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Biosynthesis of magnetic nanostructures in a foreign organism by transfer of bacterial magnetosome gene clusters

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Title:

Biosynthesis of magnetic nanostructures in a foreign organism by transfer of bacterial magnetosome gene clusters

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Introductory paragraph

The synthetic production of monodisperse single magnetic domain nanoparticles at ambient temperature is challenging. In nature, magnetosomes - membrane-bound magnetic nanocrystals with unprecedented magnetic properties - can be biomineralised by magnetotactic bacteria[3], which, however, are difficult to handle. Expression of the underlying biosynthetic pathway from these fastidious microorganisms within other organisms thus could greatly expand their nanotechnological and biomedical applications. However, this has thus far been hindered by the structural and genetic complexity of the magnetosome organelle, and the insufficient knowledge of involved biosynthetic functions. Here we show that the ability to biomineralise highly ordered magnetic nanostructures can be transferred to a foreign recipient. Expression of a minimal set of genes from the magnetotactic bacterium *Magnetospirillum gryphiswaldense* resulted in magnetosome biosynthesis within the photosynthetic model organism *Rhodospirillum rubrum*. Our findings will enable the sustainable production of tailored magnetic nanostructures in biotechnologically relevant hosts, and represent a step towards the endogenous magnetisation of various organisms by synthetic biology.

Main Text:

The alphaproteobacterium *Magnetospirillum gryphiswaldense* produces uniform nano-sized crystals of magnetite (Fe_3O_4) that can be engineered by genetic and metabolic means[8], and are inherently biocompatible. The stepwise biogenesis of magnetosomes involves the invagination of vesicles from the cytoplasmic membrane, the magnetosomal uptake of iron and redox-controlled biomineralization of magnetite crystals, as well as their self-assembly into nano-chains along a dedicated cytoskeletal structure to achieve one of the highest structural levels in a prokaryotic cell.

We recently discovered genes controlling magnetosome synthesis to be clustered within a larger (115 kb) genomic magnetosome island, in which they are interspersed by numerous genes of unrelated or unknown functions. Whereas the smaller *mamGFDC*, *mms6* and *mamXY* operons have accessory roles in biomineralization of properly sized and shaped crystals, only the large *mamAB* operon encodes factors essential for iron transport, magnetosome membrane (MM) biogenesis, and crystallisation of magnetite particles, as well as their chain-like organisation and

intracellular positioning. However, it has been unknown whether this gene set is sufficient for autonomous expression of magnetosome biosynthesis.

Using recombinogenic engineering based on phage derived recombination, we stitched together several modular expression cassettes comprising all 29 genes (26 kb in total) of the four operons in various combinations (Supplementary Fig. 1), but lacking the tubulin-like *ftsZm*. This gene was omitted from its native *mamXY* operon because of its observed interference with cell division during cloning. Regions 200-400 bp upstream of all operons were retained to ensure transcription from native promoters[13]. Our initial attempts to transfer larger parts of the magnetosome island on replicative plasmids resulted in lack of functional expression and genetic instability. Therefore, transposable expression cassettes comprising the MycoMar (*tps*) or Tn5 transposase gene, two corresponding inverted repeats, the origin of transfer *oriT,* and an antibiotic resistance gene were utilized to enable transfer and random chromosomal integration in single copy (Supplementary Tables 3 & 4). Chromosomal re-integration of all cassettes into different non-magnetic single-gene and operon deletion strains of *M. gryphiswaldense* resulted in stable wildtype (wt)-like restoration of magnetosome biomineralization, indicating that transferred operons maintained functionality upon cloning and transfer (Supplementary Fig. 2).

Next, we attempted the transfer of expression cassettes to a foreign non-magnetic host organism (Fig. 1). We chose the photosynthetic alphaproteobacterium *Rhodospirillum rubrum* as a first model because of its biotechnological relevance and relatively close relationship to *M. gryphiswaldense*[16-18] (16S rRNA similarity to *M. gryphiwaldense*: 90%). While the *mamAB* operon alone has been shown to support some rudimentary biomineralization in *M. gryphiswaldense*[6], neither genomic insertion of the *mamAB* operon alone (pTps_AB), nor in combination with the accessory *mamGFDC* genes (pTps_ABG) had any detectable phenotypic effect (Supplementary Table 1). We also failed to detect a magnetic response ("C_{mag}") in the classical light scattering assay[20] after insertion of pTps_ABG6 (*mamAB+mamGFDC+mms6*). However, the cellular iron content of *R. rubrum_ABG6* increased 2.4-fold compared to the untransformed wt (Supplementary Table 1). Transmission electron microscopy (TEM) revealed a loose chain of small (~12 nm) irregularly-shaped electron-dense particles (Fig. 2a ii) identified as poorly crystalline hematite (Fe_2O_3) by analysis of lattice spacings in high-resolution TEM images

(Supplementary Fig. 3), much as in the hematite particles previously identified in *M. gryphiswaldense* mutants affected in crystal formation. To further enhance biomineralization, we next transferred pTps_XYZ, an insertional plasmid harboring *mamX, Y* and *Z* from the *mamXY* operon, into *R. rubrum_ABG6* (Supplementary Fig. 1). The resulting strain *ABG6X* encompassed all 29 relevant genes of the magnetosome island except *ftsZm.* Intriguingly, cells of *ABG6X* exhibited a significant magnetic response (Supplementary Table 1) and were "magnetotactic", i. e., within several hours accumulated as a visible pellet near a magnet at the edge of a culture flask (Fig. 2b). TEM micrographs revealed the presence of electron-dense particles identified as magnetite (Fe_3O_4) (Fig. 2d, Supplementary Fig. 8, Supplementary Table 1), which were aligned in short, fragmented chains loosely dispersed within the cell (Fig. 2a iii). Despite their smaller sizes (average: 24 nm) the particles strongly resembled the magnetosomes of the donor strain with respect to their projected outlines and thickness contrast, suggestive of cubooctahedral or octahedral crystal morphologies (Fig. 2d). Additional insertion of the *ftsZm* gene under control of the inducible *lac* promoter had no effect on the cellular iron content and the number and size of magnetite crystals in the resulting *R. rubrum_ABG6X_ftsZm* (Fig. 2a iv, Supplementary Table 1). Magnetite biomineralization occurred during microoxic chemotrophic as well anoxic photoheterotrophic cultivation. Medium light intensity, 50 µM iron and 23 °C supported highest magnetic response ("C_{mag}") and robust growth of the metabolically versatile *R. rubrum_ABG6X*, which was indistinguishable from the untransformed wt (Supplementary Fig. 4 & 5). The magnetic phenotype remained stable for at least 40 generations under non-selective conditions with no obvious phenotypic changes.

To test whether known mutation phenotypes from *M. gryphiswaldense* could be replicated in *R. rubrum*, we constructed variants of expression cassettes in which single genes were omitted from the *mamAB* operon by deletion within the cloning host *E. coli.* The small (77 amino acids) MamI protein was previously implicated in MM vesicle formation and found to be essential for magnetosome synthesis[12]. *R. rubrum_ABG6X-dI* failed to express magnetosome particles (Supplementary Fig. 10), which phenocopied a *mamI* deletion in the related *M. magneticum*[12]. Another tested example was MamJ, which is assumed to connect magnetosome particles to the cytoskeletal magnetosome filament formed by the actin-like MamK[21]. Much as in

M. gryphiswaldense, deletion of *mamJ* caused agglomeration of magnetosome crystals in about 65% of *R. rubrum_ABG6X-dJ* cells (Fig. 2a v, Supplementary Fig. 10, Supplementary Table 1). Altogether, these observations indicate that magnetosome biogenesis and assembly within the foreign host are governed by very similar mechanisms and structures as in the donor, which are conferred by the transferred genes.

As magnetosomes in *R. rubrum_ABG6X* were still smaller than those of *M. gryphiswaldense*, we wondered whether full expression of biomineralization may depend on the presence of further auxiliary functions possibly encoded outside the canonical magnetosome operons. For instance, deletion of *feoB1* encoding a constituent of a specific ferrous iron transport system specific for magnetotactic bacteria caused fewer and smaller magnetosomes in *M. gryphiswaldense*[22]. Strikingly, insertion of *feoAB1* into *R. rubrum* strain *ABG6X* resulted in even larger, single-crystalline and twinned magnetosomes and longer chains (440 nm) (Fig. 2a vi, Supplementary Table 1). The size (37 nm) of the crystals approached that of the donor and cellular iron content was substantially increased (0.28% of dry weight) compared to *R. rubrum_ABG6X* (0.18%), although still lower than in *M. gryphiswaldense* (3.5%), partly owing to the considerably larger volume of *R. rubrum* cells (Fig. 2c).

Magnetosome particles could be purified from disrupted cells by magnetic separation and centrifugation[23] and formed stable suspensions (Fig. 3). Isolated crystals were clearly enclosed by a layer of organic material resembling the MM attached to magnetosomes of *M. gryphiswaldense*. Smaller, immature crystals were surrounded by partially empty vesicles (Fig. 3c inset) that were also seen in thin-sectioned cells (Supplementary Fig. 8) and on average were smaller (66 ± 6 nm) than the abundant photosynthetic intracytoplasmic membranes (ICM) (93 ± 34 nm (Fig. 3a, Supplementary Fig. 8)).

Organic material of the putative MM could be solubilized from isolated magnetite crystals of *R. rubrum_ABG6X* by various detergents (Fig. 3d), similar as reported for MM of *M. gryphiswaldense*[23]. Proteomic analysis of the SDS- solubilized MM revealed a complex composition (Supplementary Fig. 6), and several genuine magnetosome proteins (MamKCJAFDMBYOE, Mms6, MmsF) were detected among the most abundant polypeptides (Supplementary Table 2). An antibody against MamC, the most abundant protein in the MM of *M. gryphiswaldense*[23], recognized a

prominent band with the expected mass (12.4 kDa) also in the MM of *R. rubrum_ABG6X* (Supplementary Fig. 6).

The subcellular localization of selected magnetosome proteins in *R. rubrum* depended on the presence of further determinants encoded by the transferred genes. For example, MamC tagged with a green fluorescent protein, which is commonly used as magnetosome chain marker in *M. gryphiswaldense*[24] displayed a punctuate pattern in the *R. rubrum* wt background. In contrast, a filamentous fluorescent signal became apparent in the majority of cells (79%) of the *R. rubrum_ABG6X* background in which the full complement of magnetosome genes is present (Supplementary Fig. 7), reminiscent of the magnetosome-chain localization of these proteins in *M. gryphiswaldense*[24].

Our findings demonstrate that one of the most complex prokaryotic structures can be functionally reconstituted within a foreign, hitherto nonmagnetic host by balanced expression of a multitude of structural and catalytic membrane-associated factors. This also provides first experimental evidence that the magnetotactic trait can be disseminated to different species by only single or few events of transfer that are likely to occur also under natural conditions by horizontal gene transfer as speculated before.

As the precise functions of many of the transferred genes have remained elusive in the native magnetotactic bacteria, our results now will enable dissection and engineering of the entire pathway in genetically more amenable hosts. The approximately 30 transferred magnetosome genes constitute an autonomous expression unit which is sufficient to transplant controlled synthesis of magnetite nanocrystals and their self-assembly within a foreign organism. However, further auxiliary functions encoded outside the *mam* and *mms* operons are necessary for biomineralization of donor-like magnetosomes. Nevertheless, this minimal gene set is likely to shrink further by systematic reduction approaches in different hosts.

Importantly, the results are promising for the sustainable production of magnetic nanoparticles in biotechnologically relevant photosynthetic hosts. Previous attempts to magnetize both prokaryotic and eukaryotic cells by genetic and metabolic means (e. g.) resulted in only irregular and poorly crystalline iron deposits. This prompted ideas to borrow genetic parts of the bacterial magnetosome pathway for the synthesis of magnetic nanoparticles within cells of other organisms. Our results now set the stage for synthetic biology approaches to genetically endow both uni- and

multicellular organisms with magnetisation by biomineralization of tailored magnetic nanostructures. This might be exploited for instance as nano-magnetic actuators or *in situ* heat generators in the emerging field of magnetogenetics[30], or for endogenous expression of magnetic reporters for bioimaging[31].

Methods

Bacterial strains, media and cultivation.

Bacterial strains are shown in Supplementary Table 4. *E. coli* strains were cultivated as previously described[32]. 1 mM DL-α,ε-diaminopimelic acid was added for growth of auxotrophic strains BW29427 and WM3064.

M. gryphiswaldense strains were cultivated in FSM medium, in liquid or on plates solidified by 1.5% agar, and incubated at 30 °C under microoxic (1% O_2) conditions[33]. Cultures of *R. rubrum* strains were grown as specified (Supplementary Fig. 3).

Construction of magnetosome gene cluster plasmids and conjugative transfer

Oligonucleotides and plasmids used in this study are listed in Supplementary Table 4 and 5. Red/ET recombination was performed as previously described[14]. Briefly, a cloning cassette was PCR-amplified and transferred into electrocompetent *E. coli* cells (DH10b) expressing phage derived recombinases from a circular plasmid (pSC101-BAD-gbaA). After transfer of the cassette, recombination between homologous regions on the linear fragment and the plasmid occurred.

To stitch the magnetosome gene clusters together into a transposon plasmid (see Fig. S1), we used triple recombination[14] and cotransformed two linear fragments, which recombined with a circular plasmid. Recombinants harboring the correct plasmids were selected by restriction analysis[32].

Conjugations into *M. gryphiswaldense* were performed as described before[33]. For conjugation of *R. rubrum*, cultures were incubated in ATCC medium 112. Approximately $2*10^9$ cells were mixed with $1*10^9$ *E. coli* cells, spotted on ATCC 112 agar medium and incubated for 15 h. Cells were flushed from the plates and incubated on ATCC 112 agar medium supplemented with appropriate antibiotics for 7-10 days (Tc: 10 µg/ml, Km: 20 µg/ml, Gm: 10 µg/ml). Sequential transfer of the plasmids resulted in 10^{-6}-10^{-8} antibiotic-resistant insertants per recipient, respectively. Two clones from each conjugation experiments were chosen for further analyses. Characterized insertants were indistinguishable from wt with respect to motility, cell morphology or growth (Supplementary Fig. 5).

Analytical methods

Optical density of *M. gryphiswaldense* cultures was measured turbidimetrically at 565 nm as previously described[20]. Optical density of *R. rubrum* cultures was measured at 660 nm and 880 nm. The ratio of 880/660 nm was used to determine yields of chromatophores within intact cells (Supplementary Fig. 4). Furthermore, *bacteriochlorophyll a* was extracted from cultures with methanol. Absorption spectra (measured in an Ultrospec 3000 photometer, GE Healthcare) of photoheterotrophically cultivated *R. rubrum_ABG6X* cells were indistinguishable from that of the wt (Supplementary Fig. 4).

The average magnetic orientation of cell suspensions ("C_{mag}") was assayed with a light scattering assay as previously described[20]. Briefly, cells were aligned at different angles to a light beam by application of an external magnetic field.

Microscopy

For transmission electron microscopy of whole cells and isolated magnetosomes, specimens were directly deposited onto carbon-coated copper grids. Magnetosomes were stained with 1% phosphotungstic acid or 2% uranyl acetate. Samples were viewed and recorded with a Morgagni 268 microscope. Sizes of crystals and vesicles were measured with ImageJ software.

Chemical fixation, high pressure freezing and thin sectioning of cells was performed as previously described[17]. Processed samples were viewed with an EM 912 electron microscope (Zeiss) equipped with an integrated OMEGA energy filter operated at 80 kV in the zero loss mode. Vesicle sizes were measured with ImageJ software.

High-resolution TEM was performed with a JEOL 3010 microscope, operated at 297 kV and equipped with a Gatan Imaging Filter (GIF) for the acquisition of energy-filtered compositional maps. For TEM data processing and interpretation the DigitalMicrograph and SingleCrystal software were used[19].

Cryo-electron tomography was performed as previously described[21].

Fluorescence microscopy was performed with an Olympus IX81 microscope equipped with a Hamamatsu Orca AG camera using exposure times of 0.12-0.25 s. Image rescaling and cropping was performed with Photoshop 9.0 software.

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Author contributions I.K., D.S., Y.Z., Q. T., C.J. and R.M. planned and performed cloning experiments. I.K. and A.L. performed genetic transfers and cultivation

experiments. G.W. prepared cryo- and chemically fixed cells. S.B., O.R. and G.W. performed transmission electron microscopy and I.K. analysed the data. J.P. and O.R. performed cryo electron tomography experiments. E.T. and M.P. took high resolution TEM TEM micrographs and analyzed the data. I.K. and A.L. took fluorescence micrographs and performed phenotypisation experiments. I.K. performed Western Blot experiments and analysed proteomic data. A.B. performed Illumina genome sequencing, I.K. analyzed the data. I.K. and D.S. designed the study and wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

Supplementary information is available in the online version of the paper.

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Competing financial interests

I.K. and D.S. have filed a patent application on the process described in this work.

Figure legends:

Fig.1: Schematic representation of molecular organization of gene cassettes that were stepwise inserted into the chromosome of *R. rubrum*. Broad arrows indicate the extensions and transcriptional directions of individual genes. Different colors illustrate the cassettes inserted into the chromosome (oval shape, not to scale) as indicated by their gene names in the figure. Shown in yellow are antibiotic resistance genes (km^R: kanamycin resistance, tc^R: tetracycline resistance, ap^R: ampicillin resistance, gm^R: gentamicin resistance). Thin red arrows indicate different promoters (P) driving transcription of inserted genes (P_{km}, P_{gm}, P_{tc}=promoter of antibiotic resistance cassettes, P_{lacI}=promoter lac repressor, P_{mms}, P_{mamDC}, P_{mamH}, P_{mamXY}=native promoters of the respective gene clusters from *M. gryphiswaldense*, P_{lac}=*lac* promoter). Crossed lines indicate sites of gene deletions of *mamI* and *mamJ* in strains *R. rubrum_ABG6X_dI* and *R. rubrum_ABG6X_dJ*, respectively. IR=inverted repeat defining the boundaries of the sequence inserted by the transposase.

Fig. 2: Phenotypes of *R. rubrum* strains expressing different magnetosome gene clusters and auxiliary genes. (**a**) Transmission electron micrographs. *R. rubrum* wt (i) contains larger phosphate inclusion (P) and some small, non-crystalline electron-dense particles. *R. rubrum_ABG6* (ii), *R. rubrum_ABG6X* (iii), *R. rubrum_ABG6X_ftsZm* (iv), *R. rubrum_ABG6X_dJ* (v), *R. rubrum_ABG6X_feo* (vi). Insets show magnifications of non-crystalline electron dense particles or heterologously expressed nanocrystals from (i)-(vi). For further TEM micrographs see Fig. S10. (**b**) Unlike the untransformed wt, cells of *R. rubrum_ABG6X* accumulated as a visible red spot near the pole of a permanent magnet at the edge of a culture flask. (**c**) TEM micrograph of a mixed culture of the donor *M. gryphiswaldense* and the recipient *R. rubrum_ABG6X_feo*, illustrating characteristic cell properties and magnetosome organization. Insets show magnifications of magnetosomes from *M. gryphiswaldense* and *R. rubrum_ABG6X_feo*. Scale bar: 0.2 µm. (**d**) High resolution TEM lattice image of a twinned crystal from *R. rubrum_ABG6X*, with the Fourier transforms (i) and (ii) that show intensity maxima consistent with the structure of magnetite, respectively.

Fig. 3: Ultrastructural analysis of *R. rubrum*_ABG6X and isolated crystals. (**a**) Cryo-fixed, thin-sectioned *R. rubrum_ABG6X* harbored intracytoplasmic membranes

(ICMs) (93 ± 34 nm, n=95) and magnetic particles (MP). The inset shows a magnification of the magnetite crystals. Scale bar: 100 nm. (**b**) Cryo-electron tomography of isolated magnetic particles of *R. rubrum_ABG6X*: X-Y slice of a reconstructed tomogram (I) and surface-rendered 3D representation (II). A membrane-like structure (thickness 3.4 ± 1.0 nm, n=6) surrounds magnetic particles (red). Yellow: membrane-like structure, blue: empty vesicle. (**c** and **d**) Transmission electron micrographs of isolated magnetosomes from *R. rubrum_ABG6X* (c & d (ii), (iii), (iv)) and *M. gryphiswaldense* (d (i)) negatively stained by (**c**) uranyl acetate or (**d**) phosphotungstic acid. Insets show magnifications of respective magnetic particles. Scale bar: 100 nm. Arrows indicate the magnetosome membrane, which encloses magnetic crystals of *M. gryphis*waldense (3.2 ± 1.0 nm, n=103) and *R. rubrum_ABG6X* (3.6 ± 1.2 nm, n=100) III. Organic material could be solubilized from magnetite crystals of *R. rubrum_ABG6X* with SDS (iv) and less effectively also by Triton X-100 (iii).