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Magnesium uptake of *Arabidopsis* transporters, AtMRS2-10 and AtMRS2-11, expressed in *Escherichia coli* mutants: Complementation and growth inhibition by aluminum

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ABSTRACT

Magnesium (Mg^{2+}) plays a critical role in many physiological processes. Mg^{2+} transport systems in *Salmonella* have been well documented, but those in *Escherichia coli* have not been fully elucidated. We examined the effects of *corA*, *mgtA*, *yhiD* and *corC* gene deletion on Mg^{2+} transport in *E. coli*. We obtained every combination of double, triple and quadruple mutants. The *corA* and *mgtA* double mutant required addition of 10 mM Mg^{2+} to Luria-Bertani (LB) medium for growth, and the *corA*, *mgtA* and *yhiD* triple mutant TM2 required a higher Mg^{2+} concentration. The Mg^{2+} requirement of the quadruple mutant was similar to that of TM2. The results demonstrated that either CorA or MgtA is necessary for normal *E. coli* growth in LB medium and that YhiD plays a role in Mg^{2+} transport under high Mg^{2+} growth conditions in *E. coli*. The *Arabidopsis* Mg^{2+} transporters, AtMRS2-10 and AtMRS2-11, were heterologously expressed in TM2 cells. TM2 cells expressing AtMRS2-10 and AtMRS2-11 could grow in LB medium that had been supplemented with 1 mM Mg^{2+} and without Mg^{2+} supplementation, respectively, and cell growth was inhibited by 2 mM $AlCl_3$. The results indicated that the growth of TM2 expressing AtMRS2-10 and AtMRS2-11 reflected these AtMRS2 function for Mg^{2+} and aluminum. The *E. coli* TM2 cells are useful for functional analysis of *Arabidopsis* MRS2 proteins.

1. Introduction

Magnesium (Mg^{2+}), the second-most abundant cation in bacteria, is a cofactor of many enzymes involved in central biochemical pathways that are essential for bacterial viability [1,2]. The three distinct families of prokaryotic Mg^{2+} transport proteins are identified and cloned to date: CorA, MgtE, and MgtA/B [3,4]. In bacteria, Mg^{2+} is most likely mainly transported by CorA protein and by MgtE protein where the CorA protein is absent.

Mg^{2+} transport systems in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) have been studied bacteriologically at depth. *S.* Typhimurium has three transport systems, MgtA and MgtB, which are P-type ATPases, and CorA, which is a channel [3]. A strain carrying mutations in all three genetic loci lacks detectable Mg^{2+} transport under usual assay conditions and requires 10–100 mM Mg^{2+} in the growth medium. Introduction of the gene for any single *S.* Typhimurium Mg^{2+} transport protein or a gene encoding a putative Mg^{2+} transporter from another organism into this Mg^{2+} transport-deficient strain restores Mg^{2+} transport and confers the ability to grow without Mg^{2+} supplementation [5].

A putative Mg^{2+} transporter from *Escherichia coli* was identified in 1969 [6,7]. The locus was named *corA* because the mutant phenotype is resistance to growth inhibition by Co^{2+} (*co*balt *r*esistance). *E. coli* mutants have been recently used for heterologous expression and structure-function studies of Mg^{2+} and/or Co^{2+} transport proteins [8–11]. However, Mg^{2+} transport systems in *E. coli* have not been fully elucidated. In *E. coli*, *mgtA* is present at a location corresponding to its map position in *S.* Typhimurium; however, there is no *mgtB* present in the *E. coli* genome. *mgtB* of *S.* Typhimurium is the second gene of the *mgtCB* operon [12]. *E. coli* does not have either *mgtC* or *mgtE*.

E. coli has CorA and MgtA, but it is not known whether *E. coli* has another Mg^{2+} transport protein. We now examined the effects of deletion of *yhiD* and *corC* in addition to *corA* and *mgtA* on Mg^{2+} transport of *E. coli*. *yhiD* encodes an integral membrane protein with five transmembrane helices, and YhiD protein belongs to the MgtC family. *corC* was found as a Co^{2+} resistance locus on the *S.* Typhimurium chromosome [13]. Human ACDP2 (ancient conserved domain protein 2), a distant homologue of CorC, mediates Mg^{2+} influx [14]. An *E. coli* strain devoid of *corA*, *mgtA*, and *yhiD* genes reportedly demonstrates Mg^{2+}-auxotrophy [8], but physiological roles of YhiD and CorC in *E. coli* are not known. We demonstrate here that either CorA or MgtA is necessary for normal *E. coli* growth in LB medium without Mg^{2+} supplementation and that YhiD plays a role in Mg^{2+} transport under high-Mg^{2+} growth conditions.

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A CorA-like gene family in *Arabidopsis thaliana* was identified by two independent groups. The family has 10 members. Schock *et al.* [15] showed that one of the family members, AtMRS2-1, complemented the yeast *mrs2* mutant and named the gene family *MRS2* (*Mitochondrial RNA Splicing 2*). Li *et al.* [16] named the gene family *MGT* (*Magnesium Transport*). We refer to this family as MRS2 for simplicity. Recently, we functionally reconstituted and characterized the *Arabidopsis* Mg^{2+} transporter AtMRS2-10 in proteoliposomes [17]. We indicated that purified, reconstituted AtMRS2-10 mediates Mg^{2+} transport, and its Mg^{2+} transport is inhibited by $AlCl_3$. Here, AtMRS2-10 and another member, AtMRS2-11, were heterologously expressed in the *E. coli corA*, *mgtA* and *yhiD* deletion mutant. AtMRS2-10 and AtMRS2-11 could functionally complement the Mg^{2+} auxotroph, and the growth of *E. coli* mutant complemented by AtMRS2-10 and AtMRS2-11 was inhibited by $AlCl_3$.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli strains were grown in Luria-Bertani (LB) medium at 37 °C unless otherwise noted. Kanamycin (30 μg/ml) and ampicillin (20 or 50 μg/ml) were added as required. For strain sources and cloning strategies, please refer to Table 1. The *E. coli* Keio strains were supplied by National BioResource Project. *E. coli* K-12 W3110 was used as the wild-type strain.

2.2. Deletion mutant generation

Bacteriophage P1 transduction was used to construct deletion mutants in W3110 using BW25113-based mutants that were available from the Keio collection [18]. Briefly, to construct the W3110 *corA*::*kan* mutant with a complete deletion of *corA*, bacteriophage P1 was grown with BW25113 *corA*::*kan*, and the lysate was used to transduce the *E. coli* wild-type W3110 strain. Kanamycin-resistant colonies were selected, and the correct *corA*::*kan* mutation in the W3110 genome was confirmed by PCR and sequencing. The W3110 *mgtA*::*kan*, *corC*::*kan* and *yhiD*::*kan* strains were similarly generated with lysate that had been derived from P1 phage-infected BW25113 *mgtA*::*kan*, *corC*::*kan* and *yhiD*::*kan* strains, respectively. To construct the W3110 Δ*corA*/Δ*mgtA* double mutant, the kanamycin-resistance gene was deleted from W3110 *mgtA*::*kan* by introducing plasmid pCP20, which is an Amp^R plasmid that shows temperature-sensitive replication and thermal induction of FLP recombinase synthesis to eliminate the selective kanamycin-resistance marker [19]. The transformants containing pCP20 were grown overnight in LB containing kanamycin and ampicillin with shaking at 32 °C. The cultures were plated on LB agar without antibiotics and cultured overnight at 43 °C. Colonies were tested for kanamycin and ampicillin sensitivity: kanamycin-sensitive and ampicillin-sensitive colonies of W3110 *ΔmgtA* that lacked pCP20 were selected [20]. The *corA*::*kan* cassette was then introduced into the W3110 *ΔmgtA* mutant from BW25113 *corA*::*kan* by bacteriophage P1 transduction, which led to the construction of the double mutant W3110 *ΔmgtA*/*corA*::*kan*. The correct *ΔmgtA* and *corA*::*kan* mutation in the W3110 *ΔmgtA*/*corA*::*kan* genome was confirmed by PCR and sequencing. The other W3110 *corA*, *mgtA*, *yhiD* and *corC* double mutants, triple mutants and quadruple mutant were constructed in the same manner.

Table 1
Strains and plasmids used.

Strain and plasmid	Parent/genotype	Ref/source/construction
Strain		
JW3789	[BW25113]*corA*::*kan*	Keio collection [18]
JW4201	[BW25113]*mgtA*::*kan*	Keio collection [18]
JW0655	[BW25113]*corC*::*kan*	Keio collection [18]
JW5670	[BW25113]*yhiD*::*kan*	Keio collection [18]
SM1	[W3110]Δ*corA* (removed Km^R gene from [W3110]*corA*::*kan*)	W3110 × P1(JW3789)
SM2	[W3110]Δ*mgtA*	W3110 × P1(JW4201)
SM3	[W3110]Δ*corC*	W3110 × P1(JW0655)
SM4	[W3110] *yhiD*::*kan*	W3110 × P1(JW5670)
DM1	[W3110]Δ*corA* Δ*mgtA*	SM2 × P1(JW3789)
DM2	[W3110]Δ*mgtA* Δ*corC*	SM2 × P1(JW0655)
DM3	[W3110]Δ*mgtA* Δ*yhiD*	SM2 × P1(JW5670)
DM4	[W3110]Δ*corC yhiD*::*kan*	SM3 × P1(JW5670)
DM5	[W3110] Δ*corC corA*::*kan*	SM3 × P1(JW3789)
TM1	[W3110]Δ*corA* Δ*mgtA* Δ*corC*	DM1 × P1(JW0655)
TM2	[W3110]]Δ*corA* Δ*mgtA yhiD*::*kan*	DM1 × P1(JW5670)
QM	[W3110]Δ*corA* Δ*mgtA* Δ*corC yhiD*::*kan*	TM1 × P1(JW5670)
Plasmid		
pCP20		[19]
pECcorA	pHSG415s	*corA* gene, native promoter
pECmgtA	pHSG415s	*mgtA* gene, native promoter
pAtMRS2-10	pTV118N	AtMRS2-10 cDNA [17]

2.3. Expression plasmid construction

To construct an *E. coli corA* expression plasmid, a DNA fragment containing 1102 bp of the *corA* gene preceded by its native promoter (from 103 bp upstream of *corA* start codon) was amplified by PCR using genomic *E. coli* W3110 strain DNA as the template with the primers 5′-CATTGAATTCTTAGCGGTTGTCAGCG-3′ and 5′-CATTCTCGAGCAGACTAAGCCACCGC-3′, which also incorporated unique *Eco*RI and *Xho*I restriction sites. After double restriction enzyme digestion with *Eco*RI and *Xho*I, the resulting PCR product was cloned into pHSG415s low-copy number vector [21] for protein expression in *E. coli* and was sequenced to verify the construct. Similarly, a DNA fragment containing 2,844 bp of the *mgtA* gene preceded by its native promoter (from 97 bp upstream of the *mgtA* start codon) was amplified using the primers 5′-CATTGAATTCGATGCGTAAGGCCGTG-3′ and 5′-CATTCTCGAGTGAATCGGGGCTATCG-3′ and cloned in pHSG415s. The obtained plasmids were named pECcorA and pECmgtA, respectively. AtMRS2-10 cDNA [17] was subcloned in-frame at the *Nco*I site of the pTV118N vector (TakaraBio Inc.), in which the second amino acid of AtMRS2-10 was changed from Ser to Ala. The obtained plasmid was named to be pAtMRS2-10. The Met400 to Ile mutant of AtMRS2-10 cDNA [17] was also subcloned into pTV118N. The *Arabidopsis* sequence (stock no. ATTS2227) that encodes for AtMRS2-11 was obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). The AtMRS2-11 protein contains the longest N-terminal extension of the family. The TargetP and ChloroP algorithms predict a chloroplast leader and transit peptide cleavage site at amino acid 62 [22]. The cDNA fragment that encodes for AtMRS2-11 from Cys-63 to C-terminal Phe-459 was subcloned in-frame at the *Nco*I site of the pTV118N vector, in which Gly was inserted between N-terminal Met and Cys-63. The obtained plasmid was named to be pAtMRS2-11. The Met418 to Ala mutant of AtMRS2-11 cDNA was also subcloned into pTV118N. All plasmid sequences were confirmed by DNA sequencing.

2.4. Growth curves

Overnight cultures of W3110 mutants were grown in LB medium with an appropriate concentration of $MgCl_2$ or $MgSO_4$ if necessary, washed twice with LB and inoculated to an OD_{600} of 0.004 into liquid LB medium containing different $MgCl_2$ or $MgSO_4$ concentrations. Differences were not observed when $MgCl_2$ or $MgSO_4$ was supplemented.

2.5. Complementation assays

pAtMRS2-10, pAtMRS2-11 and their mutant plasmids were transformed into *E. coli* mutant TM2 cells. Cells were plated onto LB medium supplemented with 10 mM Mg^{2+} and 50 μg/ml ampicillin and incubated

at 37 °C overnight. TM2 transformed with empty pTV118N vector was used as a negative control. Individual transformants were grown in LB liquid medium containing 10 mM Mg^{2+} and 50 μg/ml ampicillin at 37 °C overnight and then grown in LB liquid medium containing 1 mM Mg^{2+} and 50 μg/ml ampicillin. Cells were then inoculated to an OD_{600} of 0.01 into liquid LB medium. Culture growth was monitored at 37 °C, and the OD_{600} was plotted as a function of growth time.

3. Results

Park *et al.* described in 1976 that mutations in three genes affect Mg^{2+} transport in *E. coli* [23]. Hattori *et al.* indicated that an *E. coli* strain devoid of *corA*, *mgtA*, and *yhiD* genes can survive only when supplemented with 100 mM Mg^{2+} [8]. However, differences between the roles of CorA, MgtA, YhiD and other protein(s), if any, in the *E. coli* Mg^{2+} uptake system have not been documented. We examined the effects of *corA*, *mgtA*, *yhiD* and *corC* mutations on Mg^{2+} uptake in *E. coli*.

3.1. Construction of double and triple mutant combinations and a quadruple mutant

We obtained four single W3110 mutants, five double mutants, two triple mutants and the quadruple mutant (Table 1). One double mutant (Δ*corA* Δ*yhiD*) and two triple mutant combinations (Δ*mgtA* Δ*corC* Δ*yhiD* and Δ*corA* Δ*corC* Δ*yhiD*) were not obtained because the kanamycin-resistance gene was not deleted from the source mutant *corA*::*kan* or *yhiD*::*kan*. TM2 cells (Δ*corA* Δ*mgtA yhiD*::*kan*) were then transformed with the pECmgtA plasmid to express MgtA protein, and the resulting strain was named DM6p. Similarly, QM cells (Δ*corA* Δ*mgtA* Δ*corC yhiD*::*kan*) were transformed with pECmgtA or pECcorA plasmids, and the resulting strains were named TM3p and TM4p, respectively. Thus, we obtained each double and triple mutant combination (Table 2).

3.2. Mutant growth curves

We compared the growth of the wild-type *E. coli* strain W3110 and all of the mutants obtained here in LB medium supplemented with 0-100 mM $MgCl_2$. LB medium without added $MgCl_2$ contained 0.17 mM Mg^{2+} as measured by atomic absorption spectroscopy.

3.2.1. Single mutants

No difference in growth was observed with all four single mutants (SM1, SM2, SM3 and SM4) when these mutants were cultured with 0–100 mM $MgCl_2$ and the wild-type W3110 (Fig. 1S). Thus, single *corA*, *mgtA*, *yhiD* and *corC* mutants did not demonstrate Mg^{2+} auxotrophy.

3.2.2. Double mutants

Among six double mutants, only DM1 cells (Δ*corA* Δ*mgtA*) were not grown for 8 h in LB medium with 0 and 1 mM $MgCl_2$ added (Fig. 1a). When 10 or 100 mM $MgCl_2$ was added, DM1 cells were grown, and no difference was observed in growth between DM1and W3110. These results indicated that DM1 required over 1 mM Mg^{2+} for growth. When DM1 cells were cultured with 3–10 mM Mg^{2+} for 24 h, DM1 grew to a similar level as W3110 (Fig. 1b). The DM1 growth curves, however, demonstrated that 9 to 10 mM Mg^{2+} was required for DM1 growth, which demonstrated similar growth to W3110.

When DM1 was transformed with either pECcorA or pECmgtA, the transformants were grown in LB medium that had not been supplemented with $MgCl_2$ (Fig. 1c). The results indicated that the Mg^{2+}-dependent growth of DM1 was due to the deletion of both *corA* and *mgtA* genes. The double mutants other than DM1 did not need Mg^{2+} added for growth (the DM3 and DM5 growth curves are shown in Fig. 1d and e. Those for DM2 and DM4 are not shown). These results indicated that no added Mg^{2+} was required for growth when either *corA* or *mgtA* was present.

Table 2
Combination of double (DM) and triple (TM) mutants.

Strain	*corA*	*mgtA*	*corC*	*yhiD*
DM1	−	−	+	+
DM2	+	−	−	+
DM3	+	−	+	−
DM4	+	+	−	−
DM5	−	+	−	+
DM6p	−	+	+	−
TM1	−	−	−	+
TM2	−	−	+	−
TM3p	−	+	−	−
TM4p	+	−	−	−

+, present; −, deleted.

3.2.3. Triple and quadruple mutants

TM1 and TM2 were derived from DM1 (Δ*corA* Δ*mgtA*). Growth of the cultures was monitored by OD_{600} for 8 h (Fig. 2). TM1, in which the *corC* gene was deleted, required Mg^{2+} for growth similar to DM1 (Fig. 2b). TM1 cells grew similar to W3110 when 10 mM Mg^{2+} was added, indicating that CorC itself did not work as a Mg^{2+} transporter under these conditions. In contrast, TM2, in which the *yhiD* gene was deleted, required more Mg^{2+} for growth than DM1. While the rate of cell growth and OD_{600} values measured at 8 h somewhat varied from experiment to experiment, (i) there was no significant difference in the DM1 growth in 10 and 100 mM Mg^{2+} (Fig. 1a), and (ii) there was no significant difference in growth between DM1 and TM2 when these cells were cultured with 100 mM Mg^{2+}, but (iii) the growth of TM2 in 10 mM Mg^{2+} was significantly reduced (Student's *t*-test, $P < 0.01$) than the growth of TM2 in 100 mM Mg^{2+} (Fig. 2a). These results indicated that the *yhiD* gene played a role under conditions where the *E. coli* (Δ*corA* Δ*mgtA*) cells were grown in 10 mM Mg^{2+} for 8 h. TM3p and TM4p, in which either *mgtA* or *corA* was present, did not require added Mg^{2+} for growth (data not shown).

QM did not require more Mg^{2+} for growth than TM2 (Fig. 2c).

3.3. Growth of TM2 that had been transformed with pAtMRS2-10 and pAtMRS2-11

We next examined the effect of AtMRS2-10 and AtMRS2-11 on TM2 growth. *Arabidopsis* AtMRS2-10 transported Mg^{2+} when recombinant AtMRS2-10 was reconstituted into liposomes [17]. TM2 required more than 10 mM Mg^{2+} for growth, but TM2 transformed with pAtMRS2-10 could grow in LB medium with the addition of 1 mM Mg^{2+} (Fig. 3a). When 0.5 mM or less Mg^{2+} was added, growth was greatly reduced. When TM2 cells that were transformed with pAtMRS2-10 were cultured for 24 h, growth with addition of 0 and 0.5 mM Mg^{2+} (OD_{600}) was approximately 1% and 40% of the growth with addition of 1 mM Mg^{2+}, respectively. Growth with addition of 1 mM Mg^{2+} was also observed with DM1 that had been transformed with pAtMRS2-10 (data not shown).

Previous reconstitution experiments [17] indicated that mutation in a GMN motif that was conserved in the CorA superfamily proteins (M400 to Ile of AtMRS2-10) inactivated Mg^{2+} transport. The growth of TM2 that had been transformed with M400I mutant plasmids was greatly reduced in LB medium with the addition of 1 mM Mg^{2+}.

TM2 transformed with pAtMRS2-11 could grow in LB medium, but addition of Mg^{2+} was not required (Fig. 3c). TM2 that had been transformed with AtMRS2-11 GMN motif mutant (M418A) plasmids could not grow in LB medium without Mg^{2+} supplementation.

Reconstitution experiments also indicated that the AtMRS2-10-mediated Mg^{2+} transport was inhibited by $AlCl_3$ [17]. Li *et al.* [16] indicated that AtMRS2-11-mediated uptake of Mg^{2+} into yeast cells was also sensitive to Al^{3+} inhibition. The growth of TM2 that had been transformed with pAtMRS2-10 and AtMRS2-11 was greatly reduced in the

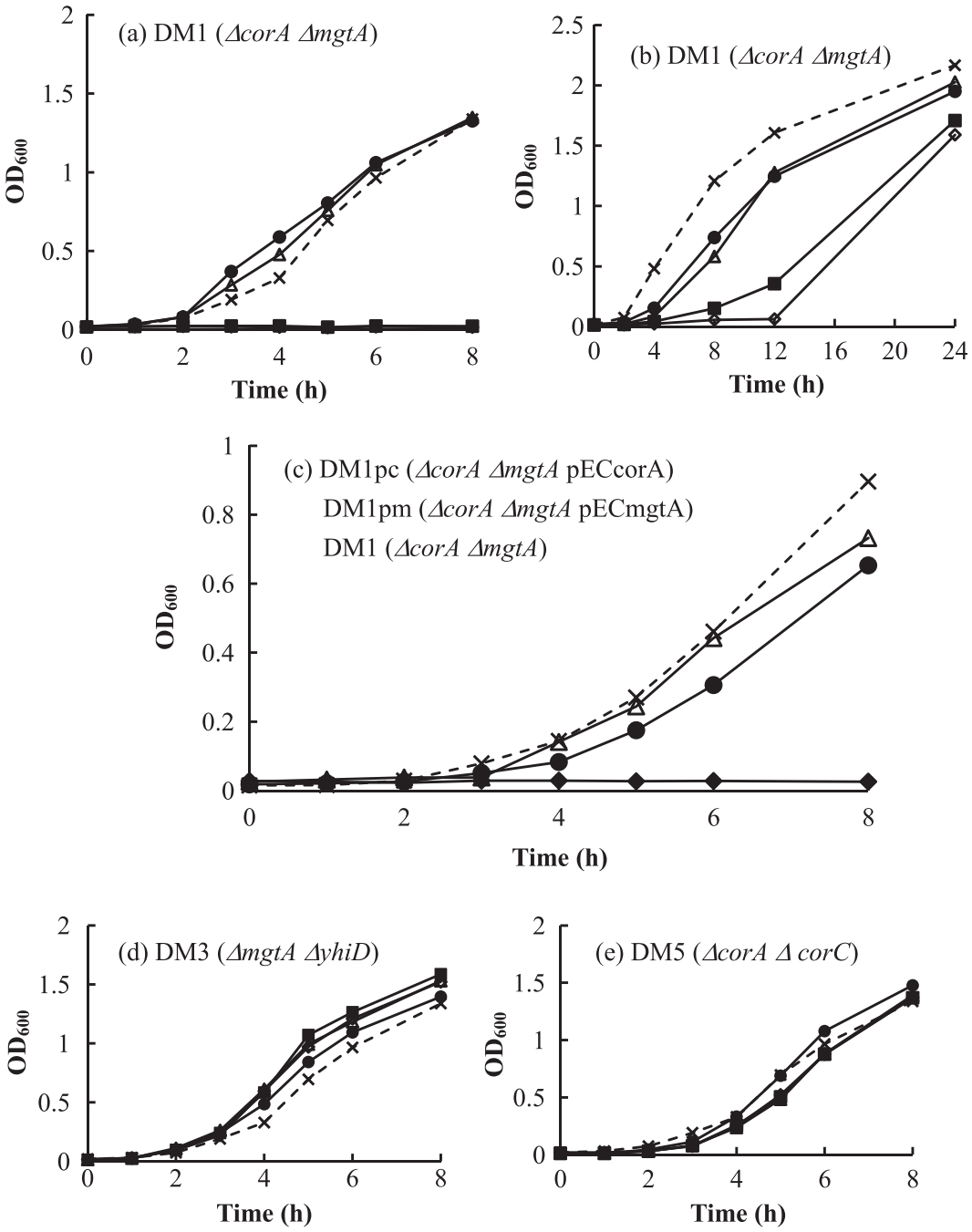

Fig. 1. Effects of Mg^{2+} concentration in LB medium on double mutant growth. Growth curves of W3110 that were not supplemented with $MgCl_2$ (×) and the double mutants indicated. (a) DM1 supplemented with 0 (◆), 1 (■), 10 (△) and 100 (●) mM $MgCl_2$. (b) DM1 supplemented with 3 (◇), 6 (■), 9 (△) and 10 (●) mM $MgCl_2$. (c) DM1 transformed without (◆) or with plasmids pECcorA (△) and pECmgtA (●). No $MgCl_2$ was added to the LB medium. (d), (e) DM3 (d) and DM5 (e) supplemented with 0 (◆), 1 (■), 10 (△) and 100 (●) mM $MgCl_2$. Cells were grown at 37 °C over 8 h as described in the "Materials and methods" section, and data shown are representative of similar results obtained from 3 independent experiments. DM2, DM4 and DM6p demonstrated similar growth curves as the single mutants as well as DM3 and DM5.

presence of $AlCl_3$. The growth inhibition demonstrated a concentration-dependent manner from 0.5 to 2 mM $AlCl_3$ (Fig. 3b, d). Growth was completely inhibited by 2 mM $AlCl_3$, but 2 mM $AlCl_3$ did not demonstrate growth inhibition of wild-type W3110 or of W3110 that had been transformed with pAtMRS2-10 (Fig. 3e, f).

4. Discussion

We examined the physiological roles of CorA, MgtA, CorC and YhiD gene products on Mg^{2+} uptake in *E. coli* by constructing deletion mutants. Bacteriophage P1 transduction and pCP20 were used to construct the deletion mutants. To obtain multiple deletion mutants, deletion of the kanamycin-resistant gene was necessary, but it was difficult for *corA*::*kan* and *yhiD*::*kan*, the reason for which was unknown. The *E. coli* CorA and MgtA expression plasmids were constructed and used to obtain some mutant combinations. Thus, we obtained and analyzed each combination of double, triple and quadruple mutants.

In *S.* Typhimurium, CorA, MgtA and MgtB can transport Mg^{2+}. Each of these is necessary and sufficient for normal growth of *S.* Typhimurium [3]. *E. coli* does not have the *mgtCB* operon. Experimental results where

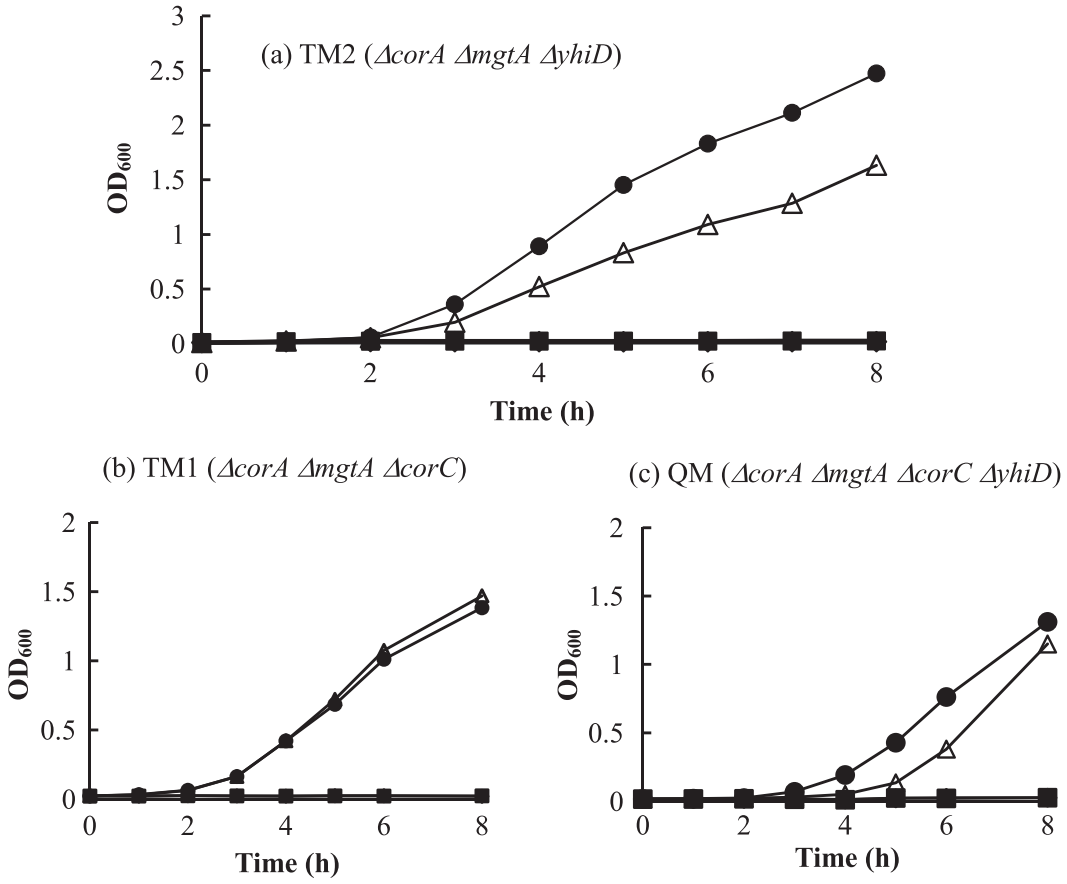

Fig. 2. Effects of Mg^{2+} concentration in LB medium on growth of the triple and quadruple mutants. Growth curves of TM2 (a), TM1 (b) and QM (c) that were supplemented with 0 (◆), 1 (■), 10 (△) and 100 (●) mM $MgCl_2$. Cells were grown at 37 °C over 8 h, and data shown are representative of similar results obtained from 3 independent experiments. TM3p and TM4p demonstrated similar growth curves to the single and double mutants other than DM1.

growth of the cultures was monitored for 8 h indicated that either CorA or MgtA was necessary and sufficient for normal growth of *E. coli* in LB medium without Mg^{2+} supplementation; all four single mutants, double mutants other than DM1 (Δ*corA ΔmgtA*), TM3p and TM4p did not show Mg^{2+} auxotrophy. Both CorA and MgtA could transport Mg^{2+} into *E. coli* sufficiently for growth when the *E. coli* were cultured in LB medium. DM1 required over 1 mM Mg^{2+} for growth. When 10 mM Mg^{2+} was added to the culture of DM1 and TM2, DM1 demonstrated similar growth as that of W3110, but the growth of TM2 was greatly reduced. These results indicated that external Mg^{2+} is transported into DM1 cells through the YhiD protein when supplemented with 10 mM Mg^{2+}. The results also indicated that YhiD can not transport Mg^{2+} sufficiently for growth when supplemented with 1 mM Mg^{2+}. The *E. coli* YhiD protein is distantly related to the *S.* Typhimurium MgtC protein. MgtC is predicted to be an innermembrane protein that is required for *S.* Typhimurium growth in a low-Mg^{2+} (0.01 mM) medium [24,25], and does not appear to be a Mg^{2+} transporter [26,27]. CorC was not involved in Mg^{2+} transport in *E. coli* under the conditions examined where cells were grown for 8 h. The quadruple mutant could survive in the presence of the 10 mM Mg^{2+} added, and it grew well on medium containing 100 mM Mg^{2+} (Fig. 2c), indicating that other low-affinity Mg^{2+} transport protein(s) were also present in *E. coli* cells.

S. Typhimurium CorA is a constitutively expressed protein whose promoter does not respond to changes in extracellular Mg^{2+} concentration [3]. The expression levels and the possible transcriptional regulation of YhiD and CorC are unknown. When cells were cultured with 3 mM Mg^{2+} at 37 °C for 16 h, DM1, TM1, TM2 and QM could grow, but the growth of TM1 and QM was significantly reduced than that of DM1 and TM2 (Fig. 2S). The results suggested that the expression of CorC was slowly induced to support growth at the later phase at 3 mM Mg^{2+}, and that other protein(s) were also present to support the growth of QM.

In a recent report, the *E. coli* MG1655 with *corA* deletion was used for a Co^{2+} transport assay of *Thermotoga maritima* CorA [10,11]. *E. coli* triple mutant spheroplasts (*ΔmgtA ΔcorA Δ yhiD*) were used for patch-clamp analysis of *Thermus thermophillus* MgtE [8]. We now used Mg^{2+}-auxotrophic TM2 for AtMRS2-10 and AtMRS2-11 *in vivo* complementation assay. The *Arabidopsis* AtMRS2 family belongs to a eukaryotic subset of the CorA superfamily proteins. The family has 10 members. Homozygous single knockouts in *A. thaliana* of AtMRS2-10 and of the related AtMRS2-1 and AtMRS2-5 genes have no significant phenotypes [28]. Phenotypes only appear after AtMRS2-1/10 double and AtMRS2-1/5/10 triple knockouts [29]. A significant phenotype was revealed for single knockout of AtMRS2-7 gene [28], and no homozygous knockout lines could be raised for AtMRS2-11 gene. Functional differences and structure–function relationship of AtMRS2 proteins are not fully understood. AtMRS2-10 and AtMRS2-11 are localized to the plasma membrane [16] and chloroplasts [22], respectively, in plants, and belong to different phylogenetic subclade of MRS2 [29]. The purified AtMRS2-10 reconstituted into liposomes transported Mg^{2+} without any accessory proteins [17]. TM2 transformed with pAtMRS2-10 and pAtMRS2-11 could grow in LB medium with the addition of 1 mM Mg^{2+} and without Mg^{2+} supplementation, respectively. Growth of TM2 that had been transformed with the GMN motif mutant plasmids was greatly reduced under these conditions. These results indicate that the exogenously expressed AtMRS2-10 and AtMRS2-11 are clearly capable of

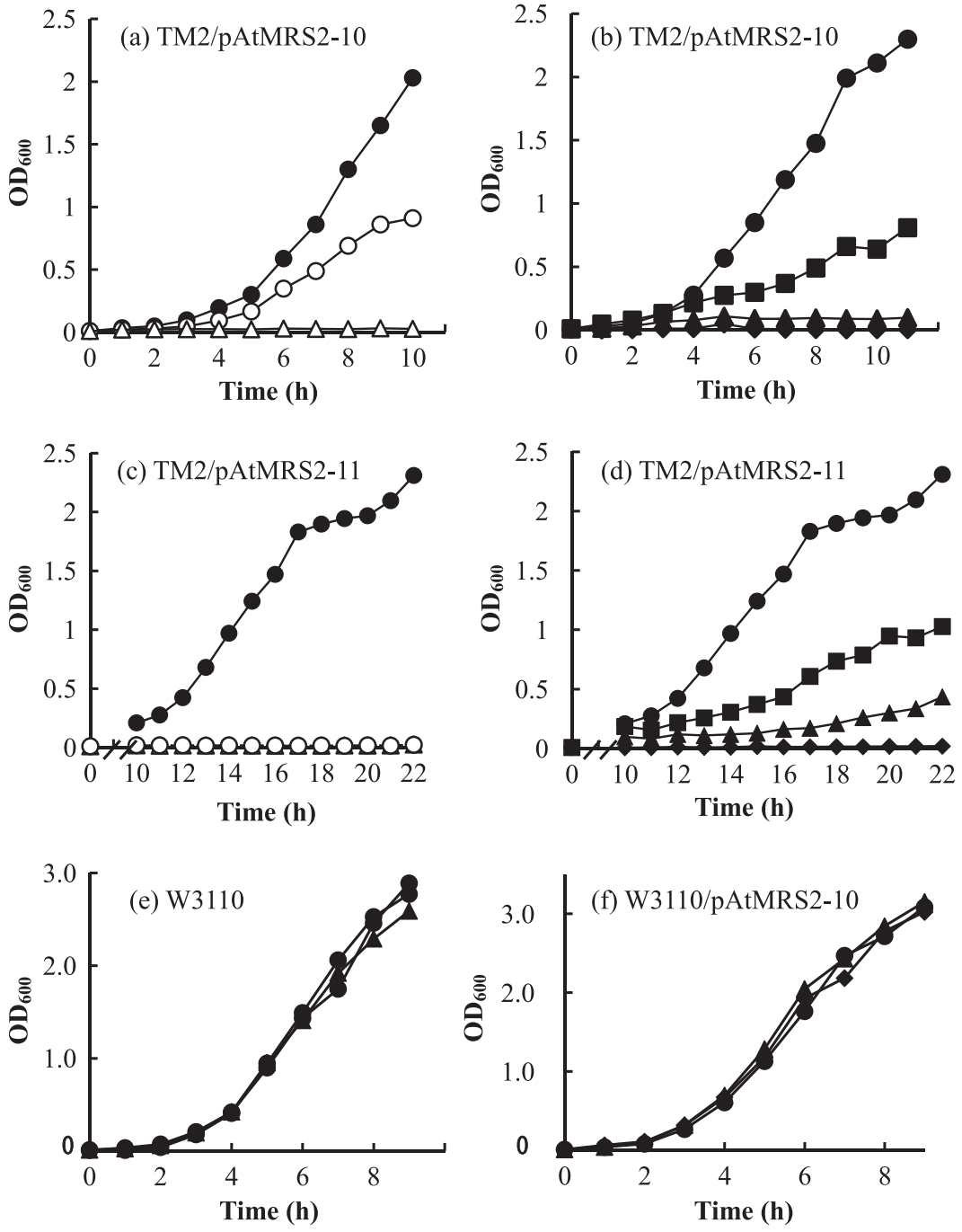

Fig. 3. Complementation of the *E. coli* TM2 by AtMRS2-10 and AtMRS2-11 and Al inhibition. (a) Growth curves of TM2 transformed with pAtMRS2-10 (●), pAtMRS2-10 M400I (○) and pTV118N vector (△). (b) Growth curves of TM2 that had been transformed with pAtMRS2-10. $AlCl_3$ was added at 0 (●), 0.5 (■), 1 (▲) and 2 (◆) mM. Cells were grown at 37 °C on LB medium that had been supplemented with 1 mM $MgSO_4$. (c) Growth curves of TM2 transformed with pAtMRS2-11 (●), pAtMRS2-11 M418A (○) and pTV118N vector (△). (d) Growth curves of TM2 that had been transformed with pAtMRS2-11. $AlCl_3$ was added at 0 (●), 0.5 (■), 1 (▲) and 2 (◆) mM. Cells were grown at 37 °C on LB medium without Mg^{2+} supplementation. (e) Growth curves of W3110 and (f) W3110 transformed with pAtMRS2-10 in the presence of $AlCl_3$. Cells were grown at 37 °C on LB medium. $AlCl_3$ was added at 0 (●), 1 (▲) and 2 (◆) mM. Data shown are representative of similar results obtained from 3 independent experiments.

transporting Mg^{2+} in *E. coli* cells and also indicate the importance of the Met residue in the GMN motif for AtMRS2-10 and AtMRS2-11 function. AtMRS2-10 and AtMRS2-11 complementation assay has also been reported using the *S.* Typhimurium mutant strain MM281, which lacks the Mg^{2+} transport system CorA, MgtA and MgtB [30]. MM281 transformed with AtMRS2-10 and AtMRS2-11 reportedly grows well on N-minimal liquid medium containing 0.01 mM Mg^{2+} [31,32]. AtMRS2-10 and AtMRS2-11 expressed here in *E. coli* had no tags. The difference of the observed Mg^{2+} requirement of AtMRS2-10 may be due to differences in the host species. The *E. coli* TM2 cells that had been transformed with the *E. coli* CorA gene could grow in LB medium without supplemented Mg^{2+} (data not shown).

The growth of TM2 that had been transformed with pAtMRS2-10 and pAtMRS2-11 was inhibited by 1–2 mM $AlCl_3$. *E. coli* aluminum toxicity is markedly dependent on pH; growth is completely inhibited with 2.25 mM Al at pH 5.4 but not at pH 6.6 [33]. In accordance with this report, 2 mM $AlCl_3$ did not inhibit wild-type W3110 growth in LB medium (pH 7.2) in our conditions (Fig. 3e).

Two possibilities can be considered regarding the $AlCl_3$-mediated growth inhibition of TM2 that had been transformed with pAtMRS2-10 and pAtMRS2-11; 1) Al^{3+} inhibits Mg^{2+} transport of AtMRS2-10 and AtMRS2-11, resulting in Mg^{2+} deficiency of the TM2 cells, or 2) Al^{3+} is transported through AtMRS2-10 and AtMRS2-11, and Al^{3+} has direct toxicity within the cells. Al^{3+} toxicity can damage membranes by causing lipid peroxidation and loss of cellular compartments. *E. coli* is resistant to 2 mM external $AlCl_3$ in LB medium. There are no reports that demonstrate Al^{3+}-mediated inhibition and Al^{3+} transport of the *E. coli* CorA. No growth inhibition was demonstrated by 2 mM $AlCl_3$ in wild-type W3110 that had been transformed with pAtMRS2-10 (Fig. 3f), which supported the former interpretation. However, the results from these experiments cannot describe which of the two possibilities caused TM2 mutant growth inhibition. Mg^{2+} transport of AtMRS2-10 and AtMRS2-11 is inhibited by Al^{3+} [16,17], but it is unknown whether AtMRS2-10 and AtMRS2-11 can transport Al^{3+}. Studies of AtMRS2-10 and AtMRS2-11 Al^{3+} transport are now in progress using *in vitro* proteoliposome assays. Even if this is the reason, the $AlCl_3$-mediated growth inhibition of TM2 that had been transformed with pAtMRS2-10 and pAtMRS2-11 reflects the function of AtMRS2-10 and AtMRS2-11 for aluminum.

5. Concluding remarks

We demonstrate here the utility of an Mg^{2+}-auxotrophic *E. coli* strain to elucidate the Mg^{2+} transport activity of plant MRS-2 proteins.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.03.005.

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