**Mycobacterium tuberculosis Expresses a Novel pH-dependent Divalent Cation Transporter Belonging to the Nramp Family**

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**Summary**

Mammalian natural resistance–associated macrophage protein (N-ramp) homologues are important determinants of susceptibility to infection by diverse intracellular pathogens including mycobacteria. Eukaryotic N-ramp homologues transport divalent cations such as Fe$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, and Cu$^{2+}$. Mycobacterium tuberculosis and Mycobacterium bovis (bacillus Calmette-Guérin [BCG]) also encode an N-ramp homologue (M-ramp).

RNA encoding M-ramp induces a 20-fold increase in $^{65}$Zn$^{2+}$ and $^{55}$Fe$^{2+}$ uptake when injected into *Xenopus laevis* oocytes. Transport is dependent on acidic extracellular pH and is maximal between pH 5.5 and 6.5. M-ramp-mediated $^{65}$Zn$^{2+}$ and $^{55}$Fe$^{2+}$ transport is abolished by an excess of Mn$^{2+}$ and Cu$^{2+}$, confirming that M-ramp interacts with a broad range of divalent transition metals.

Using semiquantitative reverse transcription PCR, we show that M-ramp mRNA levels in *M. tuberculosis* are upregulated in response to increases in ambient Fe$^{2+}$ and Cu$^{2+}$ between <1 and 5 μM concentrations and that this upregulation occurs in parallel with mRNA for y39, a putative metal-transporting P-type ATPase. Using a quantitative ratiometric PCR technique, we demonstrate a fourfold decrease in M-ramp/y39 mRNA ratios from organisms grown in 5–70 μM Cu$^{2+}$. *M. bovis* BCG cultured axenically and within THP-1 cells also expresses mRNA encoding M-ramp.

M-ramp exemplifies a novel prokaryotic class of metal ion transporter. Within phagosomes, M-ramp and N-ramp may compete for the same divalent cations, with implications for intracellular survival of mycobacteria.

**Key words:** bacillus Calmette-Guérin • *Xenopus* oocyte • metal ion • phagosome • intracellular pathogen

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1. Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; 2-DG, 2-deoxy-[14C]glucose; M-ramp, mycobacterial homologue of N-ramp; N-ramp, natural resistance–associated macrophage protein; ORF, open reading frame; RT, reverse transcription; SUM, standard uptake medium; TC, tandem competitive.
Expression is restricted to phagosomes of myelocytic cells (14). Some transition metals are also components of bacterial metalloenzymes (such as the superoxide dismutases and catalases) that protect bacteria against oxidative stresses encountered, for example, in phagosomes (15, 16).

We (17) and others (18, 19) have hypothesized that both mycobacteria and macrophages use N ramp homologues to compete for intraphagosomal metal ions. We now provide evidence that M ramp (the mycobacterial homologue of N ramp), which is a pH-dependent divalent cation transporter of broad specificity, is expressed in intracellular mycobacteria.

**Materials and Methods**

Isolation and Cloning of M ramp Sequence. We used the previously identified M. leprae sequence encoding an N ramp homologue to search the EM BL database using TBLASTN (20). PCR on genomic DNA from M. tuberculosis (H37Rv) and BCG using Pfu polymerase (Stratagene, Inc.) was carried out with primers designed to introduce BglII restriction sites and a strong eukaryotic ATG start sequence (21) and was verified by sequence analysis. Constructs in pSP, which contains 5′-untranslated N ramp (sense) and pXpmarm (antisense).

Expression of Mramp in X enopus oocytes and Fe2+ and Zn2+ uptake. X enopus oocytes were prepared as described previously (22). Capped cRNA encoding M. leprae Mramp was transcribed (MEGAscript™ SP6; Ambion) from X bal-linearized templates (pXmramp and pXpmarm), and oocytes were injected with cRNA (5 ng in 50 nl of water) or a corresponding volume of RNAse-free water. Fe2+ and Zn2+ uptake assays were performed after 48–96 h incubation in Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 15 mM Heps [pH 7.6], 0.3 mM Ca(NO3)2, 4H2O, 0.41 mM MgCl2, 6H2O, 0.82 mM MgCl2, 7H2O, 10 µg/ml penicillin, 10 µg/ml streptomycin) at 19°C (23).

65Zn2+ uptake assays were performed on batches of 10–15 oocytes washed 4 times in freshly made up standard uptake medium (SUM: 90 mM KCl, 10 mM NaCl, 10 mM morpholinoethane sulfonic acid [MES], pH 5.0–5.6; as before but with 10 mM Heps at pH 7.0) and incubated in SUM M for 1 h at room temperature with 100 µM 65ZnCl2 (N ycomed A mersham plc). Transport was terminated by washing oocytes in excess ice-cold SUM M. 65Zn2+ uptake was quantitated in individual oocytes on a Wallac Wizard 1480 gamma counter and corrected for uptake into water or antisense cRNA–injected controls.

55Fe2+ uptakes were performed with 55Fe2+ (5 µM in 100 µM total Fe2+; N ycomed A mersham plc) in SUM M containing ascorbic acid (2 mM) to maintain iron in a ferrous state, and all studies used fresh solutions to minimize reoxidation of Fe2+. The amount of ferrous iron was verified by a ferrozine assay (24).

Quantitation of 55Fe uptake was by scintillation counting (Wallac M. icrobeta Plus).

Maximal expression of both 65Zn2+ and Fe2+ uptake occurred between 2 and 4 d after microinjection. Competitions with divalent cations were performed using 65Zn2+ or Fe2+ as permeants and 100-fold excess (10 mM) concentrations of CuCl2, NiCl2, FeCl3, ZnCl2, and MgCl2.

Media for M. tuberculosis. All glassware was autoclaved after rinsing in 0.2 N HCl followed by deionized water to remove trace quantities of iron. Low Fe (~5 µM) Sauton's medium for starter cultures consisted of asparagine (15 mM), MgSO4 1 mM, sodium citrate (4 mM), KH2PO4 (7 mM), and glycerol (2% vol/vol, pH 7.1). Fe-depleted Sauton's medium (~1 µM) was prepared as above, omitting MgSO4 and stirring overnight at 4°C with Chelex 100 (10 g/l). After filter sterilization, the medium was supplemented (MgSO4 [1 mg/ml equivalent to 8.3 mM final concentration], ZnSO4.4H2O [0.2 µg/l final concentration (~1 nM)], and MCl2.4H2O [0.2 µg/l (~1 nM)] to compensate for losses during chelexation. M media containing both low Fe (~1 µM), medium Fe (4 µM), or high Fe (48 µM) and low Cu (~0.5 µM), medium Cu (5 µM), or high Cu (69.8 µM) were prepared by additionally supplementing aliquots of this medium with ferric ammonium citrate (~16% Fe content) or CuCl2. To ensure that concentrations of cations not being studied were above limiting concentrations, the Fe-modified media were supplemented with Cu (~1 µM), and Cu-modified media with Fe (4 µM). Concentrations of Fe and Cu in these media were verified by ferrozine assay (24) and atomic absorption spectrophotometry, respectively. All chemicals were obtained from Sigma-Aldrich.

Growth of M. tuberculosis in varying iron and copper concentrations. Starter cultures in Dubos broth supplemented with 10% Dubos medium albumin (Difco) were initiated from glycerol stocks and grown at 37°C to mid-log phase. These were inoculated (1:10) into low iron Sauton's medium, grown for 1 wk (37°C, 5% CO2, without shaking), and subcultured to ensure complete depletion of iron and copper. 10 ml of these cultures was inoculated into Fe/Cu-depleted Sauton's medium (10 ml to 190 ml) supplemented to give low, medium, or high concentrations of iron or copper and grown for 5 wk.

Infection of M arthropages with BCG. As BCG encodes an M ramp sequence (available from EMBL/GenBank/DBJ) under accession no. A[005699] identical to that of M. tuberculosis, we used BCG as a model to examine the expression of M ramp during intracellular infection. The human macrophage cell line, THP-1, was maintained as suspended cells and passaged at a density of 2–5 × 105 cells/ml. Before infection with BCG, the cells were passaged at least three times in antibiotic-free RPMI 1640 (ICN Biochemicals) supplemented with heat-inactivated FCS (10%), grown to a density of 2–5 × 106 cells/ml, and stimulated with PM A (20 µM; Sigma Chemical Co.) for 24 h to induce adherence. Nonadherent cells were removed by washing twice in PBS, and the resulting monolayers (~3–5 × 105 cells/ft2) were covered with supplemented RPMI 1640. Mid-log phase bacteria were pelleted from Dubos broth (500 g, 10 min), resuspended in medium, and sonicated (R inco Ultrasounds) for 15 s (five 3-s bursts at 70% amplitude) to disaggregate bacterial clumps. The sonicate was added to macrophages (10 bacilli/macrophase) and left for 24 h (37°C, 5% CO2). Extracellular mycobacteria were removed by decanting the supernatant and extensively washing the adherent cells twice in PBS. Efficiency of phagocytosis was estimated to be ~30% by counting CFU in the collected wash-
ings and microscopic examination of Ziehl-Nielsen-stained macrophages. M acrophage viability (90%) throughout these experiments was assessed by Trypan blue exclusion, with further details given in reference 25.

Recovery of RNA from Intracellular BCG. After extensive washing of the macrophage monolayer, mycobacteria were recovered from differentially lysed THP-1 cells by the addition of 20 mL g TCE solution (4 M guanidinium thiocyanate [GTC]; Fluka) containing 0.5% sodium N-lauroyl sarcosine, 25 mM sodium citrate, pH 7, and 0.1 M 2-ME to each flask (25). Total RNA was then extracted from the washed bacterial pellets as described previously (26).

Identification of Omp R and F Frames. First strand cDNA synthesis on total RNA was performed using random hexamer primers (Promega) and Moloney murine leukemia virus reverse transcriptase and on template made from RNA pretreated with DNase (Qiagen).

Precise quantification of relative amounts of cDNA for Mramp and y39 under different growth conditions was carried out by TC-PCR. A series of PCR amplifications of each gene was carried out on a template mixture consisting of a fixed quantity of cDNA and serial dilutions (at 1/4 log intervals) of competitor molecule (linearized by SacI digestion). Products derived from competitor template could be distinguished from target product by KpnI digestion and were quantified as above. The plasmid competitor/cDNA ratio was calculated for individual reactions after heteroduplex correction and analyzed as published previously (29).

Statistical Analysis. Comparison of radiolabeled uptake rates between two conditions was by the Mann Whitney U test, and comparison of uptakes at different pH values and with competitors was after Box-Cox transformation (to normalize distributions) and used MANOVA (v5.2 SYSTAT).

Results

Sequence Analysis of Mramp. A single N ramp homologue (designated Mramp) is located on cosmid M T Y 21C12 (GenBank/EMBL/DDB accession no. Z95210; gene number R v0924c) in a 5286-bp region containing 5 ORFs oriented in the same direction (30). A potential ribosome binding site is immediately upstream of the first ORF with M ramp as the fourth gene in this series. To ascertain if M ramp is cotranscribed with neighboring ORFs, we carried out RT-PCR on total RNA extracted from cultured H 37Rv using primers spanning the junction between the 3' end of M ramp and the next ORF. A product of expected size (407 bp) was obtained (data not shown), indicating that M ramp is transcribed at least as a bicistronic operon. Database searches failed to identify functionally characterized homologues of this second ORF.

M ramp encodes a predicted 428-amino acid protein with a molecular mass of 44.9 kDa and 10 transmembrane segments consistent with proposed topologies for yeast homologues (smf1 and 2) (31; Fig. 1). Compared with eukaryotic homologues, the hydrophilic N H 2-terminal region of M ramp, like those of other prokaryotic N ramp homologues, is shorter but exhibits a similar clustering of polar residues (31). The COOH terminus is also shorter and probably lacks the two final transmembrane segments predicted in some eukaryotic homologues. The amphiphilic properties of transmembrane segments M 3, M 5, and M 9, in which the polar and nonpolar residues are segregated to opposite faces of the predicted α-helices (possibly forming a transmembrane channel), are also found in M ramp.

Sequence analysis of M ramp confirms that certain amino acid residues are highly conserved between all members of the N ramp family (Fig. 1). M ramp sequences from M. tuberculosis and BCG are identical and are most closely related to other bacterial homologues (72.4, 40, and 40% sequence identities with M. leprae, B. subtilis, and E. coli, respectively), whereas comparison with eukaryotic homologues gives overall amino acid identities of 21-24%. There

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Mramp Transports Cations is an asymmetrical distribution of charged amino acid residues between the endo- and exofacial regions of Mramp. This is consistent with similar patterns of charge distribution observed in many integral membrane proteins (32).

Mramp Induces $^{65}$Zn$^{2+}$ Uptake in Oocytes. Initially we cloned Mramp into pGEM-T Easy after mutating the mycobacterial GTG start codon to ATG without strengthening the Kozak consensus sequence. Microinjection of oocytes with RNA made from this construct induced up to twofold increases in $^{55}$Fe$^{2+}$ and $^{65}$Zn$^{2+}$ uptake compared with water-injected oocytes (data not shown). To optimize Mramp expression in oocytes, we retained the modified start codon, introduced a strong Kozak consensus, and cloned Mramp into a vector containing flanking X. laevis 5' and 3' untranslated regions (see Materials and Methods).

In 10 independent experiments, RNA derived from this latter construct induced large increases (up to 22-fold) in the accumulation of $^{65}$Zn$^{2+}$ by Mramp-expressing oocytes.

Figure 1. Putative distribution of membrane-spanning segments of M ramp to illustrate alignment of conserved residues with homologues. This sketch has been derived from detailed hydropathy analyses performed by Cellier et al. (reference 31). The precise number of membrane-spanning segments and topology of the COOH-terminal region are still uncertain. M ramp sequence was analyzed using the Kyte-Doolittle algorithm (window size of 16 amino acids). The highly conserved distribution of thermodynamically unfavored charged residues within transmembrane segments (M), which is a feature of eukaryotic N ramp homologues (reference 31), is also discernible in M ramp and other prokaryotic homologues (boxed residues).

The positions of two adjacent glycine residues in M4 (region c, marked *) are known to be functionally important; in murine N ramp1, a G169D mutation is associated with the pathogen-susceptible phenotype (reference 3), while in N ramp2, a mutated neighboring glycine in the mk mouse (G165R; reference 36) and Belgrade (b) rat (reference 11) causes microcytic (iron deficiency) anemia, probably through steric and charge effects. Mramp contains a consensus transport motif (CTM) resembling the "EAA" box or "binding protein-dependent transport system's inner membrane component signature" (region f) (see reference 31). This motif [E,Q] [S,T,A] 3X,G, [L,I,V,M,F,Y,A], 4X, [F,L,I,V] is found in numerous bacterial periplasmic permeases and some eukaryotic multisubunit transporters (reference 31). M utabional analysis of three residues in this region (marked * in region f) in murine N ramp2 has demonstrated the functional importance of the first two residues (reference 10). Other highly conserved residues are shown in bold. DCT1 sequence = rat N ramp2; S. cerevisiae sequence = smf1. Sequence data are available from EMBL/GenBank/DDJB under accession nos. U15184 (M. leprae), Z99106 (B. subtilis), U00096 (E. coli), U15929 (Smf1), AF008439 (DCT1), and L32185 (N ramp1, human).

Figure 2. $^{65}$Zn$^{2+}$ uptake by oocytes expressing M ramp. (A) Induction of $^{65}$Zn$^{2+}$ uptake by M ramp in X enus oocytes injected with R NA (5 ng in 50 nl) transcribed from pX m ramp. The difference in $^{65}$Zn$^{2+}$ uptake between experimental and water (50 nl)-injected control oocytes is highly significant ($P < 0.001$). Inset shows a separate experiment in which $^{65}$Zn$^{2+}$ uptake in M ramp-expressing oocytes was compared with antisense R NA (5 ng)-injected control oocytes ($P = 0.025$). (B) $^{65}$Zn$^{2+}$ uptake in water-injected and THT1 (5 ng)-injected control oocytes. THT1 is the T. brucei hexose transporter. There was no significant difference in $^{65}$Zn$^{2+}$ uptake between water- and THT1-injected groups ($P = 0.5$). (C) 2-DOG uptake in M ramp- (5 ng), THT1- (5 ng), and water-injected oocytes. THT1 induces significantly greater 2-DOG uptake than either M ramp ($P < 0.002$) or water ($P < 0.002$). There was no significant difference in uptakes between the water- and M ramp-injected oocytes. Displayed are mean values ($\pm$ SE) of uptakes (10 oocytes per experimental condition).
compared with water-injected (Fig. 2 A) or M ramp antisense-injected controls (Fig. 2 A, inset). To confirm that these induced $^{65}$Zn$^{2+}$ uptakes were specific to M ramp, we also examined the uptake of $^{65}$Zn$^{2+}$ in oocytes injected with RNA made from the Trypanosoma brucei hexose transporter (THT1 [33]; Fig. 2 B). As expected, there was no increase in $^{65}$Zn$^{2+}$ uptake associated with expression of THT1, confirming the requirement for M ramp to induce $^{65}$Zn$^{2+}$ uptake. Conversely, we demonstrated 2'-deoxy-d[$^{14}$C]-glucose (2-D-G) uptake by THT1 but not by M ramp (Fig. 2 C). To confirm that accumulation of $^{65}$Zn$^{2+}$ continued beyond these experimental time points, we monitored $^{65}$Zn$^{2+}$ uptake for up to 4 h. The increase in uptake of $^{65}$Zn$^{2+}$ was linear during this period, which encompasses the uptake times of experiments shown (slope 3.8 ± 0.63, P < 0.001).

M ramp–induced $^{65}$Zn$^{2+}$ uptake is pH dependent. In oocytes, translocation of divalent cations by DCT1 (the rat Nramp2 homologue) depends on cotransport of protons, with maximal activity of DCT1 at an extracellular pH of 5.5. To determine if cation transport by M ramp displays a similar pH dependence, we measured $^{65}$Zn$^{2+}$ uptake by oocytes incubated in extracellular pH values between 5.0 and 7.0. In nine independent experiments, $^{65}$Zn$^{2+}$ uptake by oocytes was confined to extracellular pH values between 5.5 and 6.5 (Fig. 3) and was completely abolished at pH 7.0 (P = 0.009). There were no significant differences in uptake at pH 5.0, 6.5, and 7.0 (P > 0.1). Displayed are mean values (± SE) of uptakes (10 oocytes per experimental condition).

Figure 3. pH dependence of $^{65}$Zn$^{2+}$ uptake induced by M ramp. $^{65}$Zn$^{2+}$ uptake was assayed in M ramp-injected and control oocytes in media varying in pH (see Materials and Methods). The increase in $^{65}$Zn$^{2+}$ uptake (Fold) is shown for M ramp-expressing oocytes compared with controls. Uptakes at pH 5.5 (P < 0.001) and pH 6.0 (P ≤ 0.009) were significantly greater than uptakes under remaining conditions. There were no significant differences in uptake at pH 5.0, 6.5, and 7.0 (P > 0.1). Displayed are mean values (± SE) of uptakes (10 oocytes per experimental condition).

M ramp–induced $^{65}$Zn$^{2+}$ uptake by M n$^2^+$ in M ramp RNA–injected oocytes. Increase in uptake compared with control oocytes in the absence and presence of n$^2^+$ (10 mM as MnCl$_2$; P = 0.01). (B) Uptake of $^{55}$Fe$^{2+}$ by M ramp RNA–injected oocytes compared with water-injected controls (P < 0.001). (C) Influence of divalent cation competitors on $^{55}$Fe$^{2+}$ (100 μM) uptake by M ramp RNA–injected oocytes. Data from one experiment. Displayed are mean values (± SE) of uptakes (10 oocytes per experimental condition).

Semiquantitative analysis of PCR products from M ramp and y39 (a putative Ca$^{2+}$-translocating P-type ATPase [17]) using identical template concentrations and PCR conditions showed large increases in mRNA for M ramp (~50-fold) as Fe$^{2+}$ concentration increases from <1 to 48 μM (Fig. 5 A). As Cu$^{2+}$ concentrations increase over a similar range, mRNA for M ramp increases ~10-fold, and is maximal at 5 μM Cu$^{2+}$. There is less increase in mRNA for y39 under these conditions (~17- and 5-fold, respectively).

To investigate the regulation of M ramp and y39 transcription more precisely, we used a ratiometric PCR technique called TC-PCR to quantitate mRNA for M ramp in relation to y39 in M. tuberculosis cultured in media containing these different concentrations of Fe$^{2+}$ and Cu$^{2+}$. The mRNA ratios for M ramp/y39 fell fourfold (from 0.44 to 0.11) when Cu$^{2+}$ concentrations increased from 5 to 70
Figure 5. Growth of M. tuberculosis H37Rv in different cationic conditions. (A) RT-PCR for M ramp and y39 carried out on total RNA isolated from M. tuberculosis H37Rv cultured in media containing different Fe\(^{2+}\) and Cu\(^{2+}\) concentrations. Equal amounts of RNA template (30 ng) were used in each reaction (3 ng for 16S RNA experiments). Lane 1, low Fe\(^{2+}\) (<1 μM); lane 2, medium Fe\(^{2+}\) (4 μM); lane 3, high Fe\(^{2+}\) (48 μM); lane 4, low Cu\(^{2+}\) (<0.5 μM); lane 5, medium Cu\(^{2+}\) (5 μM); lane 6, high Cu\(^{2+}\) (70 μM). (B) Bacterial growth after 5 wk culture at each metal ion concentration. (C) Top panel, RT-PCR for M ramp on total RNA isolated from axenically cultured and intracellular BCG. Equal amounts of RNA template (135 ng) were used in each reaction. Lane 1, template from extracellular (axenically cultured) BCG; lane 2, template from intracellular BCG pretreated with RNAse A; lane 3, template from intracellular BCG; lane 4, template from intracellular BCG pretreated with RNAse A. Bottom panel, RT-PCR for rRNA (16S) using amounts of total RNA (16S) (Fig. 5 C, bottom).

Discussion

Although approximately 1.7 billion people (one third of the world’s population) are infected with M. tuberculosis at any one time, it is striking that, in the absence of coinfection with HIV, fewer than 10% of these will develop active disease during their lifetimes (34). Host genetic factors such as polymorphisms in N ramp clearly influence susceptibility to infection or disease caused by Mycobacteria species (35). For example, tuberculosis in a tribally mixed Gambian population was associated with two pairs of N ramp polymorphisms (8), and in another study involving members of Chinese and Vietnamese families with leprosy, haplotypes associated with N ramp-linked polymorphisms were observed to be distributed nonrandomly between affected and unaffected family members (9).

The N ramp family of proteins is highly conserved between bacteria and mammals, and in eukaryotic examples have been shown to transport divalent cations such as Fe\(^{2+}\) and Mn\(^{2+}\) (12, 36, 37). N ramp1 is likely to perform similar transport functions to N ramp2, which mediates pH-dependent Fe\(^{2+}\) uptake in heterologous expression studies and in vivo (12, 36). Defining the transport specificities for N ramp1 has proved difficult (38), and all studies characterizing N ramp homologues have been carried out exclusively on eukaryotic sequences. Recent studies in RAW264.7 cells overexpressing N ramp1 suggest that N ramp1 does contribute to iron mobilization from vesicles (39). N ramp1 also circumvents maturation arrest of phagosomes containing live BCG, permitting the increase in acidification normally seen in phagosomes containing killed BCG or latex beads (40). These observations point to the possibility that competition for transition metal ions may be important in determining maturational dynamics of phagosomes as well as their lethality for certain intracellular pathogens.

We studied a mycobacterial homologue of the N ramp family because of its potential relevance to intracellular survival. N ramp homologues are found in M. tuberculosis, M. leprae, M. smegmatis, and BCG (7, 17). These homologues (called Mramp) have been suggested to mediate the uptake of cations such as Fe\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\), which may be important in defence by microbial superoxide dismutase against the macrophage respiratory burst (17, 18). Our studies now provide direct evidence for a function of Mramp as a transporter of Zn\(^{2+}\) and Fe\(^{2+}\).

Furthermore, the enhanced uptake of \(^{65}\)Zn\(^{2+}\) and \(^{55}\)Fe\(^{2+}\) induced in oocytes expressing Mramp is abrogated by an excess of Mn\(^{2+}\) and Cu\(^{2+}\), but not by unrelated divalent cations such as Mg\(^{2+}\), suggesting important interactions between Mramp and these transition elements. In spite of divergence in primary sequence between N ramp1, DCT1 (a rat intestinal homologue of N ramp2), and Mramp and their diverse phylogeny, all three sequences can mediate the uptake of Fe\(^{2+}\) into Xenopus oocytes (12). DCT1 transports other members of the transition metal series, and this broad...
specificity is also observed for \( \text{M ramp} \). Therefore, \( \text{M ramp} \) represents a novel class of prokaryotic metal ion transporter with representatives in other bacteria such as \( \text{E. coli} \) and \( \text{B. subtilis} \) (Fig. 1).

We examined the pH dependence of cation transport by \( \text{M ramp} \) using \( ^{65}\text{Zn}^{2+} \) as a permeant, because at pH \( > 6.0 \) it is difficult to manipulate the equilibrium between \( \text{Fe}^{2+} \) and \( \text{Fe}^{3+} \) even in the presence of reducing agents such as ascorbic acid (41). There is a narrow range of extracellular acid pH values that allows \( \text{M ramp} \)-induced uptake of \( ^{65}\text{Zn}^{2+} \) by oocytes. This pH range (5.5–6.5) coincides with estimates of ambient pH in the microenvironment of intraphagosomal mycobacteria (42). This observation also provides evidence for the direction in which cation transport is likely to be taking place, namely from the relatively acidic phagosome into mycobacteria. By contrast, an uninfected phagolysosome (for example, one containing inert particles) has a significantly lower pH (\(< 5.5 \) [42]), and would therefore be unlikely to allow efficient transport of divalent cations by \( \text{M ramp} \).

To assess the expression of \( \text{M ramp} \) in \( \text{M. tuberculosis} \) culture, we used a precise assay to quantitate mRNA for \( \text{M ramp} \) obtained from organisms grown in media containing defined \( \text{Cu}^{2+} \) and \( \text{Fe}^{2+} \) concentrations. These studies permitted assessment of the growth characteristics of bacteria as well as the relative expression of mRNA for \( \text{M ramp} \) compared with mRNA encoding a putative \( \text{Ca}^{2+} \)-transporting P-type ATPase (\( y39 \)). \( \text{M ramp} \) is expressed poorly in bacteria grown in relatively \( \text{Cu}^{2+} \)-and \( \text{Fe}^{2+} \)-deficient media, and expression is enhanced at higher concentrations of these metal ions (\( \approx 5 \mu \text{M} \)). Similar patterns of mRNA expression are observed for the putative \( \text{Ca}^{2+} \)-transporting P-type ATPase (\( y39 \)), but in contrast, mRNA encoding an atypical heavy metal-translocating P-type ATPase (\( y39 \)) is barely detectable under any of the conditions tested (Fig. 5). This stimulation of expression of mRNA for \( \text{M ramp} \) and \( y39 \) at higher ambient concentrations of \( \text{Cu}^{2+} \) and \( \text{Fe}^{2+} \) is associated with retardation of bacterial growth by \( \approx 30\% \).

mRNA for \( \text{M ramp} \) encoded by \( \text{BCG} \) is clearly expressed in the intracellular environment (Fig. 5 C). We used BCG as a model for \( \text{M. tuberculosis} \) because \( \text{M. tuberculosis} \) is frequently cytopathic when cultured in \( \text{THP}1 \) cells, compromising yields of RNA. BCG is well recognized as a convenient model to study mycobacterial gene expression in these circumstances (25).

\( \text{M ramp} \) may act in concert with mechanisms inhibiting acidification of phagosomes to permit intracellular survival of mycobacteria. The deployment of \( \text{N ramp}1 \) in the host's phagosomal membrane is clearly important in defence against infection, as established by classical studies on the genetics of \( \text{N ramp}1 \). If \( \text{N ramp}1 \) also uses phagosomal protons to extrude cations, thereby competing with \( \text{M ramp} \), the pH dependence of this phenomenon will be critical in establishing which of the two transporters (\( \text{M ramp} \) or \( \text{N ramp}1 \)) functions most efficiently in the infected macrophage. Experiments to examine this hypothesis in greater detail can now be formulated on the basis of \( \text{M ramp} \)'s function as defined by heterologous expression. \( \text{M ramp} \) is the first mycobacterial gene to be expressed in oocytes, exemplifying the utility of this system for the functional characterization of other prokaryotic transporters (\( \text{E. coli} \) glycercol facilitator glpF, and \( \text{E. coli} \) water channel AqpZ [43]).

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