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Identification of Promoters for Efficient Gene Expression in *Magnetospirillum gryphiswaldense*▽†

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To develop an expression system for the magnetotactic bacterium *Magnetospirillum gryphiswaldense*, we compared gene expression from the widely used *Escherichia coli* P_{lac} promoter with that from known and predicted genuine *M. gryphiswaldense* promoters. With the use of green fluorescent protein as a reporter, the highest expression level was observed with the magnetosomal P_{mamDC} promoter. We demonstrate that this promoter can be used for the expression of modified magnetosome proteins to generate "antibody-binding" magnetosomes.

The formation of magnetosomes, which are subcellular compartments consisting of membrane-bounded magnetite (Fe_3O_4) nanocrystals in magnetotactic bacteria, is of interdisciplinary interest in fields of microbial cell biology, biotechnology, and nanotechnology. Despite considerable efforts by many researchers, the genetic analysis of magnetotactic bacteria is still cumbersome and many genetic tools are lacking (7). In this study we developed an expression system for *Magnetospirillum gryphiswaldense*, which is one of the most widely studied magnetotactic organisms (5, 20).

To identify genuine promoters for gene expression in *M. gryphiswaldense*, we investigated the expression of green fluorescent protein (GFP) from the *Escherichia coli* P_{lac} promoter and from putative and previously identified *M. gryphiswaldense* promoters such as P_{mamDC} and P_{mamAB}, which are highly transcribed under magnetite-inducing conditions, i.e., at microaerobiosis and in the presence of micromolar amounts of iron (19). In addition, genomic regions upstream of large ribosomal gene clusters, which potentially encode the strong P_{rpsJ} (*rpsJ*, MGR3815 ribosomal protein S10) and P_{rplK} (*rplK*, MGR3801 ribosomal protein L11) promoters, and sequences that putatively encode the P_{apdA} (P_{mms16} in "*Magnetospirillum magneticum*") and the P_{msp3} promoters, which were described previously in *M. magneticum* (26), were analyzed.

For the construction of GFP reporter vectors, the *egfp* gene encoding the GFPmut1 variant (2, 10) was PCR amplified (primers are shown in Table 1), cloned into pGEM-T Easy (Promega), sequenced, and subcloned into pBBR1MCS2 (8) downstream of the P_{lac} promoter to generate pBBRegfp (Table 2). The vector was digested with NsiI and ApaI, blunted with mung bean nuclease (New England Biolabs), and religated to remove the P_{lac} promoter and yield the promoterless GFP reporter plasmid pBBRpl (Table 2). Putative promoter regions that included the intergenic region upstream from the start codon to the next open reading frame were PCR amplified from genomic DNA of *M. gryphiswaldense* R3/S1 (21). The PCR products were cloned into pGEM-T Easy, sequenced, and subcloned into pBBRpl, resulting in the plasmids pBBRPmamDC, pBBRPmamAB, pBBRPmsp3, pBBRPapdA, pBBRPure, pBBRPrplK, and pBBRPrpsJ (Table 2). The plasmids were transferred into *M. gryphiswaldense* R3/S1 by conjugation from *E. coli* BW29427 (K. Datsenko and B. L. Wanner, unpublished data) as described previously (18, 22).

For promoter activity assays, *M. gryphiswaldense* strains expressing GFP from different promoters were cultivated in triplicate microaerobically in 3-ml culture volumes in six-well culture plates under a microoxic atmosphere (1% oxygen, 99% nitrogen) for 20 to 22 h in FSM medium (6). Cells were washed and resuspended in phosphate-buffered saline to an optical density at 565 nm of 0.5. The expression of GFP was quantified from 100-μl aliquots of the cell suspension with an Infinite 500 plate reader (Tecan). The native promoters were considerably more active than the *E. coli* P_{lac} promoter that has been used in previous studies with this organism (9, 16–18). Fluorescence quantification and immunoblot analysis (see Fig. S1 in the supplemental material) have shown that the strongest promoter in *M. gryphiswaldense* was P_{mamDC}, followed by P_{msp3}, P_{apdA}, P_{mamAB}, P_{rpsJ}, P_{rplK}, and P_{lac} (Fig. 1). Similarly to other alphaproteobacterial promoters (12, 13, 24), the tested *M. gryphiswaldense* promoters were inactive in *E. coli* (see Fig. S2 in the supplemental material).

Using GFP as a reporter, we estimated gene expression in individual cells by flow cytometry according to a previously described procedure (9). Comparison of the average fluorescence intensities confirmed the bulk measurements (Fig. 1) and showed that cells containing the P_{mamDC}-GFP construct fluoresced on average twice as much as did cells expressing GFP from P_{msp3} or P_{apdA} (P_{mms16}) (Fig. 2). In a study of gene expression in *M. magneticum*, P_{msp3} was identified in a luciferase-based assay as the strongest promoter with an activity more than threefold higher than that of the P_{mms16} promoter (26). Even though in our study a different reporter and the

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TABLE 1. Primers used in this study[a]

Primer name	Sequence[a]	Restriction site
egfpfw	**CATATG**GTGAGCAAGGGCGAGGAG	NdeI
CL2	GT**GGATCC**TTACTTGTACAGCTCGTC	BamHI
papdAfw	**CTCGAG**GAGCCTCTCCATTAAACAATG	XhoI
papdArev	**CATATG**CTTGAATTCCTCCAACCGGGGGTATG	NdeI
prplKfw	**AAGCTT**GGCATCAAGGTTTCGGAAG	HindIII
prplKrev	**CATATG**TTTACCCTACCTCTGGTCG	NdeI
prpsJfw	**CTCGAG**GTCCTTCG GGATCGCTTG AC	XhoI
prpsJrev	**CATATG**ATTCACGTCATCCGTTAAATC	NdeI
purerev	**CATATG**GTGGTTATGCGCTGCTCAAAATC	NdeI
purefw	**CTCGAG**CTTTTCTCGATCCGGGAAAAATAC	XhoI
PmamDCrev	**CATATG**CTGATCTCCGGCAAGTGTATG	NdeI
PmamDCfw	**CTCGAG**CAATGACCACCACCACCTTA AAC	XhoI
PmamABfw	**CTCGAG**ATGGCGCAAAGATGTGACGT C	XhoI
PmamABrev	**CATATG**TCCCGTCACAATTCACCTCC	NdeI
pmsp3fw	**CTCGAG**GAACTCCAAAAGCAAGGCTATTTAC	XhoI
pmsp3rev	**ATTAAT**CCGAAAGCTCCTTGAATCAAAAG	VspI
PmamDCxlrev	**CTCGAG**GATCTCCGGCAAGTGTATGCAC	XhoI
zzfw	GCTGCA**CATATG**GCGCAACACGATGAAGCC	NdeI
zzrev	CCA**TCTAGA**AATATTACCGCCAGCCATTG	XbaI

[a] Restriction sites that were incorporated in the primer are indicated in bold.

homologous promoters from *M. gryphiswaldense* were used, it was unexpected that the activities of P_{msp3} and P_{apdA} were twofold lower than the activity of P_{mamDC} and that P_{msp3} activity was almost identical to P_{apdA} (P_{mms16}) activity in *M. gryphiswaldense*. It is possible that P_{msp3}, which is a putative peroxiredoxin promoter, was downregulated due to the microaerobic growth conditions, which are required for the production of magnetosomes.

Flow cytometry also revealed that GFP was not expressed homogeneously in all cells but that a variable fraction of the cells was nonfluorescent (Fig. 2). For the weak and intermediate-strength promoters P_{lac}, P_{rplK}, P_{rpsJ}, and P_{mamAB}, the proportions of fluorescent cells (from 8.52% to 56.5%) correlated well with the increasing average fluorescence intensities, which indicates that intermediate-strength promoters activate GFP expression in a higher proportion of cells than do weak promoters. In contrast, use of the strong P_{msp3}, P_{mamDC}, and P_{apdA} promoters did not result in a further increased proportion of fluorescent cells (56.3 to 50.3%). It is unclear why a large proportion of cells was inactive with respect to GFP expression. However, inhomogeneous gene expression in an isogenic population of cells can be frequently observed in bacteria (3, 23) and might be caused by cell cycle-dependent effects, stochasticity of gene expression, or variations of growth

TABLE 2. Plasmids used in this study

Plasmid name	Description	Source or reference
pEGFPN1	GFP expression vector; Ap	BD Biotech
pGEM-T Easy	Cloning vector; Ap	Promega
pBBR1MCS-2	Mobilizable broad-host-range vector; Km	8
pGEMegfp	pGEM-T Easy + *egfp* PCR product	This study
pBBRegfp	pBBR1MCS-2 + *egfp* from pGEMegfp	This study
pBBRpl	Promoterless GFP reporter vector based on pBBRegfp	This study
pGEMPmamDC	pGEM-T Easy + P_{mamDC}-PCR product	This study
pGEMPmamAB	pGEM-T Easy + P_{mamAB}-PCR product	This study
pGEMPapdA	pGEM-T Easy + P_{apdA}-PCR product	This study
pGEMPmsp3	pGEM-T Easy + P_{msp3}-PCR product	This study
pGEMPrplK	pGEM-T Easy + P_{rplK}-PCR product	This study
pGEMPrpsJ	pGEM-T Easy + P_{rpsJ}-PCR product	This study
pBBRPmamDC	pBBRpl + P_{mamDC} from pGEMPmamDC cloned in XhoI and NdeI sites	This study
pBBRPmamAB	pBBRpl + P_{mamAB} from pGEMPmamAB cloned in XhoI and NdeI sites	This study
pBBRPapdA	pBBRpl + P_{apdA} from pGEMPapdA cloned in XhoI and NdeI sites	This study
pBBRPmsp3	pBBRpl + P_{msp3} from pGEMPmsp3 cloned in XhoI and VspI sites	This study
pBBRPrplK	pBBRpl + P_{rplK} from pGEMPrplK cloned in HindIII and NdeI sites	This study
pBBRPrpsJ	pBBRpl + P_{rpsJ} from pGEMPrpsJ cloned in XhoI and NdeI sites	This study
pEZZ18	Protein A gene fusion vector; Ap	GE Healthcare
pGEMZZ	pGEM-T Easy + ZZ protein domain PCR product	This study
pCL6	pBBR1MCS-2 + MamC-GFP	9
pBBRCZZ	The ZZ protein domain from pGEMZZ was cloned into the NdeI and BamHI sites of pCL6 to replace GFP with the ZZ domain	This study
pJETPdcx1	pJET1.2 + P_{mamDC}-PCR product	This study
pBBRPdcCZZ	pBBRCZZ + P_{mamDC} from pGEMPdcx1	This study

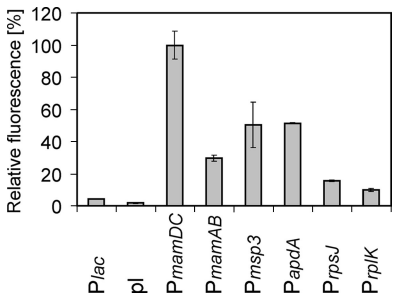

FIG. 1. Analysis of GFP expression from different promoters in *M. gryphiswaldense* by fluorometry. The excitation wavelength was 485 nm (20-nm bandwidth), and emission was recorded at 535 nm (25-nm bandwidth). The value for each sample was averaged from 10 reads over an integration period of 20 μs. The error bars reflect the standard deviations calculated from three independent experiments. pl, promoterless control.

rates and protein synthesis between individual cells (4, 15, 25). The heterogeneity of gene expression from strong promoters in *M. gryphiswaldense* is of relevance for the genetic engineering of magnetosomes for biotechnological applications, as the heterogeneity of gene expression will be reflected by heterogeneously modified magnetosomes. However, a proportion of 50 to 60% of cells that express a gene of interest is sufficient for many practical purposes.

To test the applicability of P_{mamDC} for the expression of fusion proteins in the magnetosome membrane, we expressed a MamC-ZZ fusion protein from P_{mamDC}. The *ezz* gene, which codes for the antibody-binding ZZ-protein domain (11), was PCR amplified from pEZZ18 (GE Healthcare), cloned into pGEM-T Easy, sequenced, and cloned into the pCL6 vector (9) to create the *mamC-ezz* fusion construct pBBRCZZ (Table 2). The P_{mamDC} promoter was PCR amplified, cloned into pJET1.2/blunt (Fermentas), sequenced, and cloned into pBBRCZZ to yield pBBRPdcCZZ. This plasmid was transferred to *M. gryphiswaldense*, and MamC-ZZ magnetosomes were purified as described previously (9) with the only exception that the cells were lysed with a bench top constant cell disruptor (135,000 kPa) (Constant Systems) instead of a French press. After incubation of MamC-ZZ modified, unmodified, and GFP-displaying magnetosomes with rabbit anti-GFP antibody, we detected the highest abundance of the rabbit anti-GFP antibody with a shrimp alkaline phosphatase-labeled goat anti-rabbit antibody on the surface of

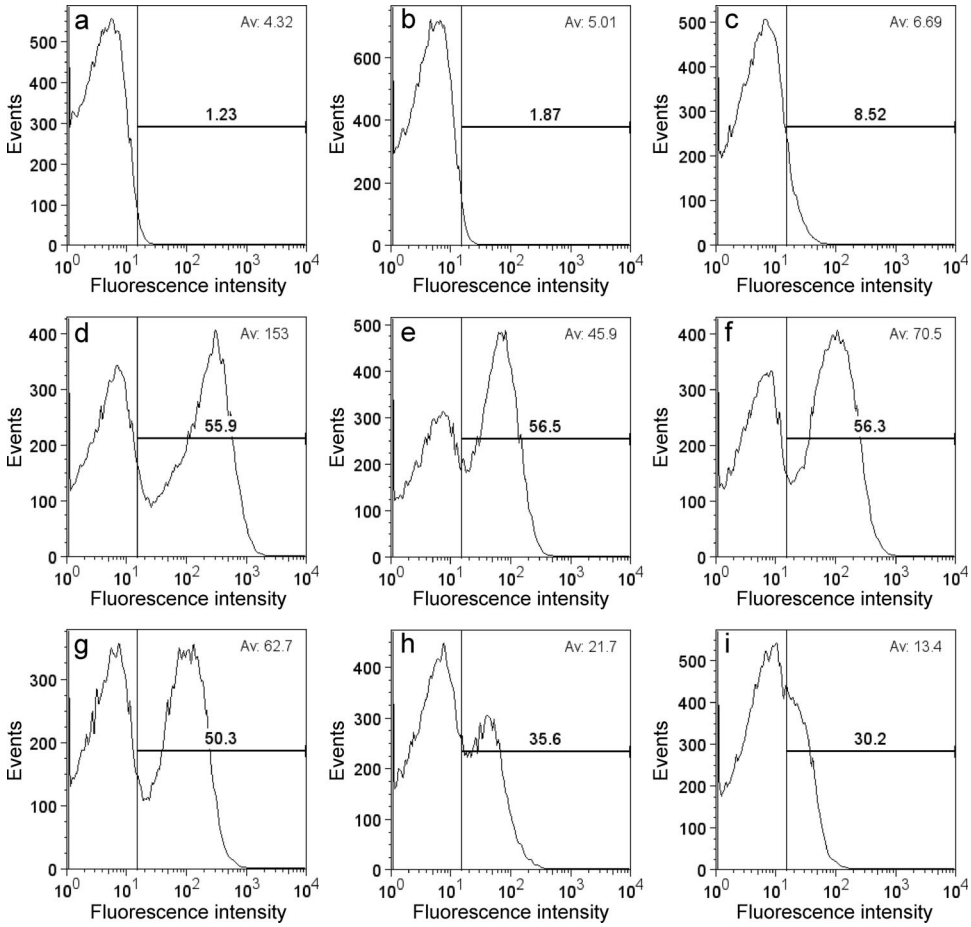

FIG. 2. Flow cytometry of *M. gryphiswaldense* (a) and strains expressing GFP from different plasmids: pBBRpl (promoterless) (b), pBBRegfp (P_{lac}) (c), pBBRPmamDC (d), pBBRPmamH (e), pBBRPmsp3 (f), pBBRPapdA (g), pBBRPrpsJ (h), and pBBRPrplK (i). The proportion of fluorescent cells is shown in bold, and the average fluorescence intensity is displayed in the upper right corner. To estimate the proportion of fluorescent cells, a threshold for fluorescence was set to the fluorescence intensity below which 99% of untransformed *M. gryphiswaldense* cells, which served as a nonfluorescent standard, were detected. Fifty thousand events were analyzed from each sample.

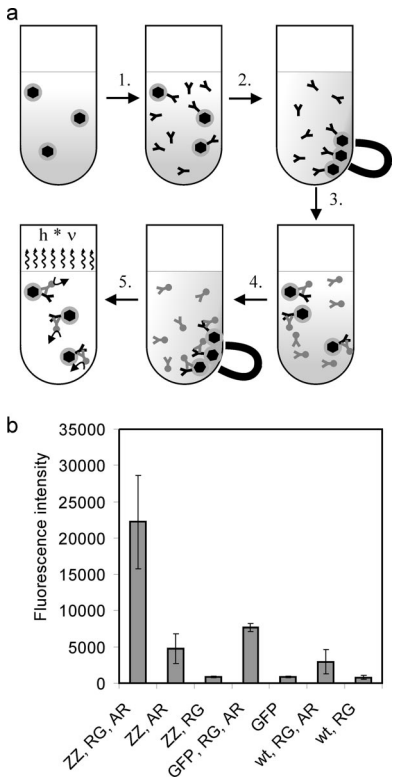

FIG. 3. Antibody-binding assay of MamC-ZZ magnetosomes. (a) Schematic illustration of the antibody-binding assay procedure. 1. MamC-ZZ modified magnetosomes and controls were diluted to a concentration of 1 mM Fe in 500 μl blocking solution (TBS [20 mM Tris, 0.5 M NaCl, pH 7.5] plus 0.5% [wt/vol] milk powder). The samples were incubated for 30 min at room temperature before rabbit anti-GFP antibody was added at a 1:2,000 dilution. 2. After incubation for 45 min, magnetosomes were magnetically collected and resuspended in TBS. 3. After a second magnetic separation step, a conjugate of shrimp alkaline phosphatase and goat anti-rabbit antibody was added in a 1:2,000 dilution in TBS. 4. After an incubation period of 45 min, the magnetosomes were magnetically separated and washed with TBS three times. 5. The particles were resuspended in 200 μl TBS, of which 100 μl was incubated with 100 μl Attophos shrimp alkaline phosphatase detection reagent (Roche) for 5 min. Fluorescence was detected with an Infinite 500 plate reader (Tecan). The excitation wavelength was 430 nm (20-nm bandwidth), and emission was recorded at 535 nm (25-nm bandwidth). (b) Antibody-binding assay of MamC-ZZ modified magnetosomes. The assay was performed with magnetosomes purified from *M. gryphiswaldense* pBBRPdcCZZ (ZZ), with MamC-GFP modified magnetosomes (GFP) (from *M. gryphiswaldense* pCL6 [9]), or with wild-type magnetosomes (wt). The magnetosomes were treated either with a primary rabbit anti-GFP antibody (RG), with a shrimp alkaline phosphatase conjugate of a goat anti-rabbit antibody (AR), or with both antibodies (RG, AR). Standard deviations calculated from three replicates are indicated.

MamC-ZZ magnetosomes (Fig. 3a and b). The signal was substantially stronger than the signal observed for MamC-GFP modified magnetosomes, which were produced by expression of the MamC-GFP fusion from the P_{lac} promoter (9) (Fig. 3b). These results demonstrate that MamC-ZZ modified magnetosomes efficiently bind rabbit antibodies. In addition, MamC-ZZ magnetosomes that were incubated only with alkaline phosphatase-labeled goat antibody yielded a weak signal (Fig. 3b). This indicates that MamC-ZZ modified antibodies also interact weakly with goat antibody. This was expected, as the staphylococcal protein A, from which the ZZ protein domain was derived, interacts weakly with goat antibodies but strongly with rabbit antibodies (14). While ZZ-modified magnetosomes have been produced in *M. magneticum* via genetic engineering of the MamC homolog Mms13 previously (27), this is the first time that antibody-binding magnetosomes from *M. gryphiswaldense* were generated by genetic engineering. One particular advantage of *M. gryphiswaldense* as a host for production of such genetically functionalized magnetosomes is that several mutant strains with an average magnetosome size of between 24 and 37 nm are available (16), which makes it possible to produce a wide range of magnetosomes with engineered bio- and physicochemical characteristics. The purified ZZ-modified magnetosomes can be used for magnetoimmunoassays (27) or for purification of antibodies, as described with ZZ-modified bacterial polyester granules (1).

In conclusion, we demonstrate that P_{mamDC} is a powerful tool for the genetic engineering of magnetosome proteins to generate functionalized magnetic nanoparticles for bio- and nanotechnological applications.

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