE44 thi1 gyrA46 recA1 lac*/F′ (*lacIqZΔM15 proAB* Tn*10*)	
S17-1	*recA pro hsdR* (RP4-2 Tc::Mu Km::Tn*7*)	31
CGSC7330	*pro-81*::Tn*10 rph-1*	*E. coli* Genetic Stock Center
Plasmids		
pNG23	*recA-recX-alaS* region cloned in the *Hin*dIII site of pUC19; identical to pNG22 (14) with the insert in the opposite orientation	
pHP45Ω-Km	pHP45 plasmid carrying the Ω-Km cassette	13
pUC18*mob*	pUC18 plasmid in which an *Sau*3A fragment carrying the *mob* region of the RP4 plasmid has been inserted in the *Bam*HI site	31
RP4	Ap^r Tc^r Km^r IncP Tra^+	10
pKT240	Ap^r Km^r (Tn*903*) IncQ Mob^+	4
pJRD215	Sm^r Km^r (Tn*5*) IncQ Mob^+	11
pJB3Km1	Ap^r Km^r (Tn*903*) IncP Mob^+	7
pUFR034	Km^r (Tn*903*) IncW Mob^+	12

[a] ATCC, American Type Culture Collection.

salt medium (2:2 liquid medium pH 5.2 to 5.4 without an energy source) supplemented with 0.5% (wt/vol) yeast extract and one antibiotic selective for the plasmid that they contained. The *T. ferrooxidans* recipient strains were usually cultured in 9K sulfur liquid medium (pH 3.5) at 30°C for 5 days to stationary phase. The cells were collected by centrifugation. *T. ferrooxidans* cells were washed three times with 2:2 basal salt medium to remove the sulfur precipitates. For matings, donor and recipient cells were combined in a 1:2 ratio. From this cell suspension (approximately 2×10^9 cells per ml), 0.1 ml was spotted on 2:2 solid medium (0.6% agar) supplemented with 0.05% (wt/vol) yeast extract, 0.5×10^{-4} M diaminopimelic acid, and 0.05% (wt/vol) $Na_2S_2O_3$. After 3 days of incubation at 30°C, the cells were harvested and suspended in 1.5 ml of 2:2 basal salt medium. The number of viable recipient bacteria was obtained by plating on 2:2 or DOP solid medium. Transconjugants were selected at 30°C on DOP solid medium containing 200 μg of kanamycin/ml, with recipient bacteria being counterselected by kanamycin and donor bacteria by pH and the absence of a carbon source in the selective medium. The plates were incubated at 30°C for 10 to 15 days. The frequencies of plasmid transfer are expressed as the "apparent transfer frequency," that is, the number of transconjugants scored on selective medium per recipient colony scored on nonselective medium after the mating period.

Stability analysis. A single colony of *T. ferrooxidans* conjugant was grown to late exponential phase in 9K ferrous iron liquid medium without antibiotic, diluted 10^3-fold in fresh 9K ferrous iron medium, and grown again to late exponential phase. The last two steps were repeated three times. An aliquot was taken at the beginning of each new culture, diluted, and plated on 2:2 solid medium with or without kanamycin. The plasmid stability was calculated as the ratio between the number of colonies observed in the presence and absence of antibiotic.

Construction of a *recA* mutant of *T. ferrooxidans* ATCC 33020. The pNG23 plasmid, carrying the ATCC 33020 *recA* and *alaS* genes cloned into pUC19 (Table 1), was digested with *Hin*dIII and religated to delete the *alaS* gene. The resulting plasmid, pUC19*recA*, replicates in *E. coli* but not in *T. ferrooxidans*. The blunt-ended *Hin*dIII fragment carrying the Km^r gene from the pHP45Ω-Km plasmid (13) was inserted into the *Sma*I site of the pUC19*recA* plasmid. The resulting plasmid, pUC19*recA*::Ω-Km, carries the *recA* gene disrupted by a 3.3-kb fragment as shown by restriction analyses, PCR, Southern hybridizations, and sequence determination. This fragment consists of the Ω-Km interposon, as expected, but also of an additional 1.3-kb fragment which corresponds to an internal region of the Ω-Km *Hin*dIII fragment. The 1.6-kb *Bam*HI fragment from pUC18*mob*, corresponding to the mobilization region of the RP4 plasmid, was cloned into the *Sca*I site of the ampicillin resistance gene of the pUC19*recA*::Ω-Km plasmid. This plasmid, called pUC19*recA*::Ω-Km*mob*, was transformed into *E. coli* S17-1 with kanamycin resistance selection. The mobilization ability of pUC19*recA*::Ω-Km*mob* was checked by transferring it by conjugation from *E. coli* S17-1 to *E. coli* CGSC7330; the selection was for tetracycline and kanamycin resistance.

The pUC19*recA*::Ω-Km*mob* plasmid was then transferred from *E. coli* S17-1 to *T. ferrooxidans* ATCC 33020 under the conditions given above except that the mating time was extended to 5 days. The selective medium was the 2:2 medium containing 200 μg of kanamycin/ml. The kanamycin plates were incubated at 30°C for 4 weeks.

Growth curves of the wild-type and *recA* ATCC 33020 strains. To determine the growth rate of the wild-type and *recA* mutant strains, liquid media were inoculated with fresh cultures of ATCC 33020 and *recA* mutant 5 and shaken at 30°C until the cultures reached the stationary phase. Samples were removed every day, diluted, and plated on solid 2:2 medium. The number of CFU was plotted against the incubation time.

UV and γ irradiation. ATCC 33020 and *recA* mutant derivatives were grown to mid-exponential phase in 9K liquid medium with ferrous iron as an energy source. Aliquots (50 μl) of 10^0 to 10^{-6} dilutions were spread on 2:2 or DOP solid medium. For UV irradiation, the plates were exposed to UV light (254 nm) at a dose rate of about 1.5 J/m²/s for 5 to 25 s. For γ irradiation, the plates were irradiated with ^{60}Co at a dose rate of 147 and 35 Gy/min. The plates were incubated for at least 2 weeks at 30°C. Survival was determined as the ratio of CFU per milliliter after irradiation to CFU per milliliter before irradiation.

DNA manipulations. General techniques were performed according to standard procedures (3) or the manufacturers' recommendations. Ultrapure plasmid DNA was obtained using the Wizard DNA purification system from Promega. *T. ferrooxidans* genomic DNA was prepared as previously described (5).

PCR. The 475-bp fragment from the rusticyanin gene of *T. ferrooxidans* was amplified with the oligonucleotides RUSNM (5′-GGCACGCTGGATTCCACA TGGAAAGAGGCG-3′) and RCX (5′-CCACTCGAGCCTTGACAATGATT TTACCAAACATACC-3′). The presence of *E. coli* cells was detected by the amplification of a 396- and a 570-bp fragment of the regulatory region of the operon encoding the major nitrate reductase of *E. coli* (6) with the oligonucleotide pairs S1 (5′-CACGGTTGGTATTGAGAAGC-3′) plus S2 (5′-CGCCGG ATTTCATTAAGAGC-3′) and S4 (5′-GCCTGCTTAAAGCTTTTCGC-3′) plus 64G (5′-TCCCCATCACTCTTGATCGTTATC-3′). The oligonucleotides KMTN5 (5′-CGATGCGCTGCGAATCGG-3′) and AKMTN5 (5′-GCAGCTG TGCTCGACGTTG-3′) were used to amplify a 531-bp fragment of the kanamycin resistance gene from Tn*5* (pJRD215), and the oligonucleotides KM1 (5′-A AGATCCTGGTATCGGTCTGC-3′) and KM2 (5′-AACATGGCAAAGGTA GCG-3′) were used to amplify a 524-bp fragment of the kanamycin resistance gene from Tn*903* (pKT240, pJB3Km1, and pUFR034). The presence of the ampicillin resistance gene of the pKT240 and pJB3Km1 plasmids was tested for by amplification of a 633-bp fragment with the oligonucleotides AAMP (5′-CC GTGTCGCCCTTATTCCC-3′) and AMP3 (5′-TGGTCCTGCAACTTTATCC GCC-3′). To amplify the region of the ATCC 33020 *recA* gene which overlaps the *Sma*I site where the Ω-Km interposon was inserted, the oligonucleotides RECA2 (5′-CGATGACGATGAGGTCC-3′) and RECA3 (5′-AAGGATGGTTACCCC TCG-3′) were chosen. The insertion junctions between the *recA* gene and the Ω-Km cassette were obtained by amplification of the DNA from Km^r clone 5 between oligonucleotides hybridizing on one side of the *recA Sma*I site, in which

the Ω-Km cassette had been inserted RECA4 (5′-CGGCTCGCTGGGTCTGG-3′) or ARECA5 (5′-CTGACAACTGGCTATGGC-3′), and an oligonucleotide corresponding to the end of the Ω-Km cassette, CKMTN5 (5′-GGAGTGGGG AGGCACGATGG-3′).

Sequence. The sequence of the PCR fragments RecA4-CKMTN5 and ARECA5-CKMTN5, corresponding to the junctions of the Ω-Km cassette insertion inside the *recA* gene (see above), were determined with the Thermo Sequenase II dye terminator cycle-sequencing premix kit from Amersham. The DNA sequences were compiled and analyzed through the World Wide Web Netscape facilities.

Southern hybridization. Genomic DNAs of strain ATCC 33020 and the kanamycin-resistant derivatives were digested with *Kpn*I and *Eco*RV restriction endonucleases, electrophoresed on agarose gel, and transferred by capillary blotting to positively charged Hybond-N membranes (Roche Biochemicals). The kanamycin resistance gene and *recA* probes were obtained by incorporation of alkali-labile DIG-dUTP (Roche Biochemicals) during PCR elongation with the oligonucleotides KMTN5 and AKMTN5 (see above) on one hand and the oligonucleotides RECA3 and RECA2 (see above), which bracket the *recA Sma*I site where the Ω-Km interposon was inserted, on the other hand. The hybridization was carried out under stringent conditions as recommended by the manufacturer.

RESULTS

Conjugative transfer of the IncP RP4 plasmid and mobilization of IncQ plasmids from *E. coli* to *T. ferrooxidans*. Although conjugation between *E. coli* and private *T. ferrooxidans* strains has been described (23), there is no report yet of this genetic transfer technique using collection strains of *T. ferrooxidans*. We first focused our study on the ATCC 33020 strain, because we have cloned several genes from this strain (1, 2, 5, 14), particularly the *recA* gene (14). Conjugation experiments between *E. coli* HB101 or MOS blue strains carrying the conjugative plasmid RP4 and *T. ferrooxidans* ATCC 33020 were carried out according to the method previously described by Peng et al. (23). Kanamycin-resistant clones were selected; *T. ferrooxidans* ATCC 33020 is sensitive to 200 μg of this antibiotic/ml on solid medium. The apparent transfer frequency obtained was lower than 10^{-8}.

Mobilization by RP4 of the broad-host-range IncQ plasmids pKT240 and pJRD215, which have been shown to replicate in *T. ferrooxidans* (17, 23) and which carry kanamycin resistance genes from different origins (Tn*903* and Tn*5*), was tested. Three- and two-partner conjugations were performed. In the first experiment, two *E. coli* strains were used: one (HB101) carries the helper plasmid (RP4), and the second (MC1061) carries the mobilizable plasmid (pKT240 or pJRD215); in the second experiment, only one *E. coli* strain was used, with the donor strain carrying the helper plasmid RP4 integrated within the chromosome (S17-1) and the mobilizable plasmid (pKT240 or pJRD215). In all the cases, the apparent transfer frequency to ATCC 33020 was approximately 10^{-7}. The mobilization of plasmid pJRD215 from *E. coli* S17-1 to the ATCC 19859 strain was also tested. In that case, the apparent transfer frequency was higher (10^{-4}).

Determination of optimal conditions for the mobilization of pJRD215 from *E. coli* S17-1 to *T. ferrooxidans* ATCC 33020. As shown above, the apparent transfer frequency obtained by the protocol of Peng et al. (23) for *T. ferrooxidans* ATCC 33020 was lower than 10^{-8} in the case of conjugative plasmids and about 10^{-7} in the case of mobilizable plasmids. We hypothesized that these low frequencies were due to the completely different growth conditions of the donor and recipient cells. Indeed, *E. coli* is a neutrophile, while *T. ferrooxidans* is an extreme acidophile; *E. coli* is a heterotroph, while *T. ferrooxidans* is a strict chemolithoautotroph. Furthermore, *E. coli* is a fast-growing microorganism (20-min generation time in Luria-Bertani medium) while *T. ferrooxidans* is a slow-growing microorganism (9-h generation time in 9K medium supplemented with ferrous iron). Transfer of genetic material by conjugation requires cell-to-cell contacts and energy for both the donor and the recipient cells. Accordingly, we sought growth media and mating medium that could minimize differences in growth conditions, thereby avoiding possible stress during mating. Rawlings et al. (28) have studied the effect of mixing Luria agar with inorganic agar medium (pH 4) containing tetrathionate on *E. coli* and *T. ferrooxidans* ATCC 33020 growth and on the mating efficiency between *E. coli* cells. They have noticed that *E. coli* was able to grow on all media tested except 100% inorganic agar but that the mating efficiency between *E. coli* cells dropped off as the percentage of inorganic agar increased. *T. ferrooxidans* was unable to grow in the presence of even a low concentration of Luria medium but remained viable. Because these results suggest that *E. coli* adapts more easily to inorganic conditions than *T. ferrooxidans* to organic conditions, the *E. coli* growth medium and the mating medium used were based on the inorganic medium described by Peng et al. (24).

The effect of adaptation of the donor and recipient cells to the mating medium on the transfer frequency was analyzed first. When *E. coli* S17-1 (pJRD215) was grown in 2:2 medium supplemented with 0.5% yeast extract instead of in Luria-Bertani medium, the apparent transfer frequency increased slightly (Table 2). The influences of the basal salt medium and of the energy source present in the *T. ferrooxidans* growth medium were also tested. As can be seen in Table 2, the best result was obtained with the 9K medium and with a sulfur compound (S^0 or thiosulfate) rather than ferrous iron as an energy source.

Different components of the mating medium were then analyzed. The effect of the pH ranging from 4.3 to 6.0 is given in Table 2. The apparent transfer frequency dropped quickly when the pH was lower than 4.6 or higher than 5.2. The concentration of thiosulfate in the mating medium was analyzed because this compound is an energy source for *T. ferrooxidans* but could be an inhibitor of *E. coli* when the concentration is too high. The highest apparent transfer frequency was obtained with a concentration of 0.05%. At higher concentrations, the frequency decreased. As already observed (23), the conjugation occurred even in the absence of an energy source for *T. ferrooxidans*. We have shown previously that diaminopimelic acid (DAP), a cell wall component, increases not only the growth rate of ATCC 33020 but also the viable cell numbers on 2:2 solid medium (19). Different concentrations of DAP in the mating medium were tested. At a concentration of 0.5×10^{-4} M, a significant increase in the apparent transfer frequency was obtained (Table 2).

We also tested different donor-to-recipient cell ratios, which is known to be important for conjugational transfer. As observed in Table 2, the apparent transfer frequency can vary from 6×10^{-7} for a 4/1 ratio to 1.2×10^{-5} for a 1/2 ratio.

By combining all the factors described above, that is, (i) by growing *E. coli* in 2:2 liquid medium with 0.5% yeast extract and growing ATCC 33020 in 9K liquid medium supplemented with sulfur as an energy source; (ii) by using the 2:2 solid medium with a pH of 4.8, 0.05% thiosulfate, and 0.5×10^{-4} M DAP as a mating medium; and (iii) by using a donor-to-recipient cell ratio of 1/2, the apparent transfer frequency of pJRD215 from *E. coli* S17-1 to ATCC 33020 can be increased 10^2- to 10^3-fold.

Mobilization of pUFR034 (IncW) and pJB3Km1 (IncP) plasmids from *E. coli* S17-1 to *T. ferrooxidans* ATCC 33020. Genetic transfer to *T. ferrooxidans* ATCC 33020 was also tested with the mobilizable plasmids from the IncW (pUFR034) and IncP (pJB3Km1) incompatibility groups. Under the optimized conditions described above, the apparent transfer frequency of

TABLE 2. Effect of the *E. coli* and *T. ferrooxidans* growth media, mating medium, and donor-to-recipient ratio on apparent transfer frequency

Parameter	Value	Apparent transfer frequency
E. coli growth medium	Luria-Bertani	0.7×10^{-6}
	2:2 (pH 5.2–5.4) + 0.5% yeast extract	2×10^{-6}
T. ferrooxidans growth medium	9K + $FeSO_4$	1×10^{-6}
	9K + S^0	3.2×10^{-5}
	2:2 + $Na_2O_3S_2$ + $FeSO_4$	2.6×10^{-6}
Mating medium		
pH	4.3	1×10^{-7}
	4.6	1.8×10^{-6}
	4.8	2×10^{-6}
	5	2.2×10^{-6}
	5.2	2×10^{-6}
	5.6	1×10^{-6}
	6	1×10^{-7}
$Na_2O_3S_2$ (%)	0	5.3×10^{-6}
	0.05	6.9×10^{-6}
	0.1	2.8×10^{-6}
	0.2	2×10^{-6}
DAP (M)	0	0.6×10^{-6}
	0.25×10^{-4}	6×10^{-6}
	0.5×10^{-4}	8×10^{-6}
	10^{-4}	4×10^{-6}
	2×10^{-4}	2×10^{-6}
E. coli/*T. ferrooxidans* ratio	1/4	4.1×10^{-6}
	1/3	7×10^{-6}
	1/2	1.2×10^{-5}
	1/1	2×10^{-6}
	2/1	1.4×10^{-6}
	4/1	6×10^{-7}

pJB3Km1 and pUFR034 was about 10 times lower than the frequency obtained with the IncQ plasmids (Table 3).

Mobilization of IncQ, IncW, and IncP plasmids from *E. coli* S17-1 to *T. ferrooxidans* ATCC 19859, BRGM1, and Tf-49. Three other *T. ferrooxidans* strains were tested as recipients: the two private strains BRGM1 and Tf-49, the latter being one of the strains tested for conjugation by Peng et al. (23), and the ATCC 19859 collection strain, from which several genes have been characterized. The results obtained under the optimized conditions described above are presented in Table 3. The IncP, IncQ, and IncW plasmids tested were all transferred to the four *T. ferrooxidans* strains tested. The apparent transfer frequency obtained depended on the *T. ferrooxidans* recipient strain and on the plasmid incompatibility group. More particularly, for all the strains tested, the apparent transfer frequency was highest with the IncQ plasmid.

TABLE 3. Apparent transfer frequency of pJRD215, pJB3Km1, and pUFR034 to *T. ferrooxidans* ATCC 33020, ATCC 19859, BRGM1, and Tf-49

Recipient	Transfer frequency of:		
	pJRD215 (IncQ)	pJB3Km1 (IncP)	pUFR034 (IncW)
ATCC33020	4.4×10^{-5}	1.2×10^{-6}	5×10^{-6}
ATCC19859	1.4×10^{-3}	6.2×10^{-7}	1×10^{-6}
BRGM1	2.5×10^{-3}	1.3×10^{-5}	2.2×10^{-6}
Tf-49	2×10^{-4}	2.7×10^{-6}	6.8×10^{-6}

Transconjugant analyses. For each conjugation experiment performed, eight kanamycin-resistant (Km^r) clones were analyzed to establish that they were true *T. ferrooxidans* transconjugants.

No contaminating *E. coli* donor cells were detected by PCR with two oligonucleotide pairs hybridizing to *E. coli* but not to *T. ferrooxidans* genomic DNA (Fig. 1). On the other hand, a fragment internal to the rusticyanin-encoding gene, which does not exist in *E. coli*, was obtained in all cases, indicating that the Km^r clones were indeed *T. ferrooxidans* cells (Fig. 1).

The presence of the kanamycin resistance gene in Km^r clones was checked by PCR with two oligonucleotides hybrid-

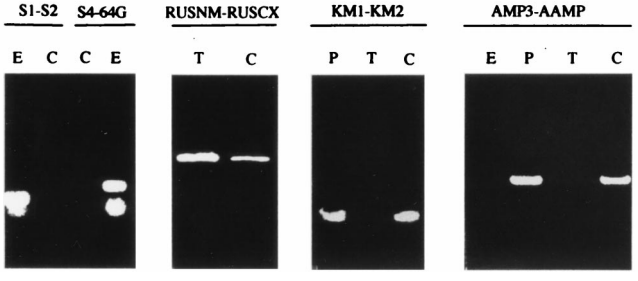

FIG. 1. PCR analyses of *E. coli* cells (E), *T. ferrooxidans*/pKT240 transconjugant cells (C), *T. ferrooxidans* cells (T), and pKT240 plasmid (P) with oligonucleotides hybridizing to the *E. coli* genomic DNA (S1 and S2, S4 and 64G), to the *T. ferrooxidans* genomic DNA (RUSNM and RUSCX), to the kanamycin resistance gene (KM1 and KM2), and to the ampicillin resistance gene (AMP3 and AAMP) of pKT240.

izing to the kanamycin resistance gene of Tn*5* in the case of pJRD215 plasmids or Tn*903* in the case of the pKT240, pUFR034, and pJB3Km1 plasmids (Fig. 1). In the same way, the presence of the ampicillin resistance gene of the pKT240 and pJB3Km1 plasmids was checked by PCR (Fig. 1).

The plasmids purified from the Km^r clones had the expected restriction sites (data not shown). Furthermore, after transformation of *E. coli* HB101 with these plasmid preparations, Km^r clones were obtained.

All these results show unambiguously that the mobilizable plasmids had been introduced successfully into *T. ferrooxidans* by conjugation and were not integrated into the chromosome.

Stability analysis. The stabilities of the different plasmids tested in the four *T. ferrooxidans* strains studied were analyzed as described in Materials and Methods. The IncQ plasmid pJRD215 was stable, especially in the two collection strains ATCC 19859 and ATCC 33020, with more than 70% retention after 40 generations without antibiotic selection (Fig. 2A). On the other hand, the plasmid pUFR034 (IncW) (Fig. 2B) and, more particularly, the pJB3Km1 (IncP) (Fig. 2C) plasmids, were unstable: after 40 generations, fewer than 10% of the clones retained pUFR034 (IncW) and all the clones had lost pJFB3Km1 (IncP).

Construction of a *T. ferrooxidans* ATCC 33020 *recA* mutant. To test whether conjugation can be used successfully as a genetic transfer technique to construct null mutants by reverse genetics, we chose the *recA* gene as a model because (i) construction of *recA* mutants by marker exchange mutagenesis has already been described in several microorganisms (22); (ii) since the RecA protein from the ATCC 33020 strain has the classical RecA biochemical activities (25, 26), a *T. ferrooxidans recA* mutant would be expected to have the same properties as the bacterial *recA* null mutants already characterized; (iii) the *recA* gene has been shown to be independently transcribed from the downstream essential *alaS* gene encoding alanyl tRNA synthetase (14), and consequently, *recA* insertional inactivation will not have a detrimental polar effect on *alaS* expression; and (iv) a *T. ferrooxidans recA* mutant is required for further genetic studies to stably maintain recombinant plasmids.

To obtain a *T. ferrooxidans recA* mutant, a mobilizable suicide plasmid carrying the *recA* gene disrupted with a cassette carrying the kanamycin resistance gene was constructed (see Materials and Methods). This plasmid (pUC19*recA*::Ω-Km*mob*), which is unable to replicate in *T. ferrooxidans*, was mobilized from an *E. coli* S17-1 strain into strain ATCC 33020 by conjugation (see Materials and Methods), and kanamycin-resistant (Km^r) clones were selected. Only five Km^r clones were obtained from two independent conjugation experiments after 4 weeks of incubation at 30°C. These clones were indeed *T. ferrooxidans* cells carrying the Ω-Km cassette as shown by PCR analyses (see Materials and Methods). Furthermore, no plasmid had been detected by plasmid purification and transformation of *E. coli*, suggesting that either the plasmid had been integrated into the chromosome by a single-crossover event or that recombination between the suicide plasmid carrying the mutated *recA*::Ω-Km allele and the chromosome carrying the wild-type *recA* allele had taken place. To increase the likelihood of this double-crossover event, the five Km^r clones were subcultured twice in liquid medium for several generations without antibiotic. Under these conditions, three clones (no. 2, 3, and 4) lost their resistance to kanamycin, a result confirmed by PCR analysis (data not shown) and Southern blot hybridization with a probe corresponding to an internal fragment of the Km^r gene (Fig. 3A). We conclude that clones 2, 3, and 4 have lost the suicide plasmid. The two other

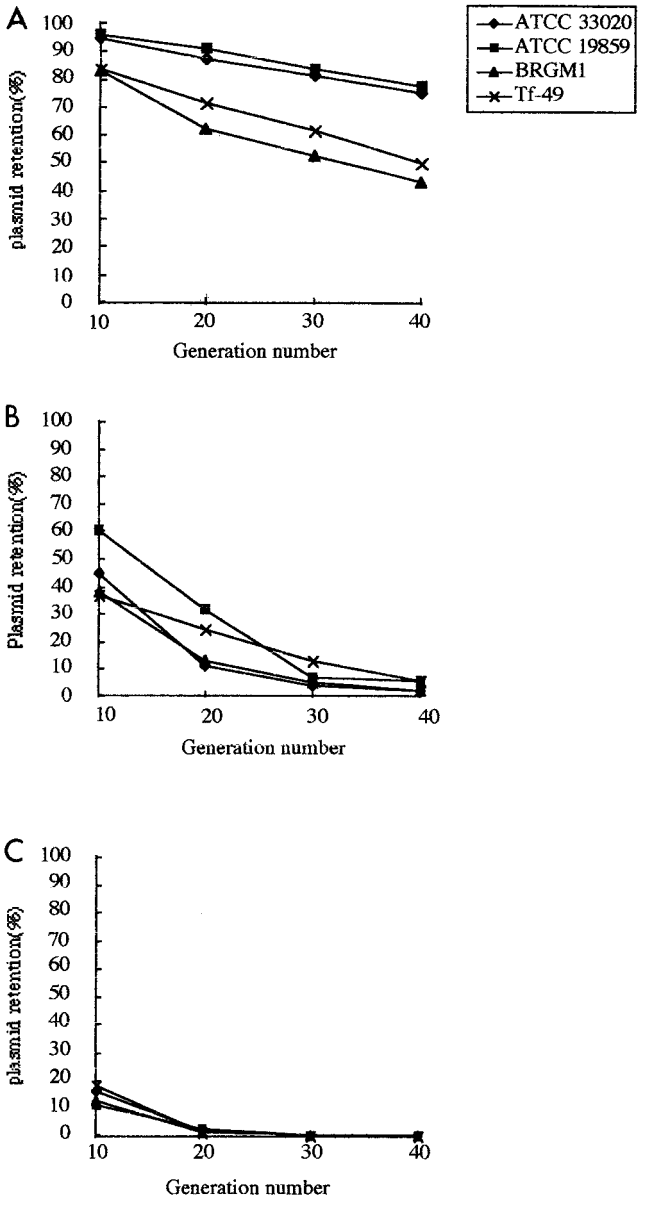

FIG. 2. Stability analysis of pJRD215 (IncQ) (A), pUFR034 (IncW) (B), and pJB3Km1 (IncP) (C) in *T. ferrooxidans* ATCC 33020, ATCC 19859, BRGM1, and Tf-49 strains.

clones (no. 1 and 5) kept the Km^r phenotype after several generations in the absence of kanamycin. The presence of the kanamycin resistance gene was confirmed by PCR analysis (data not shown). On the other hand, the ampicillin resistance gene could not be detected by PCR in these two clones. All these results suggest that a recombination event had taken place at the *recA* locus.

Characterization of *recA* mutants. To determine if *recA* is disrupted by the Ω-Km cassette in the putative *recA* mutants 1 and 5, genomic DNA was purified from these clones and compared by Southern blot hybridizations and PCR analyses to the DNA from the three Km^s clones 2, 3, and 4 and to the DNA from the wild-type ATCC 33020 strain.

When an internal fragment of the *recA* gene was used as the probe, a 4.8-kb *Kpn*I fragment and a 2.1-kb *Eco*RV fragment

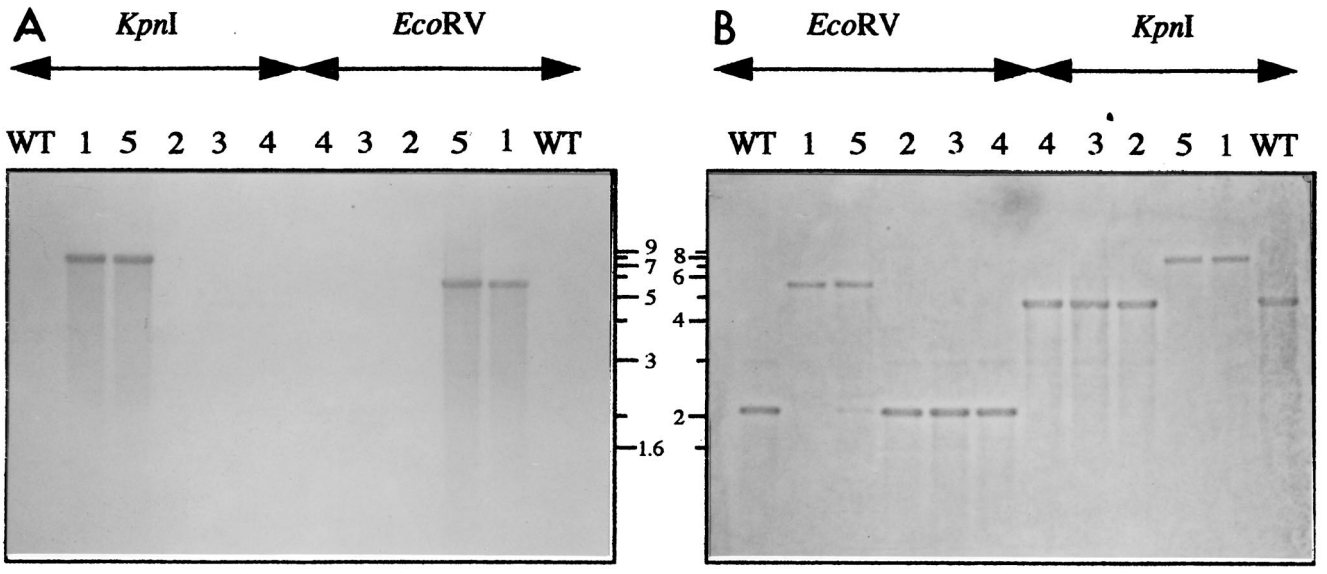

FIG. 3. Southern blot analysis of *Kpn*I- and *Eco*RV-digested DNA isolated from *T. ferrooxidans* ATCC 33020 (lane WT); Km^s clones 2 (lane 2), 3 (lane 3), and 4 (lane 4); and Km^r clones 1 (lane 1) and 5 (lane 5). The blots were probed with a PCR fragment internal to the Km gene (A) and with a PCR fragment internal to the *recA* gene (B). The numbers between panels A and B indicate the sizes (in kilobases) of some fragments from the molecular size marker from Roche Biochemicals.

were obtained with the wild type and the Km^s clones 2, 3, and 4, whereas an 8.1-kb *Kpn*I fragment and a 5.4-kb *Eco*RV fragment were obtained with the Km^r clones 1 and 5 (Fig. 3B). These results suggest the disruption of the *recA* gene in Km^r clones 1 and 5. The probe corresponding to an internal fragment of the Km^r gene did not hybridize to the DNA from the wild type or from Km^s clones 2, 3, and 4 but did hybridize to the same 8.1-kb *Kpn*I fragment and 5.4-kb *Eco*RV fragments described above in Km^r clones 1 and 5 (Fig. 3A). Altogether, the Southern hybridization results suggest that the Ω-Km cassette had been inserted within the *recA* gene in the two Km^r isolates 1 and 5.

PCR analyses and sequencing of the *recA* region from Km^r clones 1 and 5 confirmed the insertion of the Ω-Km cassette inside the *recA* gene (data not shown). Km^r clones 1 and 5 will now be referred as *recA* 1 and *recA* 5 mutants.

Properties of the *T. ferrooxidans recA* mutants. The *T. ferrooxidans recA* 5 strain showed a slightly reduced growth rate compared to the wild-type strain (data not shown), which is typically observed for *recA* mutants.

To compare the effectiveness of DNA repair mechanisms in the wild-type strain and *recA* mutants, we used sensitivity to physical DNA-damaging agents. Sensitivities to irradiation by UV and γ rays are presented in Fig. 4. As expected for *recA* mutants, *recA* 1 and *recA* 5 strains were more sensitive than the parental ATCC 33020 strain to both UV and γ radiations.

DISCUSSION

This paper reports the first construction by marker exchange mutagenesis of a mutant of the extreme acidophilic *T. ferrooxidans*. Reverse genetics was made possible by significantly improving the conditions for the transfer of plasmids from *E. coli* to the ATCC 33020 culture collection strain of *T. ferrooxidans* by conjugation. The apparent transfer frequency was shown to depend on the donor-to-recipient cell ratio and on the physiological state of the donor and of the recipient cells, two factors which are known to be important for conjugational transfer. The conjugation protocol described in this paper was followed to mobilize plasmids from the three incompatibility groups, IncQ, IncP, and IncW, to four *T. ferrooxidans* strains, BRGM1, Tf-49, ATCC 19859, and ATCC 33020. Significantly, the IncW pUFR034 and, more particularly, the IncP plasmid pJB3Km1 were unstable in the four strains tested. These plasmids may therefore be used as shuttle vectors for a marker exchange mutagenesis program. On the other hand, the IncQ plasmids, which appeared to be stably maintained, may be useful for construction of expression vectors to introduce heterologous or homologous genes into *T. ferrooxidans* and for construction of operon fusion vectors to study *T. ferrooxidans* gene expression.

We have demonstrated that conjugation under the conditions described in this study can be used successfully as a genetic transfer technique to construct null mutants by reverse genetics. The knockout of the ATCC 33020 *recA* gene was confirmed by both molecular and physiological approaches. Evidence of *recA* gene disruption by the Ω-Km cassette includes the results of Southern hybridizations, PCR analyses, and sequencing on both sides of the Ω-Km cassette. Moreover, as expected for *recA* mutants (22), the knockout mutants exhibit slightly reduced growth rates and are more sensitive to UV and γ irradiation compared to the parental strain. It is worth mentioning that the *T. ferrooxidans recA* mutants were not as sensitive to UV and γ irradiation as *recA* mutants described in other bacterial species. One could then speculate whether another *recA* gene is present in the *T. ferrooxidans* ATCC 33020 strain. This is unlikely because only one *recA* gene has been found by Southern hybridization (this paper and reference 25) and only one *recA* gene has been cloned by complementation of an *E. coli recA* mutant (25). Another possibility is that RecA-dependent DNA repair is a minor pathway in *T. ferrooxidans* compared to other repair mechanisms. In *E. coli*, the UV and γ irradiation-induced DNA lesions are primarily removed by nucleotide and base excision repair processes, respectively (see reference 30 and references therein). However, if the replication fork encounters a lesion before repair has taken place, replication stalls or collapses. Replication restarts only from recombination intermediates generated

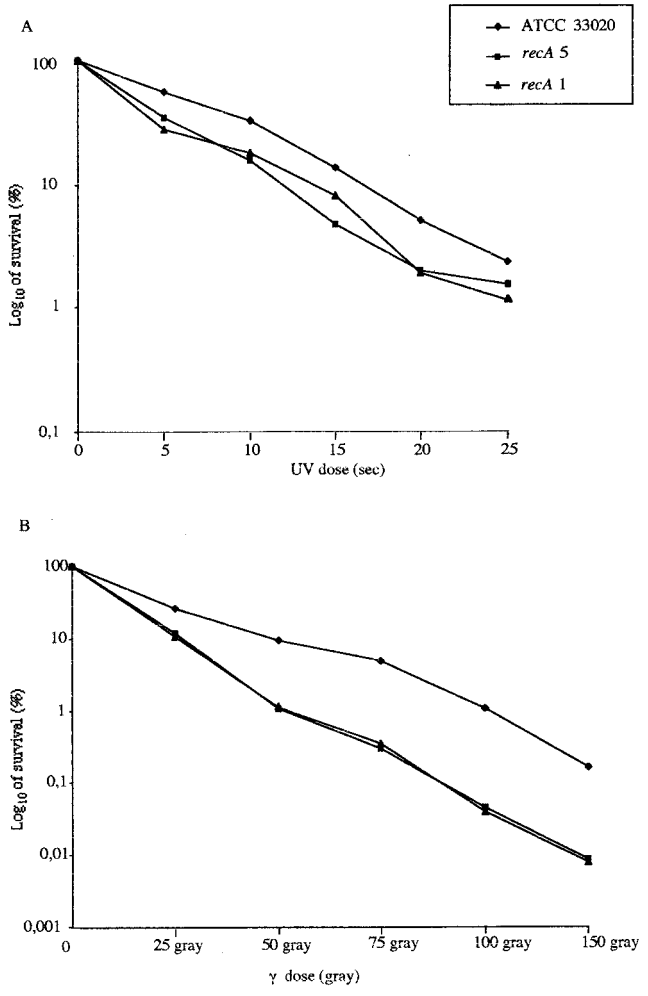

FIG. 4. Survival of strain ATCC 33020 and of the *recA* mutants after UV (A) and γ (B) irradiation.

by RecA and accessory proteins, such as RecFOR (gap repair) and RecBCD (double-strand break repair) (18; see references 9, 20, and 30 and references therein). Therefore, RecA is absolutely required for DNA repair at the level of the replication fork in *E. coli*. Because this bacterium is a fast-growing microorganism, RecA plays a key role in the survival of exponentially growing cells exposed to UV or γ irradiation. Accordingly, the basal level of RecA protein is high, and *recA* gene transcription is increased further by induction of the SOS response (see reference 32 and references therein). Because the *T. ferrooxidans* generation time is long (9 h at 30°C) but its replication rate is likely similar to that of *E. coli* (about 1,000 nucleotides per s [16]), it is reasonable to assume that in *T. ferrooxidans*, prereplicative repair processes (nucleotide and base excision repair) have sufficient time to repair most DNA damage before the arrival of the replication fork. Postreplication RecA-dependent recombinational repair processes would therefore play a minor role. In agreement with this hypothesis, the basal level of RecA protein in the *T. ferrooxidans* ATCC 33020 strain is low (25) and the *recA* gene is not induced by DNA damaging agents (26).

The construction of a *T. ferrooxidans recA* mutant should facilitate future genetic studies of this chemolithoautotrophic acidophilic microorganism by allowing the stable maintenance of plasmids in which homologous or heterologous genes have been cloned. Furthermore, the allelic replacement procedure used to produce the *recA* mutant should be applicable to the construction of null mutants of the genes encoding proteins whose physiological function have yet to be determined.

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