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A Genetic Strategy for Probing the Functional Diversity of Magnetosome Formation

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Abstract

Model genetic systems are invaluable, but limit us to understanding only a few organisms in detail, missing the variations in biological processes that are performed by related organisms. One such diverse process is the formation of magnetosomes by magnetotactic bacteria. Studies of model magnetotactic α-proteobacteria have demonstrated that magnetosomes are cubic-octahedral magnetite crystals that are synthesized within pre-existing membrane compartments derived from the inner membrane and orchestrated by a specific set of genes encoded within a genomic island. However, this model cannot explain all magnetosome formation, which is phenotypically and genetically diverse. For example, Desulfovibrio magneticus RS-1, a δ-proteobacterium for which we lack genetic tools, produces tooth-shaped magnetite crystals that may or may not be encased by a membrane with a magnetosome gene island that diverges significantly from those of the α-proteobacteria. To probe the functional diversity of magnetosome formation, we used modern sequencing technology to identify hits in RS-1 mutated with UV or chemical mutagens. We isolated and characterized mutant alleles of 10 magnetosome genes in RS-1, 7 of which are not found in the α-proteobacterial models. These findings have implications for our understanding of magnetosome formation in general and demonstrate the feasibility of applying a modern genetic approach to an organism for which classic genetic tools are not available.

Introduction

Genetic analysis historically relied on model systems that were easy to manipulate and for which genetic maps, then later full genome sequences, were available. Today many interesting organisms have their genomes sequenced, but cannot be easily manipulated due to their incompatibility with standard genetic tools. One such organism is Desulfovibrio magneticus RS-1 (RS-1).

RS-1 is a member of the magnetotactic bacteria, a phylogenetically diverse group of gram-negative bacteria that synthesize magnetic iron oxide (magnetite) or iron sulfide (greigite) crystals within complex genetically encoded intracellular organelles. Magnetotactic bacteria were independently discovered by Bellini in 1963 [1,2] and Blakemore in 1975 [3]. Both investigators observed bacteria that aligned with a magnetic field. It was hypothesized that this behavior allows the bacteria, which were isolated in the northern hemisphere, to easily swim northwards to the bottom of the water column where micro-oxic or anoxic conditions exist. This behavior is due to intracellular single-domain magnetite or greigite crystals organized in one or more chains along the length of the cell [4]. Since their discovery, magnetotactic bacteria have inspired studies in bacterial cell biology [5], biomineralization [6], and nanotechnology [7].

Magnetotactic bacteria have been isolated all over the world, and belong to the α-, γ-, and δ-proteobacteria, as well as the Nitrospirae and candidate OP3 division (Figure 1A). Different species produce different types of magnetosomes, varying in their number, shape and size of crystal, organization within the cell, and mineral composition [8,9]. In addition to the phenotypic diversity of the magnetosomes themselves, the magnetotactic bacteria are morphologically and physiologically diverse, with anaerobic and microaerophilic species; coccoids, vibrios, spirilla, and rod-shaped cells; and some obligate multicellular bacteria. Bioinformatic analyses of magnetotactic bacterial genomes have identified a genomic island that contains the genes for constructing magnetosomes [10,11], termed the Magnetosome Island (MAI). While the...
MAIs of diverse magnetotactic bacteria vary in content, they share core magnetosome forming (mam) genes [11–13]. Though the magnetotactic bacteria are dispersed among many non-magnetotactic members of the Proteobacteria and related phyla, the mam genes suggest a monophyletic origin for the magnetosome trait. The phylogenetic trees of individual mam genes are the same as the phylogenetic trees of the ribosomal 16S sequences for the bacteria that possess them [14], suggesting either that the common ancestor of all Proteobacteria, Nitrospirae and the candidate OP3 division was a magnetotactic bacterium, or that the MAI was passed to some members of these groups via ancient horizontal gene transfer.

Mechanistic analyses of magnetosome formation have been conducted largely in two closely related model organisms, *Magnetospirillum magnetica*um AMB-1 (AMB-1), and *Magnetospirillum gryphiswaldense* MSR-1 (MSR-1). Through biochemical [15–17] and genetic [18,19] analyses, the mam and mms genes were identified as participating in magnetosome formation. These genes are located within the MAI, but they make up only a small portion of it. For example, the AMB-1 MAI is 98 kilobases (kb) long, but an 18 kb portion containing only the mam genes is able to partially substitute for the whole island [20], and 26 kb of the 115 kb MSR-1 island can reconstitute magnetosome synthesis in a non-magnetotactic bacterium [21]. Because much of the MAI encodes genes that are not required for magnetosome synthesis, it is not obvious which genes in an MAI from any given magnetotactic bacterium are participating in creating magnetosomes with different phenotypes.

AMB-1 and MSR-1 are α-proteobacteria, which produce cubo-octahedral shaped magnetite crystals, while the δ-proteobacteria, represented by RS-1, produce elongated bullet or tooth shaped crystals (Figure 1B). The MAIs of δ-proteobacteria have homologs to some of the α-proteobacterial MAI genes, but not others. Conspicuously absent are genes whose mutants have small and misshaped crystals, such as the mms genes and mamS (amb0975) [12]. For comparison, the AMB-1 and RS-1 islands are shown in Figure 1C. In addition to missing some α-proteobacterial genes, RS-1 contains genes that are shared exclusively among the MAIs of magnetotactic δ-proteobacteria, designated mad genes [11].

Microscopy studies have shown that AMB-1 and MSR-1 build membrane vesicles, called magnetosome membranes, which are then the site for magnetite synthesis [13,18]. RS-1 contains numerous intracellular membranes [22], which makes looking for magnetosome membranes difficult. After release from iron starvation, RS-1 constructs another organelle composed of membrane-bound iron and phosphorus inclusions. In the same imaging study membranes were observed around these particles but not around magnetosomes [22]. These findings suggest that RS-1 may have a fundamentally different mechanism for constructing and maintaining its magnetic organelle.

These differences make a compelling argument for genetic and molecular analyses of magnetotactic δ-proteobacteria. Understanding the biological control of magnetic crystal shape and size is of particular interest to those designing magnetic nano-tools for industry [23] or health care [24]. The comparison of different systems that perform similar tasks has proved fruitful in other cases. For example research on different bacterial CRISPR systems resulted in the discovery of the simplified type II CRISPR that has become a valuable tool for manipulating eukaryotic genomes [25–27].

In this study we overcome the need for sophisticated genetic tools in RS-1 by taking advantage of modern sequencing methods to identify mutations from a classic forward genetic screen. We identified non-magnetic phenotypes for mutants of homologs of mam and mad genes, as well as genes not previously identified as being involved with magnetosome synthesis. These findings expand our understanding of magnetosome formation, both in the δ-proteobacteria and across all magnetotactic bacteria. Additionally, our results highlight the broad utility of this approach for studying interesting organisms that have previously been intractable to genetic analysis.

**Results**

**RS-1 can be transconjugated with low efficiency**

We chose RS-1 as a representative of the magnetotactic δ-proteobacteria because it can be grown as colonies on solid medium and in pure culture in liquid medium under conditions where magnetosomes are produced [22]. It also has a sequenced genome [12], consisting of a 5.2 megabase chromosome and two plasmids. In order to conduct genetic experiments, we attempted to transform RS-1 with a plasmid, pBMK7, that is designed for *Desulfovibrio* species [28]. We were successfully able to obtain RS-1 cells carrying pBMK7 by conjugation with *Escherichia coli*. However, the transconjugation rate was extremely low. With pBMK7, we obtained $10^{-5}$ transconjugates per recipient cell. For comparison, conjugation of AMB-1, an excellent genetic model system, yields $10^{-3}$ transconjugates per recipient cell [19], which results in thousands of transposon insertions per conjugation and allows for efficient screening of the entire genome.

The most comprehensive genetic analyses of the α-proteobacteria have come from genetic dissections of the AMB-1 and MSR-1 MAIs [29–31]. Systematic deletions have identified minimal sets of genes required to make magnetosomes, as well as assigned genes into groups that perform the functions of membrane remodeling, crystal nucleation, and crystal maturation. However, the tools available to create targeted deletions rely on suicide vectors that cannot replicate in the target organism such that DNA uptake and integration on the chromosome, two rare events, are selected for in one step. Attempts to move a suicide vector into RS-1 were
unsuccessful, due to the low transconjugation efficiency and perhaps also a low frequency of homologous recombination.

An alternative is a genetic screen where genes are disrupted randomly and mutants with interesting phenotypes are selected from a large pool. However, modern bacterial genetic screens are performed with transposon-based tools that allow for easy identification of the disrupted gene but are delivered on suicide vectors. Attempts to obtain mutants with a range of mariner, Tn5, Tn7, and Tn10 based transposons were also unsuccessful. To work around this inability to generate transposon insertions, we isolated non-magnetic mutants created by chemical and UV mutagenesis, then used whole genome sequencing to identify the causative genetic change.

Isolation of non-magnetic mutants
RS-1 has a doubling time of 11 hours and is an obligate anaerobe, though in our hands it is somewhat aerotolerant, and there is some evidence that it can survive near the oxic-anoxic transition zone in a gradient [32]. Screening large numbers of colonies is impractical, so we employed a two-step process. First a selection in liquid increased the proportion of non-magnetic cells in the population, then single colonies were screened for non-magnetic phenotypes. This strategy is similar to one previously used with AMB-1 [18]. To ensure that mutants with magnetosome-synthesis defects would not be accidentally discarded as magnetic because they still possessed magnetic crystals synthesized prior to mutagenesis, we began with non-magnetic cells, obtained by passaging wild type (WT) cells without iron.

We next wanted to verify the loss of magnetic particles in our cells due to this growth condition. The ability of RS-1 cells to synthesize magnetosomes can be measured by quantifying cellular alignment with a magnetic field rotated 90 degrees, using a spectrophotometer. The ratio of optical densities is called the coefficient of magnetism, or Cmag [33]. WT RS-1 has a Cmag between 1.4 and 1.6. After two passages without iron we found that the culture had a Cmag of 1, indicating that the cells no longer responded to a magnetic field. These WT, non-magnetic cells were then mutagenized with either ultraviolet radiation or ethyl methanesulfonate. In order to ensure that a sufficient level of mutagenesis was occurring, each mutagen was provided at a dose that resulted in 50% cell survival. As described in Table 1, this dose resulted in some mutants with only one genetic change, and others with dozens of changes when compared to the WT.

After mutagenesis, the cells were inoculated into fresh growth medium with iron. We expected the resultant culture to contain a mixture of mutant cells, most of which would be magnetic. To isolate the rare non-magnetic mutants, we passed the culture over a magnetized column and collected the flow-through, which contained non-magnetic cells. However, much of the flow-through was only transiently non-magnetic, and when inoculated into fresh media with iron resulted in a culture with a WT Cmag. This suggests that a proportion of a WT RS-1 culture is either naturally

Fig. 1. RS-1 is a representative of a group of bacteria that are phylogenetically and phenotypically distinct from the magnetotactic α-proteobacteria. A) 16S phylogenetic tree of magnetotactic bacteria. B) AMB-1 and RS-1 magnetite crystals visualized by TEM. Scale bar 100 nm. C) The mamAB gene clusters of AMB-1 and RS-1 shown in the context of the MAI. Pink squares represent the repeats surrounding each island. Purple arrows represent the genes that are conserved between the two.
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+++ indicates a Cmag of 1.4–1.6, ++ a Cmag of 1.1–1.3, + that some cultures have a Cmag around 1.05, near the limit of detection, and – a Cmag of 1.
ND: mamL allele 3 was the only mutant not analyzed by whole genome sequencing; its mutation was discovered by PCR and Sanger sequencing.
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non-magnetic, or lags in magnetosome formation after release from iron starvation. To isolate those cells that had a true non-magnetic phenotype, we repeated the outgrowth and column selection several times. With each repetition, the \( C_{\text{marg}} \) of the outgrowth decreased, indicating non-magnetic mutants were taking over the population. After four rounds of growth and selection the column flow-through was plated on solid media and single colonies were picked for further analysis. Those colonies with non-magnetic or low-magnetic phenotypes were analyzed by whole genome sequencing to determine the causative genetic change. The screen strategy is described in Figure 2.

Because of the number of outgrowths, we were concerned that identical mutant colonies could be isolated that all descended from one mutagenized cell. To avoid this, the outgrowth process was performed on many independent cultures, and only one colony from each culture was analyzed. After the mutation for each strain was identified, we used PCR and Sanger sequencing to check for the change. The screen strategy is described in Figure 2.

Many magnetotactic bacteria organize magnetosome synthesis genes into a genomic island, the MAI. The MAI is frequently surrounded by repetitive sequence elements that enable its excision and loss from the chromosome by homologous recombination [10,34]. In the laboratory model systems AMB-1 and MSR-1 spontaneous island deletions are often isolated during normal laboratory cultivation. As with other magnetotactic bacteria, the genome sequence of RS-1 reveals a cluster of magnetosome-related genes surrounded by repetitive sequence elements. The RS-1 MAI is an 82 kb region of DNA between nucleotides 4607747 and 4689778 [12,17]. Flanking this region is a 2.4 kb direct repeat (Figure 3C, pink regions) that consists of three genes with homology to the IS66 family of insertion sequence elements. There are a total of 12 of these IS66 operons located on the RS-1 chromosome and plasmids, including two other pairs that have 100% nucleotide identity to the pair that flanks the RS-1 MAI.

Approximately 90% of mutants recovered in this work were island deletions. Island deletions had no coverage at positions within the MAI when analyzed by whole genome sequencing (Figure S1). The deletion of the MAI was confirmed by the ability to obtain PCR products using primers just outside, but not just inside the border of the MAI. RS-1 MAI deletions could be isolated by the non-magnetic enrichment and outgrowth method used in this screen even in the absence of mutagenesis, suggesting that island loss is occurring naturally in RS-1. RS-1 MAI deletions lack the electron-dense particles found in the WT (Figure 3A and B) and have no magnetic response (\( C_{\text{marg}} = 1 \)).

In addition to the 2.4 kb repeat, portions of the MAI consist of DNA transposases, recombinases and short repetitive sequences (Figure 3C, dark grey regions). These areas divide the remaining genes into four groups, indicated in Figure 3C by Roman numerals. The largest of these, group I, contains most of the genes that have homology to genes in other magnetotactic bacteria. These include all the \( \text{mad} \) genes, which are common to magnetotactic bacteria [11–13], and some of the \( \text{mad} \) genes, which are shared among the magnetic \( \delta \)-proteobacteria [11].

 Groups II and III contain some \( \text{mad} \) genes (\( \text{mad}5 \) (DMR 41230) and \( \text{mad}12 \) (DMR 41300)), as well as some genes that share homology with signaling or transcriptional regulation genes (DMR 41220, DMR 41310). Group IV contains genes associated with motility, such as a cheW homolog and genes encoding a methyl accepting chemotaxis protein and a GGDEF domain protein (DMR 41440, DMR 41450, and DMR 41480, respectively). There is a copy of the 2.4 kb repeat that has 94% nucleotide identity to those surrounding the MAI and is located directly upstream of group IV, suggesting that these genes might compose a motility module that could be lost by recombination, though this was not observed during this study.

Functional Diversity of Magnetosome Formation

Fig. 2. Screen strategy. Pink, yellow, and brown lines represent genes. "X" represents a mutation. In this example, a mutation in the yellow gene resulted in a magnetic defect, whereas a mutation in the brown gene did not. doi:10.1371/journal.pgen.1004811.g002
Three mutants with deletions of a portion of the island were recovered (Figure 3C, Table S1). In each case the region between groups I and II formed one edge of the deletion. This region contains many 46 base, 12 base, and 10 base direct repeats; however these repeats do not border the deletions, suggesting they were not created by homologous recombination with the repeats. In deletion 1, a portion of group I is missing, removing many mam genes, such as mamB (DMR_41110) and mamQ (DMR_41130), whose deletions in the α-proteobacteria result in non-magnetic cells [29,30]. The other two deletions remove areas outside of group I. One includes most of group II, and the other includes the entire right side of the island, groups II, III, and IV. All three deletions have non-magnetic phenotypes, with a Cmag of 1 and no particles visible by transmission electron microscopy (TEM, Figure S2). These results show that there are genes in addition to the mam and mad genes of group I that are necessary for RS-1 to form magnetite crystals.

RS-1 intracellular membranes and iron-phosphorus organelles do not require the MAI

RS-1 cells are filled with intracellular membranes [22]. Intracellular membranes are rare among bacteria, often being involved in photosynthesis or energy production [35]. As RS-1 does not photosynthesize, the function of its extensive intracellular membrane network remains mysterious. In our previous work we were not able to see membranes surrounding RS-1 magnetite crystals, even though many intracellular membranes are visible throughout the cell and surrounding the iron-phosphorus inclusions that RS-1 transiently produces upon release from iron starvation [22]. However, it is possible that membranes do surround RS-1 crystals, but that they are so closely associated that they are not visible by microscopy. Indeed, Matsunaga and coworkers observed some material by TEM around purified magnetosomes that was removed by detergent treatment, which they interpreted as magnetosome membranes [17]. Some of the intracellular membranes of RS-1 have an elongated shape that vaguely resembles the elongated shape of RS-1 magnetite crystals although their dimensions are significantly different. In previous work no magnetite crystals were observed within these intracellular membranes yet a connection between them and magnetite formation could not be ruled out [22].

In the α-proteobacteria, the genes for remodeling the inner membrane to produce magnetosome membranes are encoded within the MAI, and in the absence of these specific genes or of the entire MAI no magnetosome membranes are synthesized [29]. To determine if the intracellular membranes of RS-1 depend on the
MAI, we compared an MAI deletion strain to WT by preparing the cells using cryo-ultramicrotomy and imaging them with TEM. As shown in Figure 4A, both strains contain elongated intracellular membrane structures, suggesting that RS-1 magnetosomes and intracellular membranes are unrelated. It is possible, however, that RS-1 synthesizes crystals using membranes that were native to the bacterium before the acquisition of the MAI, or that the genes responsible for creating and maintaining the intracellular membranes were originally part of the MAI but have since been moved to a different part of the genome.

In addition to making magnetosomes, RS-1 makes an iron and phosphorus containing organelle [22]. In the hours after release from iron starvation, hundreds of intracellular nanometer-sized iron phosphorus particles surrounded by membranes appear throughout the cell, and then disappear over the course of three days [22]. As their disappearance corresponds with the synthesis of magnetosomes, we initially hypothesized that they were magnetosome precursors. However, a pulse-chase experiment showed that the iron in the iron-phosphorus organelles did not end up in magnetosome crystals [22], suggesting that they are unrelated to magnetosome synthesis. To determine if the iron-phosphorus organelles require magnetosome genes, we subjected WT and MAI deletion cells to iron starvation, and then followed them by TEM after the re-introduction of iron. As shown in Figure 4B, both strains produced the iron-phosphorus organelles, confirming our original finding that these are unrelated processes that occur in RS-1.

The mutants are complemented with WT MAI genes

Some of the isolated mutants differed from the WT genome sequence at only one position, while others contained many changes (Tables 1, S2). In these cases, we predicted that the change in a gene located in the MAI was responsible for the observed phenotype. To test this, we complemented each of the mutants by expressing a WT copy of the mutated MAI gene from plasmid pBMK7. Some genes were expressed from the RS-1 mamA (DMR_41160) promoter, while others were expressed from plasmid pBMK7. Strains were transformed with an empty plasmid control (purple bars) or a plasmid expressing the gene mutated in each strain (pink bars). Error bars indicate the standard deviation from multiple replicates (for details see table S3).

Fig. 5. Complementation of mutants. Strains were transformed with an empty plasmid control (purple bars) or a plasmid expressing the gene mutated in each strain (pink bars). Error bars indicate the standard deviation from multiple replicates (for details see table S3). doi:10.1371/journal.pgen.1004811.g005
their own promoters (Tables S3, S4). Though complementation efficiency differed, expression of the candidate MAI gene increased the C\textsubscript{mag} in all cases compared to the same mutant that was carrying the empty vector (Figure 5).

In some cases different mutant alleles of the same gene exhibited varying levels of complementation. For instance, unlike other alleles of mamB, allele 1 was complemented at a very low level. This allele is a transposon insertion that may have polar effects on downstream genes. By contrast, the transposon insertion in tauE allele 3 complemented to the same level as the other tauE alleles. The gene downstream of tauE is on the opposite strand, so in this case no polar effects would be predicted, mamL allele 1 (E63K), and fmbB allele 2 (a frameshift) could not be complemented even though the other alleles of mamL and fmbB could, suggesting that these are dominant negative alleles. Alternatively, one or a combination of the three or two additional mutations in these respective strains could be responsible for the phenotype. Surprisingly, fmbB allele 2 contains a frameshift at the same amino acid as fmbB allele 1, which is complementable. The alleles are the gain (allele 1) or loss (allele 2) of an extra cysteine in a poly-cysteine tract, and encode proteins that end with different polypeptides. These may impart different biochemical properties on the FmpB mutant proteins, perhaps resulting in degradation of one and toxic build up of the other.

tauE was the only single gene we found outside of group I whose mutants have a non-magnetic phenotype. The location of tauE could account for the phenotypes of large deletions 2 and 3, both of which lack tauE. We investigated whether tauE was sufficient to complement the loss of most of group II or the loss of groups II–IV. We found that tauE alone was able to complement deletion 2 to a similar level as single tauE mutations but had little effect on the magnetic response of deletion 3 (Figure 5). This indicates that while much of group II besides tauE is dispensable for magnetosome formation, there is another factor or factors in groups III and IV that are required for magnetosome synthesis in RS-1 but were not identified in this screen.

Point mutations in mamB and mamL highlight functional regions of the encoded proteins

Due to their functional importance in the \(\alpha\)-proteobacteria and their conservation in every magnetotactic bacterium investigated, we expected to find some mam gene mutants with non-magnetic phenotypes. We isolated mutations in mamB, mamL, and mamQ. MamB is a member of the cation diffusion facilitator (CDF) family, which exports divalent cations from cells. In magnetosome synthesis MamB and another CDF protein, MamM, are thought to be involved in the transport of iron [36]. MamB is also required for magnetosome membrane formation in AMB-1 [29]. Five alleles of mamB were isolated, four with non-magnetic phenotypes. As shown in Figure 6A, G20 is conserved among the \(\delta\)-proteobacterial MamBs. MamB\textsubscript{G20D} has a less severe phenotype than the other alleles of mamB suggesting that this residue may not be required for MamB function, even among the \(\delta\)-proteobacteria. MamB\textsubscript{G20Y} is of particular interest as the mutated cysteine, along with neighboring residues C6 and K2, is conserved across all magnetosome MamB proteins, but varies among CDF family homologs from other species (Figure 6A). Together with the non-magnetic phenotype of MamB\textsubscript{G20Y}, this conservation pattern suggests the amino-terminus of MamB is important to its role in magnetosome formation.

We isolated three mamL alleles, which initially appeared to have no magnetic response, as shown in Figure 5. However, occasional cultures of all three mamL mutants had a C\textsubscript{mag} of about 1.05, nearing our limit of detection. We interpret this to mean that these mutants are so impaired in magnetosome formation that only rare cultures had enough magnetic cells to produce a measureable C\textsubscript{mag}. This is supported by the rare particles observed in some cells of mamL allele 1 by TEM (Figure S2). Though no particles were observed for the other alleles of mamL, these strains’ occasional magnetic response implies that crystals can be present, though not observed in our experiments. In the \(\alpha\)-proteobacteria, mamL deletion results in complete loss of crystals and magnetosome membranes [29]. Together, this suggests that either all three alleles isolated for mamL retain partial function, or mamL is not required for the synthesis of magnetite in RS-1.

The mamL alleles are all point mutations. As shown in Figure 6B, the I37 position contains mainly aliphatic amino acids, but in mamL\textsubscript{E63K}, an aromatic phenylalanine is at this position. RS-1 E63 is in a group of acidic amino acids that are present only in \(\delta\)-proteobacterial MamLs, but in mamL\textsubscript{E63K} this acidic stretch is disrupted with a basic lysine residue. The conservation pattern of this acidic stretch and its phenotype in RS-1 points to this area of the protein as being important for MamL’s ability perform \(\delta\)-proteobacteria-specific functions.

Some mad genes have non-magnetic phenotypes

The mad genes were recently identified using comparative genomics. They are defined as being conserved uniquely in the MAI regions of magnetotactic \(\alpha\)-proteobacteria [11]. It was hypothesized that due to this conservation pattern they must be important for magnetosome formation in the \(\delta\)-proteobacteria. However, no mad gene phenotype has been observed and nothing is known about their activity or function. 31 mad genes were identified, and RS-1 has homologs of 25 of them [6,11]. We were interested in whether the screen uncovered mutations in any mad genes that resulted in non-magnetic phenotypes. Indeed, mutations of mad1 (DMR 41150), mad2 (DMR 41120), and mad6 (DMR 41050) were isolated. This is the first demonstration of phenotypes for mad genes.

The mad6 mutant had a limited magnetic response. Mad6 consists mostly of a NapH nitrate reductase domain, though RS-1 does not reduce nitrate [37]. In MSR-1, deletions of genes affecting nitrate reduction have effects on magnetite biomineralization [38,39]. Mutations of mad1 and mad2 resulted in cells with no magnetic response. Mad1 and Mad2 have no obvious homology to any characterized proteins. Mad1 contains three CXXCH heme-binding motifs. Two adjacent CXXCH motifs is a feature common to a number of magnetosome proteins, and has been termed the magnetochrome domain [40,41]. The sequences of Mad1 and Mad6 suggest they both perform redox functions, a feature found in a number of magnetosome proteins from the \(\alpha\)-proteobacteria. This aspect of the \(\delta\)-proteobacterial genes mad1 and mad6, as well as fmbB (described in the discussion), suggest that redox activity is just as important to magnetosome synthesis, if not more so, in the \(\delta\)-proteobacteria.

Some mutants synthesize misshaped particles

Given the unique morphology of RS-1 crystals, we were particularly interested in mutants that produced misshaped magnetic particles. Thus, we examined the mutants with whole-cell TEM. As described in Table 1 and shown in Figures 7 and S2, some non-magnetic mutants contained no electron-dense particles. This suggests that MamB, MamQ, and TauE are absolutely required for magnetite synthesis.

As expected, mutants with low C\textsubscript{mag} values were found to contain electron-dense particles consistent with magnetite. As observed above for mamL allele 1, the shape, size, and organization of these particles appear similar to WT; however...
the number of particles per cell was much lower (Figures 7, S2). In mutant cultures with $C_{\text{muc}}$ values above one, most cells had no particles. Those mutant cells that had particles contained between one and four per cell, while WT RS-1 cells have $10^{+}/-4$ crystals per cell. These results indicate that Kup, MamL, Mad6 and FmpB contribute to efficient magnetite formation, but that WT-like crystals can be produced at a lower rate if they are impaired.

In contrast, mad1 and mad2 mutants and cells with fmpA allele 2 contained rare, electron-dense particles even though they have no magnetic response. These particles appeared less elongated than in the WT (Figure 7 mad1), and they sometimes had sharp corners or jagged-looking edges (Figure 7 mad2 allele 3).

**Mad1 is required for stable, single-domain magnetite crystallization**

We wondered why some mutants, such as the mad1 mutant, have no magnetic response despite the presence of electron-dense particles. To detect if the particles in mad1 mutants are magnetite, we performed high-resolution transmission electron microscopy (HRTEM) on these samples, finding small, multi-domain crystals (Figure 8A). The different angles of the lattice lines in Figure 8A indicate that the crystal is composed of multiple domains, and the crystal lattice line spacing indicates that the crystal is magnetite. This is reminiscent of the phenotype of a deletion of mamS, a gene found in AMB-1 but absent in RS-1, where many small crystals are found together in one magnetosome membrane [29]. These results suggest that Mad1 may regulate crystal nucleation, limiting each developing crystal to one nucleus, or that it functions to cull multi-nucleate crystals down to one as the crystals grow in size.

The multi-domain nature of the magnetite crystals in the mad1 mutant offers an explanation for why these cells are unable to turn in a magnetic field. It is thought that the many small, aligned magnetic moments of the magnetite crystals in magnetotactic bacteria combine to produce a magnetic moment strong enough to turn the cells in a magnetic field [42]. As the crystals of mad1 mutant cells are multi-domain, each domain may be too small to hold a stable dipole moment. Or, if large enough, the unaligned magnetic moments may work against each other. To observe the effect this has on the magnetic moment of the entire cell, we employed a wide-field optical magnetic microscope using nitrogen-vacancy (NV) color centers in diamond [43] to observe the magnetic field produced by WT and mutant cells. This technique relies on the fluorescence produced by NV centers in diamond that have been spectroscopically probed by microwaves in order to visualize local magnetic fields under ambient conditions. Because most mad1 mutant cells lack any electron-dense particles, we enriched the mutant population for those containing magnetite by passing the culture over a magnetized column and imaging only the 1–2% of cells that were retained on the column.

We carried out the initial NV-diamond magnetic imaging of wild-type RS-1 and mad1 mutant cells in a small externally-applied bias field of 1.2 mT. WT cells dried on the surface of the diamond chip were measured with the NV technique, and found to produce dipolar magnetic fields on the order of 0.6 µT (consistent with an average magnetic moment in the range of $10^{-11}$ to $10^{-17}$ A m$^2$, and approximately an order of magnitude smaller than the average magnetic moment measured for wild-type AMB-1 [43]), whereas almost none of the mad1 mutant cells produced fields above the measurement noise floor of $\sim$0.1 µT (Figure 8B). To test for possible paramagnetic behavior, the same WT and mutant cells (in the same fields of view) were then measured at a higher bias field of $\sim$20.5 mT. In this case, only a
scalar magnetic field projection along a single diamond crystal axis was measured, instead of the full vector field recorded in the first set of experiments. At the larger bias field, most WT cells still produced magnetic field patterns consistent with randomly oriented, fixed dipoles in the plane of the diamond (Figure 8C). However, a significant fraction of the mad1 mutant cells now also produced dipolar field patterns, predominantly aligned along the direction of the applied bias field (Figure 8C). Approximately one third of the mutant cells produced detectable fields in the 20.5 mT bias, compared to less than 10% of the same population at 1.2 mT bias. This paramagnetic behavior is consistent with the formation of many small, superparamagnetic magnetite domains in the mad1 mutants, instead of the larger, blocked ferrimagnetic particle chains observed in the WT cells.

The RS-1 MAI-encoded Kup is a potassium transporter

Our screen for non-magnetic mutants uncovered two genes in the RS-1 MAI that are homologous to common bacterial genes. Mutations in tauE and kup (DMR_40800) resulted in non-magnetic phenotypes even though RS-1 has additional copies of each of the effected genes outside the MAI. tauE encodes a putative anion transporter [44] found in many bacteria. RS-1 has an island copy (DMR_41280) and an extra-island paralog (DMR_40120) of tauE. TauE domains are found fused to the protease domains of MamE in RS-1 (DMR_41080) and MamO in AMB-1 (amb0969), though no standalone TauE protein has been identified as being important for magnetosome synthesis. Like tauE, kup is a common bacterial gene of which RS-1 contains an MAI-specific paralog (DMR_40800) and an extra-island paralog (DMR_15830). We isolated three alleles of kup (DMR_40800), all of which are associated with rare cells containing electron-dense particles and few cultures having a measurable Cmag. Kup proteins are ubiquitous potassium transporters that have been characterized in E. coli and plants [45]. Most bacteria possess one copy of kup, though some, like RS-1, have two.

We were surprised to find a phenotype for kup (DMR_40800), as potassium transport has not previously been implicated in magnetosome synthesis. An alignment of RS-1 Kup proteins DMR_40800 and DMR_15830 with the well-studied KupE coli (YP_026244) shows a significant amount of conservation (Figure 9A). Though the structure and mechanism of potassium transport for Kup remain undetermined, a recent study highlighted several negatively charged amino acids that are required for transport in E. coli, D23, E116, E229, and D408 [46]. As shown in Figure 9A, these are also conserved in both RS-1 proteins, consistent with the idea that kup (DMR_40800) encodes a potassium transporter.

To see if Kup (DMR_40800) can transport potassium, we tested whether it was able to rescue the growth of a strain of E. coli, TK2446, in which all potassium transporters have been deleted. TK2446 is capable of growth in the presence of high potassium (120 mM), but requires the expression of a potassium transporter to grow in the presence of low potassium (15 mM). As shown in Figure 9B, expression of KupE coli allows TK2446 to grow on low potassium, while cells carrying the control vector that expresses Green Fluorescent Protein cannot grow. As with KupE coli, expression of Kup (DMR_40800) in TK2446 results in growth on low potassium.

These findings suggest that Kup (DMR_40800) has the ability to transport potassium. In E. coli, Kup spans the inner membrane and transports potassium ions into the cytoplasm. General import of potassium into the cell cannot be the only function of Kup (DMR_40800), as the expression of a second copy of Kup (DMR_15830) was not able to complement the magnetic

Fig. 7. Electron-dense particles are found in some mutants. TEM of WT and mutant cells show WT-like particles in some mutants (fmpB, fmpA) and unusual-looking particles in others (mad1, mad2). Scale bar 200 nm for whole cell images, 50 nm for enlargements. doi:10.1371/journal.pgen.1004811.g007
The phenotype of *kup* allele 1 RS-1. *KupDMR_13030* is functional for potassium transport, as it was also able to rescue TK2446 growth. Whether *KupDMR_140900* is transporting potassium at a special location or time for magnetosome synthesis, or whether it plays an entirely different role while retaining some potassium transport capability remains to be determined.
Discussion

Magnetotactic bacteria are a diverse group but our mechanistic understanding of magnetosome synthesis is based only on the model α-proteobacteria. Relying on these model systems fails to elucidate the divergent magnetosome phenotypes of RS-1 and other non-α-proteobacterial magnetotactic bacteria. RS-1 is well placed to expand our understanding of magnetosome synthesis because it is a representative of a larger group of δ-proteobacteria, which produce bullet-shaped magnetite crystals.

As some of the mam genes are conserved among all sequenced members of magnetotactic bacteria, it appears that magnetosome formation evolved once. Whether the most recent common ancestor of all Proteobacteria, Nitrospirae and the OP3 division was a magnetotactic bacterium, or whether these genes moved through horizontal gene transfer remains under debate [14]. Within the most deeply branching clades of magnetotactic bacteria, including the one containing RS-1, bacteria synthesize bullet-shaped crystals, suggesting this is an ancestral form of magnetosome synthesis [14]. By studying this process, as we have undertaken here, we can increase our understanding of the diversity of the genes and mechanisms behind magnetosome formation, as well as begin to understand its origins.

Saturation and limitations of our screen

In this study we isolated 26 single-gene mutants of RS-1 with magnetic phenotypes. The causative mutation for each mutant was identified with whole genome sequencing and confirmed with complementation analysis. We found mutations in 10 genes so that, on average, 2.6 alleles were isolated per mutated gene. Using the Poisson distribution, we calculated that with a frequency of 2.6 mutations per genetic locus there is probability of 0.074 that other viable targets have not been found in our screen. Therefore, within the constraints of our genetic strategy, 92.6% of the non-essential genes that could produce the desired phenotype when disrupted have been identified.

In addition to the screen not being fully saturated, there are several reasons that it could have failed to identify genes important
for the magnetic phenotype of RS-1. For example, mutations in essential genes would not be identified by this method. Although the entire MAI is dispensable for growth under laboratory conditions, one could imagine a situation where the loss of one gene, but not the whole MAI, resulted in toxic magnetosome intermediates and a growth defect, though this has not been identified in the genetic dissections of the $\alpha$-proteobacterial MAIs. Even if the growth defect of a mutant were slight, the multiple rounds of selection and outgrowth used here ensure that it could not be isolated. In addition to growth defects, genetic redundancy and the potentially reduced susceptibility of some loci to mutation could prevent the identification of mutants with interesting phenotypes. There is reason to suspect that at least a couple genes are missing from this screen. mamI (amb0962), a gene essential for membrane remodeling in AMB-1 [29], was not identified in this study. However, several mamI homologs have been identified in RS-1 (mamI-L, DMR_41140, DMR_41040) [11], suggesting that there could be redundancy among the RS-1 mamI genes. mamM (DMR_41020), mamO (DMR_41070), and mamE (DMR_41080) are additional genes that play important roles in the $\alpha$-proteobacteria but whose RS-1 homologs were not mutated in our screen. In addition, one or more factors present in groups III and IV that are required for RS-1 magnetosome synthesis are clearly missing from this study, as deletion 3 could not be complemented by tauE alone.

The mutagenesis dosage used in this screen (50% cell survival) resulted in some mutants with only one change from WT and others with dozens of changes. Because the location of a mutated gene within the MAI could be used in this case to pick the candidate causative mutation, the high number of changes in some mutants was not a problem. If no such information is available to help identify candidates, lower doses of mutagenesis might result in individuals with fewer mutations. Alternatively, candidate causative mutations could be identified as those shared by a number of individuals with similar phenotypes and many mutations. Such a strategy has been pursued to identify attenuated mutants of the obligate intracellular pathogen Chlamydia trachomatis [47].

Fewer Magnetic Particles (fmp) genes

Two genes identified in this screen, DMR_41090 and DMR_41100, had previously been named based on their homology to other magnetosome genes. DMR_41090 encodes a protein consisting of three PDZ domains, a protein-protein interaction domain found in magnetosome proteins including MamP and MamE. DMR_41090 was originally annotated mamP (amb0970) [17], however DMR_41090 lacks the heme-binding domain that all other MamP proteins have. More recently it was suggested that DMR_41090 be re-annotated mamE-Cter [11], a name that does not follow the conventional nomenclature for bacterial genes. As PDZ domains are not a unique feature of MamP and MamE proteins, we have renamed DMR_41090 fmpA for Fewer Magnetic Particles A.

Based on sequence, DMR_41100 has two domains with redox activity, an amino-terminal magnetochrome domain and a carboxy-terminal NiX/NiB domain. NiX/NiB domains are involved in the synthesis of the iron molybdenum cofactor, and bind redox-active iron-sulfur clusters. Due to its magnetochrome domain, DMR_41100 was originally annotated mamT (amb0976) [17], but reannotated mamP-like because it also contains a PDZ domain [11]. However, these features are not unique to MamP and are also found in combination in $\alpha$-proteobacterial MamEs. NiX/NiB is a motif not found in other magnetosome proteins. For these reasons we have renamed DMR_41100 fmpB for Fewer Magnetic Particles B.
that is not thought to involve an organic matrix [52], so it is difficult to imagine potassium playing a similar role in RS-1.

Finally, a number of our mutants had intermediate phenotypes, with magnetic responses below WT levels. This is also found for a number of genes in the α-proteobacteria. These biomineralization mutants in AMB-1 and MSR-1 have full chains of crystals like WT, but each crystal is either too small or the wrong shape [20,29,30,48]. We originally expected RS-1 biomineralization mutants would have similar phenotypes, with full chains of poorly synthesized crystals. However, without exception RS-1 mutants with a magnetic response contained WT-like crystals in much fewer number than the WT. One explanation for this discrepancy is that such α-proteobacterial style mutants exist but were not identified in this screen because the isolated mutants had to be able to flow through a magnetic column, so only strains where some individual cells contained no magnetic particles could be isolated. However, another possibility is that this is due to a fundamental difference between how the α- and δ-proteobacteria synthesize magnetosomes.

When the α-proteobacteria are reintroduced to iron after iron starvation, they synthesize a full chain of crystals all at once, first possessing many small crystals, then many large crystals [18]. However, when RS-1 is reintroduced to iron after iron starvation, cells first possess one, then two or three full-sized crystals, with more at later time points [22]. Even in the absence of iron, AMB-1 is known to contain empty magnetosome membranes [18], each poised to mineralize its own crystal. Perhaps RS-1 crystals are made one at a time from a single magnetosome factory. In the case of biomineralization mutants, the chain of α-proteobacterial membranes will each try to build a crystal with some inefficiency,
resulting in a full chain of small crystals. In an RS-1 biomineralization mutant, the central factory is the only point of synthesis, so the inefficiency stemming from the mutation results in many fewer crystals produced. The dissociation of magnetosome crystals from their point of synthesis could also explain why many magnetosome proteins in RS-1 are integral membrane proteins, but no membranes have been observed around magnetosome crystals. In this model, the magnetosome factory would be imbedded in a membrane, but mature crystals would be free to move away from this membrane after synthesis. This new model for δ-proteobacterial magnetosome synthesis is described in Figure 10B.

Modern sequencing tools and the new accessibility of interesting biological diversity

In this work we applied classical mutagenesis combined with modern sequencing to query a previously opaque system: the magnetic δ-proteobacteria. A genetic analysis of these bacteria was not previously possible, as the genetic tools that allow the construction of targeted deletions or the mapping of mutations are not accessible for bacteria with low rates of transformation. With the minimal genetic tools available for RS-1 we were also able to complement the mutations, the gold standard in genetics for demonstrating that a mutation in a gene is responsible for a phenotype. This strategy confirmed and extended our understanding of the genetic and molecular mechanisms at play in magnetotactic bacteria as understood from the α-proteobacterial model systems, suggesting roles for unexpected proteins such as Kup, and reemphasizing the importance of overlooked domains, such as the TauE domains fused to MamO and MamE proteins. We have also discovered phenotypes for some mad genes, which had previously only been tied to magnetosome synthesis through bioinformatics analysis. These insights have been possible because of the depth of understanding of the model α-proteobacteria combined with the interesting differences between these bacteria and RS-1. Based on our success and the success of similar genetic analyses with the even more recalcitrant Chlamydia trachomatis [47], we envision that the strategy outlined here can be applied to any culturable organism to make and identify mutations of interest. With this and other high-throughput genetic tools that are emerging, such as Tn-organism to make and identify mutations of interest. With this and other high-throughput genetic tools that are emerging, such as Tn-seq [53], diverse and obscure systems like the magnetotactic bacteria are sure to be fertile ground for new genetic and mechanistic insights.

Materials and Methods

Strains

Cloning was performed in E. coli DH5α λ-pir. Matings, described below, were performed with E. coli WM3064 [54]. Potassium growth experiments, described below, were performed with E. coli TK2446 [F thi rha lacZ nagA Δkdp EAB 5 trkD1 trkG [kan] trkH [cam] ΔtrkA-uncL], gift of Ian Booth). The WT RS-1 used in this work (AK8) is a spontaneous mutant of the ATCC RS-1 (AK8) that was isolated in our lab. Unlike AK8, AK30 is non-motile and does not make biofilms, allowing us to take Cmag measurements more easily. The differences in AK8 and AK30 from the published RS-1 genome [12] are listed in Table S5. RS-1 was grown in RS-1 Growth Media as described previously [22] except that 12 mM Hepes buffer was included in the media and the pH set to 6.7 with sodium hydroxide. For RS-1 carrying a plasmid, 125 ug/mL kanamycin sulfate was added to the media.

Mutagenesis

Wild type RS-1 was passaged two times in liquid without supplied iron. Cells were mutagenized either with 10 mJ of ultraviolet radiation or with 30% ethyl methane sulfonate for one hour. Both treatments resulted in about 50% survival. Table S6 indicates which mutants resulted from which treatment. After mutagenesis, cells were grown in liquid in the presence of iron. The resultant cultures were passed over a magnetized MACS MS column (Miltenyi Biotec), and the flow-through was inoculated into fresh liquid media. This outgrowth and enrichment was performed four times. By the last enrichment, most cells were non-magnetic and were found in the flow-through of the column, which was diluted and plated for single colonies. The resultant colonies were screened by PCR for loss of the magnetosome island (see below). Those colonies that had a magnetic phenotype and possessed the island were saved for further analysis.

Cmag assays

Cmag assays were performed as previously described [18], except that the optical density was measured at 650 nm. For strains carrying a plasmid, kanamycin (which is in the form of kanamycin sulfate) was not included in the culture to be measured for Cmag as sulfate reduction interferes with magnetosome formation in RS-1 [22].

Electron microscopy

Whole-cell transmission electron microscopy and high-resolution transmission electron microscopy were performed as previously described [22]. Cryo-ultramicroscopy was performed as previously described [18].

Sequencing

Genomic DNA was isolated from 10 mL of RS-1 culture with the Qiagen Blood and Tissue kit. Library prep was performed by the Functional Genomics Laboratory at the University of California, Berkeley by following the standard Illumina-compatible library preparation protocol by IntegenX (now Wafergen). Samples were fragmented using the Covaris S220 to a target insert size of 250 bps and sample fragmentation length was confirmed using an Agilent Bioanalyzer. Samples were then loaded on the IntegenX Apollo 324 system. IntegenX PrepX Library kits were used to undergo end-repair, A-tail addition, adapter ligation, and size selection using AMPure XP beads. Samples were quantified using the Qubit and PCR amplified to incorporate indexes and flow cell binding regions. Final libraries were quantified using the Qubit, Bioanalyzer and qPCR. The indexed libraries were combined up to 35 per lane then sequenced with a 50 base pair, single-end run on a HiSeq2000 instrument using V3 chemistry and standard Illumina analysis software (RTA/Casava). Reads were aligned to the reference genome (NCBI Reference Sequence: NC_012796.1) with the BWA aligner [55], and single nucleotide polymorphisms were called with Freebayes [56]. Deletions and insertions were visualized with the Integrative Genomics Viewer [57]. Whole genome coverage was visualized with Qualimap [58]. Sequencing data is available on the NCBI Sequence Read Archive accession number SRP045907.

PCR genotyping of mutants

PCR and agarose gel electrophoresis or Sanger sequencing were used to check for the presence of the MAI and to confirm mutations identified by whole-genome sequencing. Primers are listed in Table S7. In the case of the MAI test, two PCR products of varying sizes, one inside and one outside the island, were...
produced together in one PCR reaction then their presence or absence visualized on a gel. To confirm the large deletions, PCR was performed spanning the deletion scar so that a PCR product was possible only in the case of the putative deletion. To test for putative transposon insertions, a difference in size between the WT and mutant PCR products of each gene was observed. To confirm point mutations, the candidate gene was amplified by PCR then sequenced with Sanger sequencing.

**Multiple sequence alignments**

MamB homologs from non-magnetotactic bacteria were identified with BLAST [59]. MamB, MamL, Kup sequences, and the 16S sequences used in Figure 1A were aligned with Clustal Omega [60]. Alignments were visualized with JalView [61].

**Plasmids and cloning**

For a list of plasmids used in this work, see Table S4. Cloning was performed using the Gibson method [62], pBMK7 [28] was digested with HindIII and Sall to create pLR6 and with Sall to create the remaining pBMK7-based plasmids. pLR6 was digested with Sall to create the pLR6-based plasmids. pMscSH6 [63] was digested with Ncol and Xhol to create the pMscSH6-based plasmids. All inserts were amplified with the indicated primers from RS-1 genomic DNA except for kupE. coli, which was amplified from DH5α cells, and gfp, which was amplified from pAK22 [64].

**Transconjugation**

RS-1 was transformed by conjugation with E. coli strain WM3064. WM3064 cells carrying the plasmid to be transformed into RS-1 were grown overnight in Luria Broth with 50 ug/mL kanamycin sulfate and 0.3 mM DAP. 500 ul of WM3064 culture was washed once in Luria Broth then combined with 3 to 10 mL of RS-1 culture in 25 ul LS-1 Growth Media with 0.3 mM DAP for 4 to 6 hours. The cells were plated on RS-1 Growth Media Agar with kanamycin.

**Magnetic field imaging using nitrogen-vacancy centers in diamond**

The magnetic fields produced by wild type and mad 1 mutant RS-1 bacteria were measured using nitrogen-vacancy (NV) color centers in diamond [43]. This magnetic imaging technique employed a 2.5×2.5×0.5 mm diamond chip produced using chemical vapor deposition, and engineered to contain a dense layer of NV centers at the diamond surface (surface density ~3×10^11 NV/cm^2, depth ~20 nm). Bacteria were placed on this diamond surface and allowed to dry. The magnetic fields sensed by the NV centers in the vicinity of each bacterium were then measured using optically detected magnetic resonance (ODMR) spectroscopy [43], where the magnetic field is determined from the NV electronic spin-flip resonance frequencies by monitoring the spin-state dependent fluorescence signal from the NV centers while simultaneously applying laser excitation and a microwave field with variable frequency. By imaging the fluorescence from the NV centers onto a sCMOS camera, images of the magnetic field at the diamond surface were recorded with sub-micron resolution over 400 μm fields of view. These magnetic images were co-registered with optical transmission images of the bacteria on the diamond surface using the same camera.

For one set of measurements, all three vector components of the magnetic field at the diamond surface were determined by independently measuring the magnetic field projections along each of four possible NV crystallographic orientations, in the presence of a 1.2 mT bias field produced by a set of Helmholtz coils. The absolute magnitude of the magnetic field signal in the vicinity of each bacterium within a field of view was then computed and used to estimate the relative magnetic moments within the sampled wild type and mad 1 mutant RS-1 bacteria populations, and also to estimate the average magnetic moment of the wild type RS-1 bacteria by comparing the signals with previous measurements on AMB-1 bacteria [43]. In another set of measurements, only one projection of the magnetic field vector was measured across the diamond surface, along a single NV crystallographic direction while applying a uniform 20.5 mT bias field along the same direction using a pair of permanent magnets. The two sets of measurements were conducted on the same bacteria within a given field of view to determine whether or not the magnetic signal near each bacterium changed as a function of the magnitude of the uniform bias field.

**Potassium growth experiments**

E. coli TK2446 carrying various plasmids were grown in LK medium (10 g tryptone, 5 g yeast extract, and 6.4 g KCl in 1 L) with 25 μg/mL carbenicillin. To test for growth on low potassium, cells from LK starter cultures were normalized for optical density, washed three times with K_a buffer [65] (16.47 g Na_2HPO_4*12 H_2O, 3.13 g NaH_2PO_4*2 H_2O, and 1.05 g (NH_4)_2SO_4 in 1 L), then plated on K_12 or K_20 plates (15 g agar autoclaved in 800 mL water, then 200 mL 3X K_a buffer added, supplemented with 0.2% glucose, 0.0001% thiamine, 0.4 mM MgSO_4, and 6 μM iron and 15 or 120 mM potassium chloride) with 12.5 μg/mL carbenicillin and 0.4 mM IPTG as 10 ul drops of 1:10 serial dilution series.

**Supporting Information**

S1 Fig. Coverage across the genome for WT and an MAI deletion strain. (TIF)

S2 Fig. TEM of WT and mutant cells isolated in the screen. Scale bar 200 nm for whole cells and 100 nm for insets. (TIF)

S1 Table Plasmids used in this study.

S2 Table Additional mutations in each mutant.

S3 Table Plasmid carried, replicate number, and C_magnitude value for each strain in Figure 5.

S4 Table Primers used in this study.

S5 Table Mutations identified in RS-1 Wild Type strains.

S6 Table Mutagen used for each mutant.

S7 Table Primers used in this study.

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