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## Development of a genetic system for *Magnetospirillum gryphiswaldense*

Received: 25 July 2002 / Revised: 2 October 2002 / Accepted: 8 October 2002 / Published online: 15 November 2002

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**Abstract** Genetic analysis of bacterial magnetosome biomineralization has been hindered by the lack of an appropriate methodology for cultivation and genetic manipulation of most magnetotactic bacteria. In this report, a genetic system for *Magnetospirillum gryphiswaldense* is described. The system includes a plating technique that allows the screening of magnetic vs non-magnetic colonies, and a protocol for the transfer of foreign DNA by electroporation and high-frequency conjugation. Various broad-host-range vectors of the IncQ, IncP, and pBBR1 groups were found to be capable of replication in *M. gryphiswaldense*. Several antibiotic resistance markers that can be expressed in *M. gryphiswaldense* were identified. Tn5 transposons delivered on a suicide plasmid showed transpositional insertion into random chromosomal sites.

**Keywords** Broad-host-range vectors · Conjugation · Electroporation · *Magnetospirillum gryphiswaldense* · Magnetotactic bacteria · Magnetosome biomineralization · Transposon mutagenesis

### Introduction

Magnetotactic bacteria are capable of forming magnetosomes, which in most magnetotactic bacteria are nanometer-sized, membrane-bound magnetic particles consisting of the iron mineral magnetite (Fe<sub>3</sub>O<sub>4</sub>) (Bazylinski 1995; Balkwill et al. 1980). The unique characteristics of bacterial magnetosomes have attracted a broad interdisciplinary research interest. Their superior crystalline and magnetic properties make them potentially useful as a highly ordered biomaterial in a number of applications, e.g. in the immobilization of bioactive compounds, magnetic drug-targeting, and as contrast agent for magnetic resonance

imaging (Schüler and Frankel 1999). Understanding bacterial magnetosome formation is expected to provide insights into more complex biomineralization systems in higher organisms (Kirschvink and Hagadorn 2000). The characteristics of bacterial magnetosomes have been recently discussed as tentative biosignatures to identify presumptive ancient life on Mars (Buseck et al. 2001).

The formation of magnetosome particles is achieved by a complex mechanism that involves the uptake of large quantities of iron and the deposition of the mineral particle within a membranous compartment (Schüler 1999). However, little is known about the pathways that regulate the formation of magnetosomes at the molecular level, and the genetic basis of magnetite biomineralization has remained mostly unknown. Recently, the almost complete genome sequences of two magnetotactic bacteria (*Magnetospirillum magnetotacticum* and the magnetic coccus strain MC-1) have become available ([http://www.jgi.doe.gov/JGI\\_microbial/html/index.html](http://www.jgi.doe.gov/JGI_microbial/html/index.html)). An initial analysis of genome data suggested that magnetosome formation likely involves a considerable number of unknown gene functions (Grünberg et al. 2001). Thus, there is an urgent need to augment physiological, biochemical, and genomic level studies with a genetic approach to elucidate specific gene functions involved in biomineralization. However, progress in the genetic analysis of magnetotactic bacteria has been severely hampered by a general lack of an appropriate methodology for their genetic manipulation. This has been largely due to difficulties with their cultivation, namely their fastidious and microaerophilic mode of growth. Consequently, adequate techniques for clonal selection, genetic markers, methods for DNA transfer, and isolation of mutants have not yet been established for most magnetotactic bacteria, despite the considerable efforts of various laboratories (Dubbels et al. 1998; Bertani et al. 1997; Berson et al. 1991; Eden and Blakemore 1991; and others). Conjugational gene transfer has been described for one species, *Magnetospirillum* sp. AMB-1, which has subsequently been used for the isolation of several mutants defective in magnetosome formation by transposon mutagenesis (Matsunaga et al. 1992; Wahyudi et al. 2001). How-

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ever, these experiments were difficult to replicate and have not yet fully disclosed the apparently complex genetic determination of bacterial magnetite biomineralization.

The magnetotactic  $\alpha$ -proteobacterium *Magnetospirillum gryphiswaldense* can be cultivated more readily than most other magnetotactic bacteria. The strain, which was isolated from a freshwater sediment (Schüler and Köhler 1992), intracellularly forms a chain of up to 60 cubo-octahedral magnetosomes and has been used as model organism in a number of studies (Schüler and Baeuerlein 1996, 1998; Schüler et al. 1995, 2000). Recently, the biochemical analysis of the magnetosome membrane in this organism led to the detection of a number of genes encoding magnetosome proteins (Grünberg et al. 2001). In addition, this study revealed that *M. gryphiswaldense* is highly similar at the genomic level to *M. magnetotacticum*, for whole genome data are available. Here, we report on the establishment of a system for the transfer of various broad-host-range plasmids via electroporation and conjugation, and the delivery of transposons in *M. gryphiswaldense*.

## Material and methods

### Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured as described in Sambrook et al. (1989). Liquid cultures of *M. gryphiswaldense* strains were grown in liquid medium (Schüler and Baeuerlein 1996) at 28 °C containing 50  $\mu$ M ferric citrate essentially as described before (Grünberg et al. 2001).

Cells grown on solid activated charcoal agar (ACA) medium were incubated at 28 °C in anaerobic jars (Ochs, Bovenden-Lengler) under an O<sub>2</sub>:CO<sub>2</sub>:N<sub>2</sub> (0.5:5:94.5, by volume) atmosphere, which was replaced every 48 h. Colonies of about 1 mm were visible after 3–5 days of growth on ACA medium; after 7 days of incubation, colonies up to 5 mm in size were observed. ACA medium, whose composition was optimized during this study, contained per l deionized water: 2.38 g HEPES, 3 g sodium pyruvate, 0.1 g yeast extract, 3 g soybean peptone (Merck), 0.34 g NaNO<sub>3</sub>, 0.1 g KH<sub>2</sub>PO<sub>4</sub>, 0.15 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 3 g activated charcoal, 15 g agar (Difco). The pH was adjusted to 7.0 with NaOH. Ferric citrate (500  $\mu$ M) and 1,4-dithiothreitol (DTT; 1 mM) were added aseptically after autoclaving. Unless indicated otherwise, all chemicals were from Sigma. For the determination of minimal inhibitory concentrations (MIC), dilutions of mid-exponential phase cultures of *M. gryphiswaldense* strains were spotted onto ACA medium containing various concentrations of ampicillin (Ap), chloramphenicol (Cm), gentamicin (Gm), kanamycin (Km), rifampicin (Rif), tetracycline (Tc), and streptomycin (Sm). The MIC was defined as the concentration that completely inhibited growth after 10 days of incubation.

### Isolation of antibiotic-resistant *M. gryphiswaldense* strains

Spontaneous mutants of *M. gryphiswaldense* that were resistant to Rif or to Sm, were isolated after plating of approximately 10<sup>9</sup> cells onto ACA plates containing either Rif (300  $\mu$ g/ml) or Sm (20  $\mu$ g/ml). After incubation for up to 10 days, putative mutant colonies appeared at a frequency of approximately 10<sup>-7</sup>. The colonies were restreaked on ACA medium containing the appropriate antibiotic.

### Mating experiments

Strains of *E. coli* S17–1 containing the appropriate plasmid served as donors in agar-plate matings. Overnight cultures of the *E. coli* donor and the Rif<sup>r</sup> *M. gryphiswaldense* R11 recipient strain (48 h

**Table 1** Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Reference or source
<b>Bacterial strains</b>		
<i>Escherichia coli</i> S17–1	<i>thi pro hsdR recA</i> with RP4–2[Tc::Mu-Km::Tn7]	Simon et al. (1983)
<i>Magnetospirillum</i> sp. strain AMB-1	Wild-type	Matsunaga et al. (1992) ATCC 700264
<i>Magnetospirillum magnetotacticum</i> MS-1	Wild-type	Schleifer et al. (1991) ATCC 31632
<i>Magnetospirillum gryphiswaldense</i> MSR-1	Wild-type	Schleifer et al. (1991) DSM 6361
<i>Magnetospirillum gryphiswaldense</i> R11	Rif <sup>r</sup> spontaneous mutant	This work
<i>Magnetospirillum gryphiswaldense</i> S1	Sm <sup>r</sup> spontaneous mutant	This work
<b>Plasmids</b>		
pBBR1MCS	4.7 kb ND <sup>a</sup> Cm <sup>r</sup> <i>lacZ</i> $\alpha$	Kovach et al. (1994)
pBBR1MCS2	5.1 kb ND <sup>a</sup> Km <sup>r</sup> <i>lacZ</i> $\alpha$	Kovach et al. (1995)
pBBR1MCS3	5.2 kb ND <sup>a</sup> Tc <sup>r</sup> <i>lacZ</i> $\alpha$	Kovach et al. (1995)
pBBR1MCS4	4.9 kb ND <sup>a</sup> Ap <sup>r</sup> <i>lacZ</i> $\alpha$	Kovach et al. (1995)
pBBR1MCS5	4.7 kb ND <sup>a</sup> Gm <sup>r</sup> <i>lacZ</i> $\alpha$	Kovach et al. (1995)
pRK415	10.5 kb incP-1 (RK2) Tc <sup>r</sup> <i>lacZ</i>	Keen et al. (1988)
pKT231	12.8 kb incQ/P-4 (RSF1010) Km <sup>r</sup> Sm <sup>r</sup>	Bagdasarian et al. (1981)
pSUP1021	pACYC184 Cm <sup>r</sup> Tc <sup>r</sup> Tn5 Nm <sup>r</sup>	Simon et al. (1986)
pLAFR1	21.6 kb incP-1 (RK2) Tc <sup>r</sup>	Friedman et al. (1982)

<sup>a</sup>ND, the incompatibility group of pBBR1MCS plasmids has not been defined (Antoine et al. 1992); compatible with IncP, IncQ and IncW group plasmids and with ColE1- and P15a-based replicons

cultures) grown as described above were harvested by centrifugation and were each resuspended in liquid medium (Schüler and Baeuerlein 1996) to approximately  $10^8$  CFU/ml. A 10- $\mu$ l aliquot of the donor and of the recipient cell suspension were mixed and spotted onto dried ACA medium in six-well plates (Nunc, Wiesbaden; 9.6 cm<sup>2</sup>/well). After incubation for 8 h at 28 °C under microoxic conditions (0.5% oxygen in the headspace), cells were flushed from the agar surface into sterile liquid medium containing Rif (150  $\mu$ g/ml) to counterselect the *E. coli* donor and incubated for 2 h at 28 °C for maximum expression of the acquired antibiotic resistance. Transconjugants were diluted appropriately and aliquots were plated onto ACA medium containing Rif (300  $\mu$ g/ml) and the appropriate concentration of the antibiotic whose resistance was specified by the plasmid to be transferred. The total number of recipient cells was determined by plating the mating mixture onto ACA medium devoid of antibiotics. For intraspecific conjugations, the mating mixtures were plated onto ACA medium with Km (10  $\mu$ g/ml) and Sm (10  $\mu$ g/ml) when *M. gryphiswaldense* S1 was used as the recipient to select against the *M. gryphiswaldense* R11 donor strain harboring pBBR1MCS-2.

#### Preparation of electrocompetent *M. gryphiswaldense* cells and electroporation procedures

All manipulations were carried out on ice. For each pulsing experiment, 30 ml of a late-exponential phase culture ( $OD_{565}=1$ , equivalent to about  $10^{10}$  cells) was harvested by centrifugation (14,000 $\times$ g for 10 min). The cells were washed twice using an equal volume of electroporation buffer (1 mM HEPES, pH 7.0, 1 mM MgCl<sub>2</sub>, 200 mM sucrose) and resuspended to a final volume of 400  $\mu$ l using the same buffer. An appropriate amount (0.2–5  $\mu$ g) of plasmid DNA was added. After incubation for 5 min on ice, the mixture was loaded in a chilled 0.2-cm cuvette and subjected to a single pulse using a Gene pulser and a pulse controller apparatus (Biorad, Munich; settings: 1.2 kV, 600 Ohm, 25  $\mu$ F). Pre-warmed liquid growth medium (4.5 ml) was added immediately, and the cells were incubated for 3 h at 28 °C for regeneration before they were plated onto ACA medium supplemented with the appropriate antibiotic.

#### Assessment of plasmid stability under nonselective conditions

A liquid culture of Km<sup>r</sup> transconjugants harboring pBBR1MCS-2 was repeatedly transferred in the absence of selective pressure, and the ratio of total CFU to Km<sup>r</sup>-CFU was determined at different time points by plating on non-selective and selective medium. The number of generations between time points was determined from the total CFU/ml.

#### DNA techniques

PCR, DNA isolation, transformation, and DNA manipulations essentially followed standard methods (Sambrook et al. 1989). For

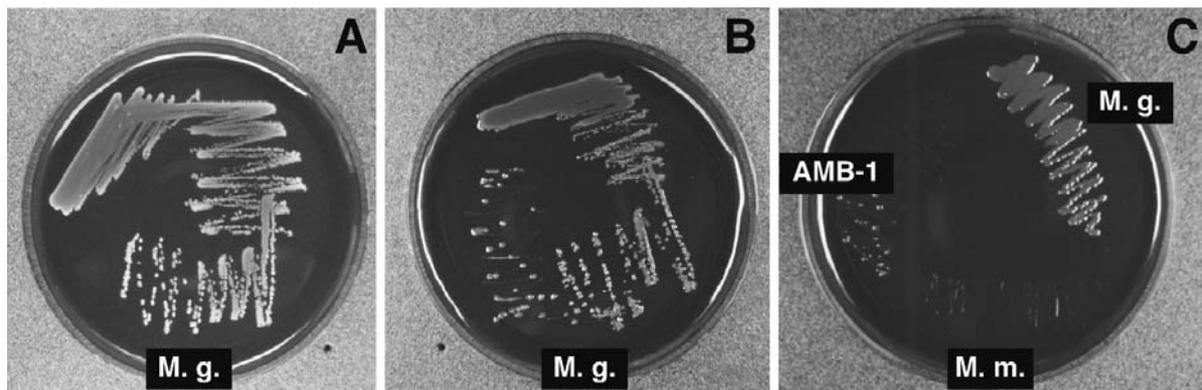
detection of transposition by Southern blot hybridization, a 413-bp probe was generated by PCR using the primers Tn5foIII (ATCTG-GACGAAGAGCATCAGGG) and Tn5rwIII (TTTCATAGAAG-GCGGCGGTG) and pSUP1021 DNA as template.

## Results and discussion

### Growth on solid medium

A seemingly trivial prerequisite for genetic experiments is that bacteria can be grown as colonies originating from single cells. However, this has been notoriously difficult to achieve with a number of microaerophiles such as magnetotactic spirilla. Although colony formation has been reported for *M. magnetotacticum* MS-1 (Blakemore et al. 1979) and *Magnetospirillum* sp. AMB-1 (Matsunaga et al. 1991), no or poor growth on solid medium could be achieved in our hands for these strains. However, medium improvements such as the use of activated charcoal, DTT as a reductant and elevated concentrations of iron resulted in ACA medium that supported colony formation of *M. gryphiswaldense* under both oxic and microoxic conditions with a plating efficiency of more than 90%. The efficiency of charcoal to stimulate growth of fastidious microaerophiles is probably due to its potential to scavenge and decompose toxic oxygen compounds (Hoffman et al. 1983; Krieg and Hoffman 1986). Plating on ACA medium allowed visual screening of the magnetic vs. non-magnetic phenotype (Fig. 1a, b). The two other *Magnetospirillum* strains, for which colony formation had been previously described, were also examined for growth on ACA. Strain *M. magnetotacticum* MS-1 failed to form colonies on ACA plates under both microoxic and oxic conditions, while only a small fraction of *Magnetospirillum* spec. AMB-1 cells appeared to grow, forming small non-magnetic colonies (Fig. 1c).

**Fig. 1** Growth of non-magnetic (a) and magnetic cells (b) of *Magnetospirillum gryphiswaldense* (*M. g.*) on ACA medium. While colonies of *M. gryphiswaldense* grown in the presence of air had a white-to-creamy appearance (a) and consisted of non-magnetic cells, cells incubated under an atmosphere containing 0.5% oxygen were magnetic and formed colonies that were dark-brown in color (b). For comparison, *M. gryphiswaldense* (*M. g.*), *Magnetospirillum magnetotacticum* (*M. m.*), and *Magnetospirillum* sp. strain AMB-1 (*AMB-1*) were streaked on ACA medium (c)



**Table 2** Minimal inhibitory concentrations (MICs) of selected antibiotics for *Magnetospirillum gryphiswaldense* strains

Antibiotic	Minimal inhibitory concentrations ( $\mu\text{g/ml}$ )			
	Liquid medium		Solid medium (ACA)	
	Wild-type	Transconjugant/resistant mutant	Wild-type	Transconjugant/resistant mutant
Ampicillin	0.5	20	1	>50
Kanamycin	0.25	>60	5	>150
Gentamicin	0.5	60	10	>60
Chloramphenicol	0.25	15	10	>60
Tetracycline	0.6	60	15	120
Streptomycin	0.5	>50 <sup>a</sup>	1	100 <sup>a</sup>
Rifampicin	0.9	900 <sup>a</sup>	50	600 <sup>a</sup>

<sup>a</sup>Resistance was by spontaneous mutation

### Examination of antibiotic sensitivity and identification of genetic markers

Growth of *M. gryphiswaldense* on ACA plates was inhibited by a number of commonly used antibiotics (Table 2). MICs determined on ACA were generally higher than those observed in liquid medium, probably due to the previously reported inactivation of antibiotic activity by charcoal (Pendland et al. 1997; Barker and Farrell 1986).

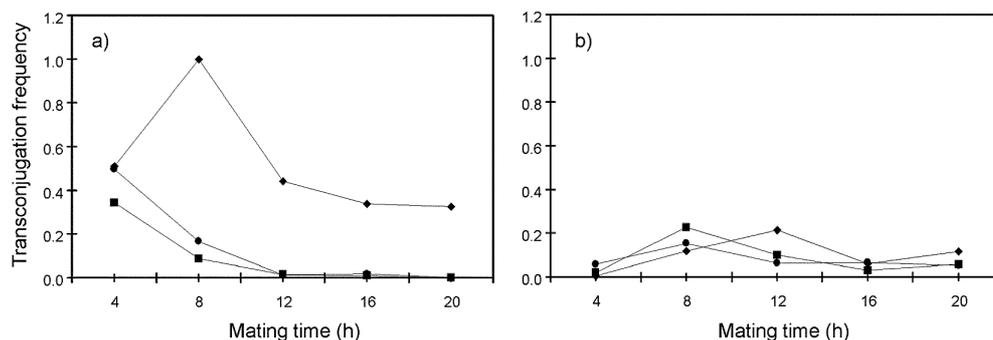
### Development of a conjugative plasmid transfer procedure and identification of genetic markers

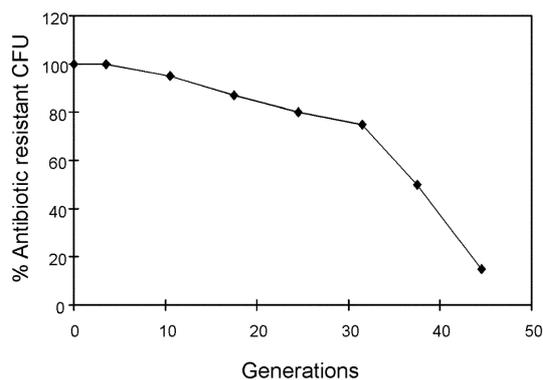
Heterospecific biparental matings were set up on ACA medium using *E. coli* S17-1 as the donor strain, which provides the *tra* functions of the broad-host-range plasmid RP4 residing in the chromosome to prevent its self-transfer (Simon et al. 1983). The Rif<sup>r</sup> mutant strain *M. gryphiswaldense* R11 was used as the recipient in conjugation experiments to allow for efficient counterselection against the *E. coli* donor, which can readily grow on ACA medium. The conjugation conditions were optimized at different donor-recipient ratios and at various mating times using the broad-host-range cloning vector pBBR1MCS-2 (Kovach et al. 1995). Km<sup>r</sup> transconjugant colonies appeared on plates after about 7 days. Spontaneous Rif<sup>r</sup> *E. coli*

colonies could be easily distinguished by their conspicuous colony morphology. A maximum transfer frequency approaching 100% of recipient *M. gryphiswaldense* cells was obtained when a donor-recipient ratio of 1:1 and a mating time of 8 h were used (Fig. 2a). This indicates that conjugational transfer of this plasmid to *M. gryphiswaldense* was highly efficient, although it cannot be excluded that replication of cells during the mating and recovery step might have led to an apparently increased transfer frequency. Nevertheless, these efficiencies are among the highest so far demonstrated in Proteobacteria (e. g. Pattaragulwanit and Dahl 1995; Dehio and Meyer 1997; Coppi et al. 2001). For comparison, maximum reported transfer frequencies in the closely related strain *Magnetospirillum* sp. AMB-1 were  $3\text{--}4.5 \times 10^{-3}$  transconjugants per recipient (Matsunaga et al. 1992). The presence of pBBR1MCS-2 in transconjugants was confirmed by PCR (data not shown). Plasmid DNA isolated from *M. gryphiswaldense* cells exhibited restriction patterns identical to those isolated from *E. coli* (not shown) and could be efficiently transferred back to *E. coli* via transformation, indicating that no deletions or genetic rearrangements had occurred. To test whether the observed high frequencies could have resulted from secondary conjugation events between *M. gryphiswaldense* cells, homospecific conjugation experiments were set up between Km<sup>r</sup> transconjugants harboring pBBR1MCS-2 as the donor and the Sm<sup>r</sup> strain *M. gryphiswaldense* S1 as recipient. No colonies resistant to both Km and Sm were obtained in several experiments, indicating that conjugational transfer of the Km resistance marker did not occur at a detectable frequency.

A variety of other plasmids were further tested for their ability to replicate in and confer antibiotic resistance upon *M. gryphiswaldense*. The transfer of the plasmids pBBR1MCS (Cm<sup>r</sup>), pBBR1MCS-3 (Tc<sup>r</sup>), pBBR1MCS-4 (Ap<sup>r</sup>), pBBR1MCS-5 (Gm<sup>r</sup>), which are derivatives of pBBR1MCS-2 carrying different antibiotic-resistance cassettes (Kovach et al. 1994, 1995), yielded transconjugants resistant to the respective antibiotic (Table 2), with similar efficiencies as pBBR1MCS-2. Heavy background growth of satellite colonies was observed around Ap<sup>r</sup> colonies harboring pBBR1MCS-4 on ACA-Ap, which might limit the practical use of the Ap marker in genetic experiments. The presence of antibiotics did not affect the ability of transconjugants to produce magnetosomes. Overall, these results indicate that it is possible to select for the acquisition

**Fig. 2** Conjugation frequencies in *M. gryphiswaldense*. Plasmids pBBR1MCS-2 (a) and pRK415 (a) were transferred at different donor-recipient ratios [1:1 (◆), 5:1 (■), 10:1 (●)] and incubation times. Frequencies are given as transconjugants per recipient cell





**Fig. 3** Stability of pBBR1MCS-2 in *M. gryphiswaldense* in the absence of antibiotic selection

of multiple antibiotic resistance markers by *M. gryphiswaldense*.

In addition, the transfer frequencies of the broad-host-range vectors pRK 415 (Fig. 2b), which is based on the RK2 replicon (IncP1, Keen et al. 1988), the RSF1010-derived vector pKT231 (IncQ, Bagdasarian et al. 1981), and the mobilizable Inc P1 cosmid pLAFR1 (Friedman et al. 1982) were investigated. Transfer of these vectors occurred at lower frequencies ( $0.1\text{--}2 \times 10^{-1}$ ) than pBBR1MCS derivatives, which might be explained by their larger sizes (Table 1). In the absence of antibiotic selection, pBBR1MCS-2 had a half-life of about 40 generations (Fig. 3).

#### Development of an electrotransformation procedure

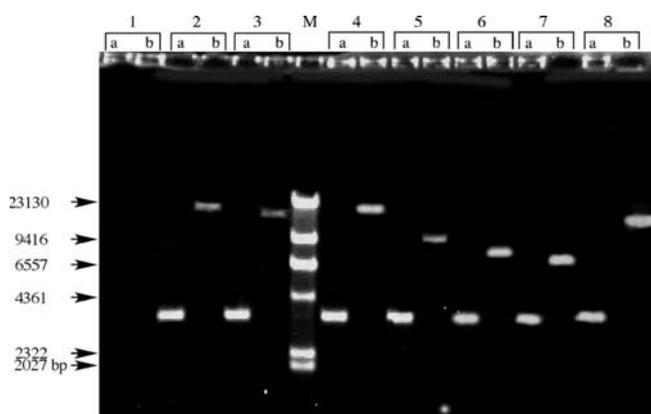
A protocol for the electrotransformation of *M. gryphiswaldense* was developed by modifying existing protocols (Eden and Blakemore 1991; Coppi et al. 2001; Garg et al. 1999; and others). The presence of  $\text{MgCl}_2$  and sucrose in the electrotransformation buffer was critical for maintaining the viability of *M. gryphiswaldense* through the washing and electrotransformation procedure. Electrotransformation with the broad-host-range plasmid pBBR1MCS-2 resulted in the growth of  $\text{Km}^r$  colonies after 8–10 days of incubation. Optimum conditions for electrotransformation were a field strength of 6 kV/cm and a time constant of approximately 7 ms. Under these conditions, electrotransformation lethality was roughly 90%. A maximum of  $1.5 \times 10^3$  transformants was obtained per  $\mu\text{g}$  plasmid DNA. The use of plasmid DNA isolated from *M. gryphiswaldense* instead of *E. coli* did not increase the transformation efficiency, suggesting that the transfer of foreign DNA to *M. gryphiswaldense* was not limited by a restriction barrier. However, there was a considerable variation of transformation frequencies between different individual electrotransformation experiments, despite our extensive efforts to further optimize the procedure for cell treatment and electrotransformation. The poor reproducibility was attributed to the high sensitivity of cells during the washing and pulsing procedure. In addition, subtle variations in the cultivation and harvesting of cells appeared to have drastic effects onto the electrocompe-

tence of cells. In conclusion, electrotransformation can be used for the introduction of plasmids into *M. gryphiswaldense* as an alternative to conjugation. However, considering the problems mentioned above, electrotransformation seems to be of limited use for experiments in which high transformation efficiencies are required.

#### Delivery of transposons

Transposon mutagenesis has proven to be an extremely useful tool in the genetic analysis of many bacteria. In order to establish transposon mutagenesis in *M. gryphiswaldense*, Tn5-based transposons were delivered on the suicide vector pSUP1021 (Simon et al. 1986) by conjugational transfer under slightly modified conditions. The mating step (8 h) was followed by an extended incubation step (12 h) in liquid medium containing only Rif to allow for efficient transposon delivery, before the mixture was plated onto selective medium containing both Rif and Km.  $\text{Km}^r$  transconjugants appeared with frequencies of approximately  $10^{-4}$  to  $10^{-5}$  per recipient. To confirm that the  $\text{Km}^r$  colonies were the result of transposition and the vector carrying  $\text{Cm}^r$  was lost, the colonies were replicated onto ACA-Cm. The random insertion of the transposon was confirmed by Southern blotting of digested genomic DNA from seven randomly chosen clones and hybridization using a fragment homologous to an internal Tn5 sequence as probe (Fig. 4).

In conclusion, our study provides the first description of a reliable plating technique as well as the first demonstration of plasmid transfer by electrotransformation and conjugation in the magnetotactic bacterium *M. gryphiswaldense*. The achieved conjugation frequencies are the highest so far reported for a magnetotactic bacterium. This work has extended the range of vectors and genetic markers useful in



**Fig. 4** Southern hybridization analysis of DNA from seven Tn5-insertion mutants of *M. gryphiswaldense* R11 probed with a fragment of transposon Tn5. Genomic DNA isolated from *M. gryphiswaldense* R11 (lane 1) and seven mutants carrying independent chromosomal insertions of Tn5 after transposition from the suicide plasmid pSUP1021 (lanes 2–7) was digested with *Hind*III, which generates an internal fragment of 3.4 kb (a) and *Eco*RI, which does not cleave within the transposon (b)

magnetotactic bacteria and has established powerful tools applicable to *M. gryphiswaldense*. In particular, Tn5 mutagenesis may aid in the identification and analysis of novel genetic determinants for magnetite biomineralization, and mobilizable broad-host-range plasmids should allow the in trans expression of cloned genes in *M. gryphiswaldense*.

**Acknowledgements** This work was supported by the Federal Ministry of Education and Research (BMBF) and the Max-Planck-Gesellschaft. The excellent technical assistance of Cornelia Stumpf is acknowledged. We are grateful to Doug Bartlett (La Jolla), Thomas Hurek (Bremen), Matthias Keller and Alfred Pühler (Bielefeld), and Gerrit Voordouw (Calgary) for their kind gifts of plasmids and bacterial strains.

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