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Gene Transfer in Magnetic Bacteria: Transposon Mutagenesis and Cloning of Genomic DNA Fragments Required for Magnetosome Synthesis

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Broad-host-range IncP and IncQ plasmids have been transferred to the aerobic magnetic bacterium *Aquaspirillum* sp. strain AMB-1. Conjugal matings with *Escherichia coli* S17-1 allowed high-frequency transfer of the RK2 derivative pRK415 (4.5×10^{-3} transconjugant per recipient cell) and the RSF1010 derivative pKT230 (3.0×10^{-3} transconjugant per recipient). These plasmids successfully formed autonomous replicons in transconjugants and could be isolated and transformed back into *E. coli*, illustrating their potential as shuttle vectors. A mobilizable plasmid containing transposon Tn*5* was transferred to *Aquaspirillum* sp. strain AMB-1 and also to the obligately microaerophilic magnetic bacterium *Aquaspirillum magnetotacticum* MS-1. Five nonmagnetic kanamycin-resistant mutants of *Aquaspirillum* sp. strain AMB-1 in which Tn*5* was shown to be integrated into the chromosome were obtained. Different genomic fragments containing the mutagenized regions were cloned into *E. coli*. Two genomic fragments were restriction mapped, and the site of Tn*5* insertion was determined. They were shown to be identical, although derived from independent transposon insertions. One of these clones was found to hybridize strongly to regions of the *A. magnetotacticum* MS-1 chromosome. This is the first report of gene transfer in a magnetic bacterium.

Magnetic bacteria synthesize intracellular particles of magnetite (Fe_3O_4) which are aligned in chains and enveloped by a membrane (17). These structures, known as magnetosomes, impart a magnetic dipole to the bacterial cell, which is then sensitive to externally applied magnetic fields. The ecological significance of these biological magnets remains unclear, although it has been proposed that sensitivity to the geomagnetic field allows oxygen-sensitive magnetic bacteria to swim downward into oxygen-poor sediments, their preferred habitat (22).

Biogenic magnetite was originally identified as a strengthening mineral in the radula teeth of chitons (*Mollusca* and *Polyplacophora* spp.) (15). In addition, magnetic particles have also been found in the honeybee (8) and also in higher animals such as the pigeon (23) and in migratory fishes (14, 25). This magnetite is involved in the sensory perception of the geomagnetic field and in the recognition of direction and location (11, 13, 24). Although biomineralization of magnetite is clearly important from an ecological and evolutionary standpoint, the molecular mechanisms by which magnetite crystals are formed remain unknown. In order to begin to understand such mechanisms, we have chosen as a model organism for magnetite biomineralization the freshwater magnetic bacterium *Aquaspirillum* sp. strain AMB-1 (18).

The application of recombinant DNA techniques to study the genetic control of intracellular magnetite crystallization has been prevented by difficulties in the laboratory culture, purification, and colony formation ability of magnetic bacteria (16, 17). Since their discovery in 1975 (4), only three isolates have been obtained as axenic cultures: an obligate microaerophile, *Aquaspirillum magnetotacticum* MS-1 (5); a vibroid marine bacterium designated MV-1 (2); and an aerobic magnetic bacterium, *Aquaspirillum* sp. strain AMB-1 (18). Colony formation has not been reported for MV-1, and although colony formation by *A. magnetotacticum* MS-1 has been reported (6), in our hands, colony formation with this species is difficult to achieve. However, the recently isolated *Aquaspirillum* sp. strain AMB-1 readily forms colonies on agar plates (18). This has facilitated the isolation of a number of nonmagnetic mutants which appear as white colonies rather than the dark brown to black appearance of wild-type colonies.

To investigate further the mechanism of magnetite biomineralization in *Aquaspirillum* sp. strain AMB-1, a molecular genetic approach has been used. Initial studies confirmed that AMB-1 did not possess endogenous plasmids from which shuttle vectors could be constructed. In addition, attempts to transform this species by a variety of calcium chloride-mediated procedures proved unsuccessful. We therefore used conjugation to attempt gene transfer, initially of the plasmid pSUP1021, a pBR325 derivative containing the kanamycin resistance marker transposon Tn*5* (20). In addition, conjugation procedures were optimized to give maximum transfer frequencies by using the broad-host-range plasmids pRK415 (12) and pKT230 (1).

We describe here the first example of gene transfer in a magnetic bacterium and report the isolation of three genomic fragments from *Aquaspirillum* sp. strain AMB-1 involved with the synthesis of magnetosomes. Two of these fragments were restriction mapped, and the position of the transposon insertion was determined. In addition, hybridization studies have indicated that homology exists between the *Aquaspirillum* sp. strain AMB-1 sequences and the chromosome of *A. magnetotacticum* MS-1.

MATERIALS AND METHODS

Strains and plasmids. Table 1 lists the strains and plasmids used in this study. pKT230 is an IncQ plasmid with an

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Plasmids		
pUC19	Ap^r, cloning vector	26
pKT230	Km^r Sm^r *mob*$^+$	1
pRK415	Tc^r *lacZ mob*$^+$	12
pSUP1021	Km^r Cm^r Mob Tn*5*	20
pSUP202	Ap^r Mob	20
pCN3	8.8-kb *Eco*RI fragment from *Aquaspirillum* sp. strain AMB-1 nonmagnetic mutant NM3 cloned into pUC19	This work
pCN5	8.8-kb *Eco*RI fragment from *Aquaspirillum* sp. strain AMB-1 nonmagnetic mutant NM5 cloned into pUC19	This work
E. coli strains		
S17-1	Pro r^- m^+ $RecA^-$ Tp^r Sm^r ΩRP4-Tc::Mu-Kn::Tn*7*	20, 21
DH5α	*supE44 lacU169 hasR17* (ϕ80 *lacZΔM15*) *recA1 endA1 gryA96 thi-1 relA1*	9, 10, BRL Inc., Tokyo, Japan
Magnetic bacteria		
Aquaspirillum sp. strain AMB-1	Wild type	18
A. magnetotacticum MS-1	Wild type	6, ATCC 31632

[a] Resistance abbreviations: Ap, ampicillin; Tc, tetracycline; Tp, trimethoprim; Sm, streptomycin; Cm, chloramphenicol; Km, kanamycin, Mob, P-type specific recognition site for mobilization (Mob site).

RSF1010 origin of replication (1). The IncP plasmid pRK415 is a derivative of RK2 which contains a pUC19 polylinker (12). Both plasmids are broad-host-range vectors and stably maintained in a wide variety of gram-negative bacteria. pSUP202 is a mobilizable pBR325 derivative which contains the Mob site from RP4 (20, 21). The plasmid pSUP1021, which has a pACYC184 replicon, also contains transposon Tn*5*. *Escherichia coli* strains were grown on Luria broth at 37°C (19). Antibiotics were used at the following final concentrations: kanamycin, 25 $\mu g \cdot ml^{-1}$; tetracycline, 12.5 $\mu g \cdot ml^{-1}$; and ampicillin, 25 $\mu g \cdot ml^{-1}$. Magnetic bacteria were grown at 25°C on a previously described chemically defined medium, MSGM (6), from which resazurin had been omitted. For magnetic bacteria, antibiotics were used at the following final concentrations: kanamycin, 10 $\mu g \cdot ml^{-1}$; tetracycline, 5 $\mu g \cdot ml^{-1}$; and ampicillin, 10 $\mu g \cdot ml^{-1}$.

Plasmid transfer by transconjugation. *E. coli* S17-1 was used to mobilize plasmids into both species of magnetic bacteria. S17-1 was transformed with the appropriate plasmid (19). Several freshly transformed colonies were suspended in 50 μl of MSGM medium, and the cell concentration was determined with a hemocytometer. This cell suspension was diluted with MSGM medium, and the appropriate volume was mixed with 50 μl of a mid-exponential-phase culture of magnetic bacteria, containing approximately 5×10^7 cells per ml. The mating suspensions which contained different donor-recipient ratios were spotted onto dried MSGM agar plates. When the recipient was *A. magnetotacticum* MS-1, plates contained catalase (30 U/ml) (6). Plates were incubated at 25°C for 6 h. Cells were then harvested with liquid medium and inoculated onto MSGM agar plates containing appropriate antibiotics. Transconjugants were observed after 7 to 10 days of growth at room temperature. Antibiotic-resistant colonies of magnetic bacteria were picked up and purified by serial dilution in MSGM medium in which the donor *E. coli* could not grow. The magnetic or nonmagnetic phenotype was assayed by observing the effect of an applied external magnetic field on the cells under the microscope. The identity of nonmagnetic mutants was confirmed by light microscopy, by transmission electron microscopy, and by a comparison of the carbon source requirements of the mutants with those of the wild-type cells (data not shown).

Analysis of DNA. Plasmid extraction from transconjugants, restriction enzyme digestions, and agarose gel electrophoresis were carried out as described elsewhere (19). Hybridization reactions were performed by using the nonradioactive Chemiprobe procedure (Orgenics Ltd., Yavne, Israel) according to manufacturer's instructions. Plasmid DNA extracted from magnetic bacterial transconjugants was transformed into *E. coli* DH5α cells by the Hanahan method (9, 10, 19). Plasmid DNA extracted from *E. coli* transformants was subjected to hybridization analysis as described above.

Genomic DNA from magnetic bacteria was isolated by standard procedures (19). Southern hybridization analysis was carried out by using an *Xho*I restriction fragment of Tn*5* as a probe. *Eco*RI digests of total DNA from each kanamycin-resistant (Km^r) mutant were shotgun cloned into pUC19 and transformed into *E. coli* DH5α. Tn*5* containing genomic DNA was then isolated from Km^r colonies. The region of chromosomal DNA flanking the transposon was then isolated by restriction digestion with *Eco*RI and *Hpa*I. Digestion by *Hpa*I was employed to remove Tn*5*. The fragment was used as a probe during subsequent Southern hybridization analysis. Following agarose gel electrophoresis, DNA fragments were removed from the gel and purified by dialysis (19).

RESULTS

Conjugal gene transfer between *E. coli* and *Aquaspirillum* sp. strain AMB-1. We chose a previously described broad-host-range mobilization system to attempt gene transfer in *Aquaspirillum* sp. strain AMB-1 (21). We have used the transfer functions of the broad-host-range plasmid RP4 to mobilize plasmids from *E. coli* to *Aquaspirillum* sp. strain AMB-1. To prevent self transfer of RP4, the RP4 DNA has been immobilized into the chromosome of the donor strain S17-1. This strain is devoid of the *E. coli* K-12-specific DNA restriction system which allows efficient uptake of foreign DNA. Furthermore, the kanamycin resistance gene from

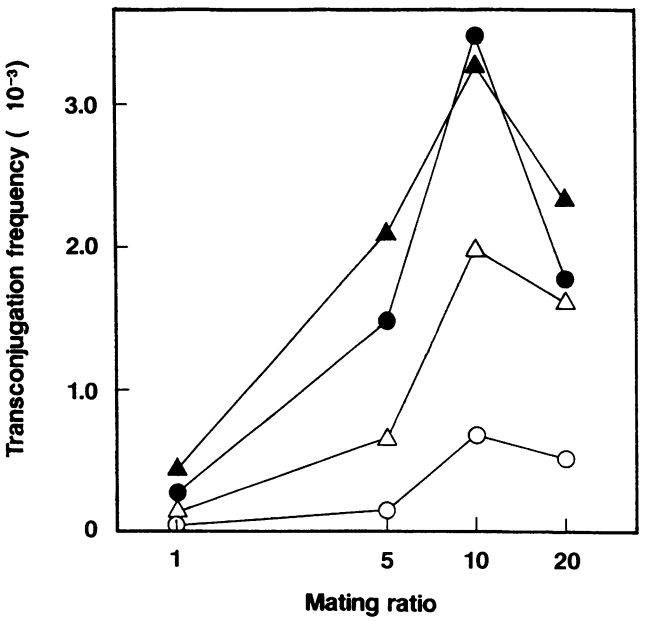

FIG. 1. Conjugation frequencies in *Aquaspirillum* sp. strain AMB-1. Plasmid pKT230 was transferred at different donor-recipient (mating) ratios and incubation times. Frequencies are given as transconjugants per recipient cell. Mating times: 2 h (○), 4 h (△), 6 h (●), and 8 h (▲).

RP4 has been inactivated, allowing selection for kanamycin resistance plasmids or transposons. Using the conditions described in Materials and Methods, we optimized transfer of pKT230 by performing matings at different donor-recipient ratios and for different times. The results are shown in Fig. 1. Kanamycin-resistant transconjugants were detected at frequencies of up to 3×10^{-3} per recipient cell. Similar experiments were carried out for transfer of pRK415 (data not shown). Optimum conditions for this vector occurred at a donor-recipient ratio of 100:1 and for 6 h at 25°C. Frequencies of the order of 4.5×10^{-3} per recipient were obtained for pRK415 transfer. The presence of antibiotics did not affect the ability of transconjugants to produce magnetosomes. Spontaneous mutations to kanamycin or tetracycline resistance were not observed.

pSUP202 and pSUP1021, which has a pACYC184 replicon, were also transferred to *Aquaspirillum* sp. strain AMB-1. Ampicillin-resistant colonies were obtained following pSUP202 transfer. However, no subsequent growth could be obtained in the presence of antibiotic. When pSUP1021 was transferred to *Aquaspirillum* sp. strain AMB-1, kanamycin-resistant colonies were obtained. To determine whether pSUP1021 replicated in AMB-1, total DNA was then purified and dot blots were probed with an *Eco*RI-*Bam*HI restriction fragment from the vector region of plasmid pSUP1021. No hybridization was detected (data not shown).

Analysis of *Aquaspirillum* sp. strain AMB-1 transconjugants. Agarose gel electrophoresis revealed the presence of plasmids pRK415 and pKT230 in transconjugants. They appeared as faint bands of the correct molecular weight, so Southern analysis was carried out to confirm that these bands were indeed the transferred plasmids (Fig. 2). Plasmid DNA from putative pKT230 transconjugants was cut with either *Pst*I or *Eco*RI and probed with the pKT230 plasmid (Fig. 2, lanes 3 and 4). All of the transconjugants tested contained bands of the correct size which hybridized with the probe. In addition, plasmid preparations from AMB-1 transconjugants were used to transform *E. coli*. Plasmids were then recovered from *E. coli* and shown to have the same restriction profile. Total chromosomal DNA from AMB-1 did not contain sequences which hybridized to the plasmid probes. To confirm that the transconjugants were indeed *Aquaspirillum* sp. strain AMB-1, several tests were performed. Microscopic analysis verified the spirillum morphology, and the ability to grow on MSGM medium suggested that the transconjugants were *Aquaspirillum* sp. strain AMB-1, since donor *E. coli* could not grow on this medium. In addition, carbon source utilization profiles of transconjugants were found to be identical to that of wild-type AMB-1 (data not shown).

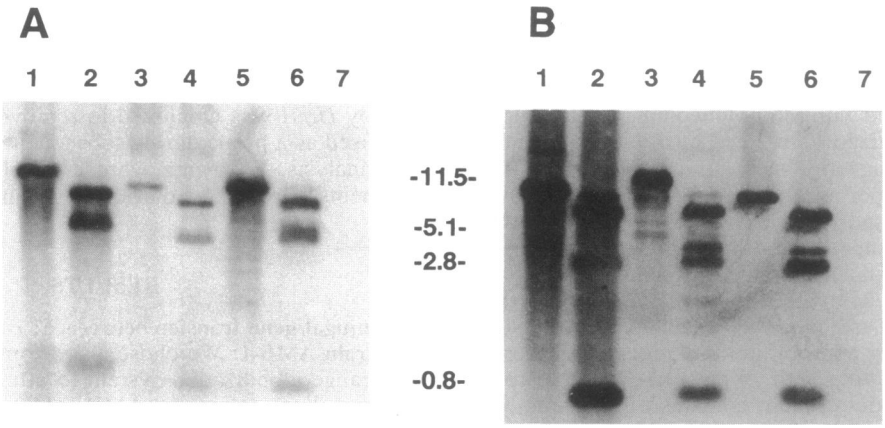

FIG. 2. Southern hybridization analysis of transconjugants. Probes used were pKT230 (A) and pRK415 (B). (A) Lanes 1 and 2, pKT230 purified from *E. coli* by ultracentrifugation; lanes 3 and 4, pKT230 purified from AMB-1 transconjugant; lanes 5 and 6, pKT230 from AMB-1 transformed and reisolated from *E. coli* DH5α; lane 7, genomic DNA from wild-type AMB-1. DNA samples in lanes 1, 3, 5, and 7 were digested with *Eco*RI, and samples in lanes 2, 4, and 6 were digested with *Pst*I. (B) Lanes 1 and 2, pRK415 purified from *E. coli* by ultracentrifugation; lanes 3 and 4, pRK415 extracted from AMB-1 transconjugant; lanes 5 and 6, pRK415 transferred back into *E. coli* DH5α from AMB-1 transconjugant; lane 7, genomic DNA from wild-type AMB-1. DNA samples in lanes 1, 3, 5, and 7 were digested with *Eco*RI, and samples in lanes 2, 4, and 6 were digested with *Sma*I. Sizes are indicated in kilobases.

Conjugal transfer of pSUP1021 to *A. magnetotacticum* MS-1. Conjugations were performed using *E. coli* S17-1 containing the mobilizable plasmid pSUP1021 (20), which contains transposon Tn*5*. Optimum conditions for transfer of this plasmid to *Aquaspirillum* sp. strain AMB-1 were determined (see below), and these conditions were also used for matings with *A. magnetotacticum* MS-1. However, the matings were harvested from the agar plates (which contained catalase) and inoculated into liquid MSGM medium as well as onto fresh MSGM plates containing kanamycin. We were unable to observe kanamycin-resistant colonies or colonies on control plates containing no antibiotic. However, when liquid cultures were observed, kanamycin-resistant *A. magnetotacticum* MS-1 cells grew to high cell concentrations ($>10^8$ cells per ml) after 5 to 7 days of growth. Control matings with S17-1 which did not contain pSUP1021 did not give rise to kanamycin-resistant cells.

Random transposon mutagenesis of *Aquaspirillum* sp. strain AMB-1 and cloning of chromosomal regions involved in synthesis of magnetosomes. The plasmid pSUP1021 (20) was used to introduce the transposon Tn*5* into *Aquaspirillum* sp. strain AMB-1. This plasmid contains the RP4-specific Mob site and can therefore be mobilized with high frequency into *Aquaspirillum* sp. strain AMB-1 by using *E. coli* S17-1. pSUP1021 also has a narrow-host-range basic replicon from pACYC184 and is therefore unable to replicate in *Aquaspirillum* sp. strain AMB-1. Thus, transposition events can be selected for by screening for kanamycin resistance, while the initial transposon carrier plasmid is eliminated (21). Transposon Tn*5* was selected for these studies because it has demonstrated very little site specificity and produces polar insertion mutations (3). The optimum mating conditions for Tn*5* transfer to *Aquaspirillum* sp. strain AMB-1 were determined. A donor-recipient ratio of 10:1 gave maximum transfer frequencies of 1.9×10^{-4} per recipient. Matings were incubated on MSGM agar at room temperature for 6 h. Kanamycin-resistant colonies were selected, purified, and assayed for magnetic sensitivity. Of the 118 kanamycin-resistant colonies tested, 5 were found to be completely nonmagnetic while 2 showed reduced magnetite production compared with that of the wild type (data not shown). The nonmagnetic transconjugants were denoted NM1, NM2, NM3, NM5, and NM7. The nonmagnetic phenotype of these mutants was confirmed by transmission electron microscopy. To verify that kanamycin resistance was due to the insertion of Tn*5* into the AMB-1 chromosome, we first confirmed the loss of the transposon carrier plasmid pSUP1021 by performing dot blot analysis and hybridizing total DNA with a pSUP1021-specific probe. No hybridization was observed. From this we conclude that pSUP1021 has been lost because of the inability of the pACYC184 replicon to function in *Aquaspirillum* sp. strain AMB-1. In addition, genomic DNA from each mutant was probed with a Tn*5*-specific *Xho*I restriction fragment purified from pSUP1021 (Fig. 3). Chromosomal insertion of Tn*5* into four different *Eco*RI genomic fragments was obtained. Four Tn*5*-containing chromosomal *Eco*RI fragments were cloned into *E. coli* DH5α as described in Materials and Methods. Three clones, plasmids pCN1, pCN3, and pCN5, were obtained from nonmagnetic transconjugants NM1, NM3, and NM5, respectively. One clone, plasmid pCN3, contained an 8.8-kb *Eco*RI insert consisting of approximately 3 kb of AMB-1 chromosomal DNA interrupted with Tn*5*. This fragment was restriction mapped, and the site of Tn*5* insertion was determined. A second clone, pCN5, also contained an 8.8-kb *Eco*RI fragment with an identical restriction pattern, although this fragment was obtained from a independent transposition event (Fig. 4). AMB-1 chromosomal DNA was purified from pCN3 by restriction digestion and used to probe *A. magnetotacticum* MS-1 genomic DNA (Fig. 5). Hybridization signals in lane 4 indicate that significant homology exists between the pCN3 AMB-1-specific DNA and regions of the *A. magnetotacticum* MS-1 chromosome. The wild-type 3-kb *Eco*RI fragment is seen in lane 3. Nonspecific binding of AMB-1 DNA to pUC19 DNA from pCN3 in lane 1 has resulted in a faint signal at 2.7 kb.

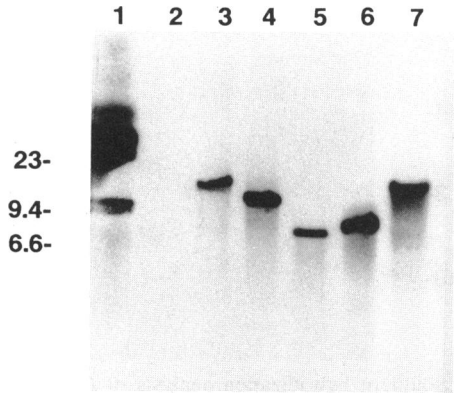

FIG. 3. Southern hybridization analysis of *Eco*RI-digested genomic DNA from nonmagnetic mutants with Tn*5* as a probe. DNA was digested with *Eco*RI and subjected to electrophoresis. Transferred DNA samples were probed with a Tn*5* *Xho*I restriction fragment purified from pSUP1021. Lane 1, pSUP1021 (undigested); lane 2, wild-type genomic DNA from *Aquaspirillum* sp. strain AMB-1; lanes 3 to 7, total DNA from the nonmagnetic mutants NM1, NM2, NM3, NM5, and NM7, respectively. Sizes are indicated in kilobases.

DISCUSSION

We have demonstrated high-frequency conjugal transfer of broad-host-range cloning vectors in two species of magnetic bacteria; the aerobic *Aquaspirillum* sp. strain AMB-1 and the obligate microaerophile *A. magnetotacticum* MS-1. Results from preliminary transposon mutagenesis experiments indicate that at least three regions of the AMB-1 chromosome are required for the successful synthesis of magnetosomes.

High conjugative transfer frequencies were obtained at optimum donor-recipient ratios and incubation times. Conjugation conditions which favored the recipient gave higher frequencies than conditions which suited the donor. The use

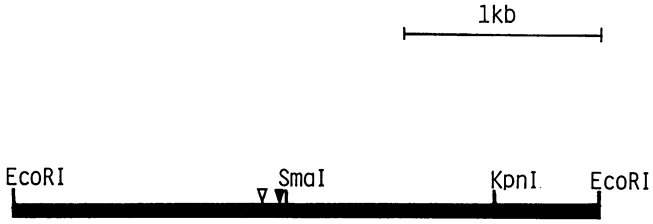

FIG. 4. Partial restriction map of the 8.8-kb *Eco*RI insert from pCN3. Only restriction enzymes useful for cloning into unique sites in the pUC19 polylinker were determined. Sites of transposon insertions are shown as arrowheads. The filled arrowhead is Tn*5* from pCN3, and the open arrowhead is Tn*5* from pCN5.

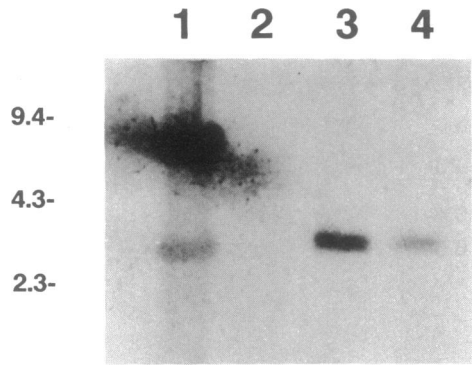

FIG. 5. Southern hybridization analysis of *Eco*RI-digested genomic DNA from wild-type magnetic bacteria. DNA was probed with two AMB-1 chromosomal *Eco*RI-*Hpa*I restriction fragments from pCN3. Lane 1, pCN3 digested with *Eco*RI; lane 2, *E. coli* DH5α genomic DNA; lane 3, AMB-1 genomic DNA; lane 4, *A. magnetotacticum* MS-1 genomic DNA. Sizes are indicated in kilobases.

of relatively short mating times (6 h) with these conditions resulted in the highest transfer frequencies so far obtained for an *Aquaspirillum* species (7). The Tc^r and Km^r colonies obtained after the matings were true transconjugants, since they contained the correct plasmids as demonstrated by Southern hybridization and were spirilla with carbon source utilization profiles identical to that of the wild type. Thus, these plasmids could exist as autonomous replicons, and the encoded antibiotic resistance genes were successfully expressed. These plasmids are therefore excellent candidates for introducing cloned DNA into AMB-1. The conjugative plasmid pSUP202, which has a pBR325 replicon, and pSUP1021, which has a pACYC184 replicon, were also unable to replicate in AMB-1. We did not determine the ability of these plasmids to replicate in MS-1. However, growth of MS-1 in the presence of kanamycin indicates the successful transfer of transposon Tn*5*. This gene transfer was obtained in MS-1 without requiring anaerobic or microaerobic conditions during conjugation. Conjugations were carried out for 6 h in an air atmosphere. Determination of the frequencies of transfer and the isolation of individual clones were hampered by difficulties in the colony formation ability of this species.

The number of Tn*5* insertions into AMB-1 was high (10^{-4} Km^r transconjugant per recipient). This may be due to the transfer of more than one copy of the multicopy vector plasmid (21). The frequency of occurrence of nonmagnetic transconjugants was about 4.3% and, although rather high, could be due to nonrandom insertion of Tn*5* into "hot spots" present in regions of the chromosome required for synthesis of magnetosomes. Indeed, two nonmagnetic mutants had insertions into the same part of the chromosome, although not at the same point. Genetic lesions may give rise to a nonmagnetic phenotype indirectly. However, the extent of this cannot be estimated, since the number of genes required directly for magnetosome production remains unknown. Therefore, the cloned DNA may or may not be directly involved in synthesis of magnetic particles. The demonstration of homology between MS-1 and AMB-1 sequences suggests that similar genes relevant to magnetosome synthesis exist in *A. magnetotacticum* MS-1.

Further work is currently being carried out to establish the nature of the genes which have been interrupted. Sequencing of the cloned regions together with mutant complementation should contribute to the further understanding of the genetic control of magnetite biomineralization.

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