Deletion of a Cation Transporter Promotes Lysis in *Streptococcus pneumoniae*  
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*Streptococcus pneumoniae* is a significant human pathogen which causes respiratory and serious invasive diseases. Mg2+ is essential for life, and its concentration varies throughout the human body. Magnesium uptake plays an important role in the virulence of many bacterial pathogens. To study the Mg2+ uptake of *S. pneumoniae* strain D39, a mutant was generated in SDP1383, a P-type ATPase with homology to the *Salmonella* Mg2+ transporter MgtA, which has also been shown to be a Ca2+ exporter in strain TIGR4. Under low-Ca2+ conditions, mutation led to a growth defect in complex medium and the gene was nearly essential for growth under low-Mg2+ conditions. Addition of Mg2+ restored the normal growth of the mutant in all cases, but the addition of other divalent cations had no effect. Addition of Ca2+, Mn2+, and Zn2+ in the presence of high Mg2+ concentrations inhibited restoration of growth. The mutant was unable to proliferate in blood, which was also alleviated by the addition of Mg2+. The protein was located in the membrane and produced in various *S. pneumoniae* strains and pathogenic streptococcal species. Surprisingly, mutation of the gene led to an elevated toxicity for endothelial cells. This was caused by an increased amount of pneumolysin in the medium, mediated by elevated lysis of the mutant. Thus, in this study, we uncovered a role for SPD1383 in Mg2+ toxicity and hypothesize that the protein is a Mg2+Ca2+ antiporter. Furthermore, a disturbance in Mg2+ homeostasis seems to promote lysis of *S. pneumoniae*.  

Infection with the Gram-positive bacterium *Streptococcus pneumoniae* is a common cause of respiratory disease and serious invasive diseases, such as pneumonia, septicemia, and meningitis; in particular, infants, the elderly, and immunocompromised individuals are at risk. Annually, it is estimated that over 1 million people, including young children, die of pneumonia and meningitis and that, in the United States alone, approximately 40,000 deaths are caused by pneumococcal pneumonia or meningitis (43). *S. pneumoniae* meningitis has high mortality and morbidity rates compared to those caused by other meningeal pathogens (30). Current vaccines do not protect against all serotypes, and resistance to commonly used antibiotics is on the rise. Thus, there is a need for new targets for the development of new antimicrobials and protein vaccines against this bacterium.  
Mg2+ is essential for life due to its important role in multiple types of cellular processes. The concentration of Mg2+ varies throughout the human body, and half of the total amount is sequestered in bone. The mean serum concentration is ~1 mM, and it is estimated that half of this amount is free and thus readily available; in interstitial fluid, the concentration is estimated to be 0.5 mM (11). The amount of (free) Mg2+ in the healthy human mucosa of the respiratory tract is largely unknown. Sputa of patients suffering from cystic fibrosis have been reported to contain approximately between 0.1 mM and 1 mM Mg2+, depending on the method used for measuring (13, 27); the sputa of patients suffering from other bronchopneumopathologies were reported to contain around 0.5 mM Mg2+ (13).  

Mutants in Mg2+ homeostasis genes in a wide variety of pathogenic bacteria are attenuated for virulence (10, 16, 18, 36, 45). Magnesium concentrations within eukaryotic vesicles are not precisely known but are thought to be even lower than in interstitial fluid. Several pathogens experience Mg2+ limitation inside eukaryotic cells and induce the expression of Mg2+ transporters (3, 12, 18). Thus, the availability of free Mg2+ in the host is thought to be an important signal for pathogenic bacteria to denote in which compartment they reside (17). Extensive studies of Gram-negative bacteria showed that several transporters are involved in Mg2+ homeostasis (18, 37, 42). One well-studied Mg2+ transporter is the primary uptake transporter CorA, which is thought to be a constitutively active pore in the membrane. The Mg2+ transporter MgtE is a highly selective ion pore. The P-type ABC transporters MgtA and MgtB are induced upon encountering Mg2+-limiting conditions and import Mg2+ against the gradient at the expense of ATP (37, 42). In comparison, little work has been done on Mg2+ transport in Gram-positive bacteria, except for the MgtE protein, a highly selective ion pore (8, 49).  

Throughout colonization and infection, *S. pneumoniae* might encounter various environments with changing Mg2+ concentrations: relatively high concentrations in blood, lower concen-
Grown as a standing culture in M17 broth (Oxoid) (53) containing 0.5% glucose. Plasmids homologue. Here we show that the protein, which has also interaction with eukaryotic cells, we studied a putative MgtA play an important role in the lifestyle of S. pneumoniae transporters, among them a CorA homologue and several P- reported to respond to Mg2+ interestingly, in contrast to the PhoPQ system, which has been transporters in this organism have been characterized. Inter- to 10 mM (15), the CsrRS (CovRS) system responds to a component system, CsrRS (CovRS), has been reported to respond to external Mg2+ concentrations (19, 20). However, so far no transporters in this organism have been characterized. Interestingly, in contrast to the PhoPQ system, which has been reported to respond to Mg2+ concentrations ranging from 8 μM to 10 mM (15), the CsrRS (CovRS) system responds to a much narrower range, i.e., from 1 to 10 mM Mg2+ (20). Analysis of the published S. pneumoniae genomes (25, 35, 54) revealed the presence of several putative homologues of Mg2+ transporters, among them a CorA homologue and several P-type ATPases, indicating that Mg2+ homeostasis might also play an important role in the lifestyle of S. pneumoniae. To characterize the role of Mg2+ homeostasis in S. pneumoniae and determine its role in infection, in particular during its interaction with eukaryotic cells, we studied a putative MgtA homologue. Here we show that the protein, which has also been reported to be a Ca2+ exporter (48), is essential for growth under low-Mg2+ conditions and is a determinant of pneumococcal lysis.

### MATERIALS AND METHODS

#### Bacterial strains and growth conditions.

Strains used in this study are listed in Table 1 and were stored in 12% glycerol at −80°C. S. pneumoniae was routinely grown as a standing culture in M17 broth (Oxoid) (53) containing 0.5% glucose (GM17) at 37°C. Lactococcus lactis NZ8000 was grown in the same medium with conditions at 30°C, and Escherichia coli was grown in LB broth in shaken cultures at 37°C. The composition of the chemically defined medium (CDM) was the same as described before (28) and contained 500 μM MgCl2. Chloramphenicol and erythromycin were used at concentrations of 2.5 and 0.25 μg/ml for S. pneumoniae and 5 and 4 μg/ml for L. lactis, respectively. Trimethoprim was used at a concentration of 15 μg/ml (28). For S. pneumoniae growth on plates, 3% defibrinated sheep blood (Johnny Rottier, Klokerozande, Netherlands) was added to GM17 agar or Columbia agar (Oxoid). All staphylococcal and streptococcal species were grown in standing tryptic soy broth (TSB) cultures at 37°C; overnight cultures were diluted 1:40 or 1:50 and grown to late exponential phase. Streptococcal species were isolated during a routine procedure at the University Medical Center Groningen by the Medical Microbiology Department. Mg2+ and Ca2+ concentrations in the media were determined by the microanalytical group of the University of Groningen using inductively coupled plasma mass spectrometry (ICP-MS).

#### Generation of mutations in S. pneumoniae.

Strain D39 misRK was generated by the introduction of the misRK genes into the hgaA locus of D39 as described by Klokerozande et al. (28). SPD1383 was deleted from the genome by allelic-replacement mutagenesis. In short, primers SP1382for/SP1382rev and SPD1384for/SPD1384rev (Table 2) were used to generate PCR products of approximately 500 bp of the 3’ and 5’ regions flanking SPD1383. These fragments were fused to an erythromycin resistance gene amplified with primers eryf and eryrev (Table 2) from plasmid pOR13 by means of overlap extension PCR (51). The resulting PCR product was transformed into S. pneumoniae as described before (28). Deletion of mgtA from the genome was verified by PCR using primers contdmgta.fw and contdmgta.rev (Table 2), located outside the region used for the generation of the overlap PCR. (Table 2) and/or Southern blot. The pneumolysin gene was deleted from the genome in the same manner, using primers spr1740for/spr1740rev and spr1738for/spr1738rev fused to a spectinomycin cassette generated with primers specfor and specrev (Table 2) by means of overlap PCR. Deletion of ply was confirmed using primers contdplyf and contdplyrev (Table 2), located outside the region used to delete the gene, and by Western blot analysis using an anti-Ply antiserum. S. pneumoniae D39cps was produced by replacement of the capsule locus by a kanamycin resistance gene, as described by Pearce et al. (46).

#### Construction of a pNZ8048 derivative that produces MgtA.

For complementation analysis, a pNZ8048 (9) derivative that contained the mgtA gene under the control of nisin-inducible promoters was constructed. The mgtA gene was amplified from the chromosome of D39 using primers mgtAstart and mgtAstop, which contain XbaI and NcoI sites, respectively (Table 2). The resulting 2,749-bp PCR fragment was cloned into the pNZ8048 plasmid using NcoI and XbaI and harbors the nisRK genes in between.

#### Growth experiments.

S. pneumoniae was grown in GM17 to an optical density at 600 nm (OD600) of 0.2 to 0.3; glycerol was added to 12%, and the culture was frozen in 1-ml aliquots at −80°C. For the growth experiments, S. pneumoniae strains containing the mgtA deletion were grown for aliquots with 10 mM MgCl2 in the growth medium to ensure that the bacteria were equally viable and contained comparable numbers of CFU at that stage of growth. Using this approach, the presence of several putative homologues of Mg2+ transporters, among them a CorA homologue and several P-type ATPases, indicating that Mg2+ homeostasis might also play an important role in the lifestyle of S. pneumoniae. To characterize the role of Mg2+ homeostasis in S. pneumoniae and determine its role in infection, in particular during its interaction with eukaryotic cells, we studied a putative MgtA homologue. Here we show that the protein, which has also been reported to be a Ca2+ exporter (48), is essential for growth under low-Mg2+ conditions and is a determinant of pneumococcal lysis.

#### TABLE 1. Strains and plasmids used in this study

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<th>Strain or plasmid</th>
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<td>pPP2 containing the putative promoter of SPD1383 in front of lacZ</td>
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* Km’, kanamycin resistance; Em’, erythromycin resistance; Tm’, trimethoprim resistance; Sp’, spectinomycin resistance; Cm’, chloramphenicol resistance.
predicted ATG of mgtA was amplified from the genome of strain D39 using primers pmgtAlacZ5 and pmgtAlacZ3 (Table 2) and cloned into the XbaI sites of the pPP2 plasmid (21). The resulting plasmid was introduced into the bgaA locus of the S. pneumoniae genome. The activity of the promoter was assayed by measuring β-galactosidase activity as described before (28).

Microarray analyses. For DNA microarray analysis, the D39 Δrep wild type and its isogenic mgtA mutant were grown as three biological replicates in GM17 to which no extra cations were added and harvested at an OD595 of approximately 0.2. All other procedures regarding microarray analyses were performed as described before (29). A gene was considered differentially expressed when its expression levels deviated from those of the control by more than two-fold and when the difference was statistically significant (5% of significance level).

Endothelial cell line and culture conditions and infection studies. Immortalized human brain microvascular endothelial cells (HBMEC) (52) were used up to passage 36. Cells were routinely cultivated in RPMI 1640 (Biochrom), which was supplemented with 10% fetal calf serum (FCS) (Biochrom), 10% Nu-Scrum (BD Biosciences), 2 mM l-glutamine (Gibco), 1 mM Na-pyruvate (Gibco), 1% minimal essential medium (MEM)-vitamins (Gibco), and 1% nonessential amino acids (Gibco) at 37°C and 5% CO2. The cells were subcultured every third or fourth day, diluted 1:6, and seeded for further growth. For adherence and invasion studies, HBMEC were seeded at 2 × 104 cells per well in a 12-well plate and incubated for 2 days to 100% confluence. For infection, the cell layer was washed twice with 500 μl phosphate-buffered saline (PBS)/well and incubated for 1 h with culture medium containing 10 ng/ml tumor necrosis factor alpha (TNF-α). Subsequently, various numbers of S. pneumoniae CFU/well were added to the cells. After 2 h, the HBMEC were washed twice with Dulbecco PBS (DPBS) (Cambrex) to remove nonadhered bacteria. Pictures were taken using a Leica inverted microscope (model DMI 3000 B) using the LAS software and a Leica DFC350FX camera.

Production and purification of the MgtA protein and generation of anti-MgtA antibodies. To generate antibodies, an MgtA protein containing a C-terminal His6 tag was generated by amplifying the mgtA gene from the D39 genome using the primers mgtAstart and mgtAstop (Table 2). The resulting PCR product was cloned into the pNZ8048 plasmid using the XbaI and NcoI sites and introduced into L. lactis strain NZ9000. The protein was produced by the induced strain and harvested at 100,000 × g; subsequently, the pellet was solubilized by incubation in solubilization buffer (300 mM NaCl, 20 mM sodium phosphate buffer, pH 8.0, 0.2% Tween 20, 10% glycerol, 6 M urea) for 2 h at 4°C, and the insoluble fraction was removed by centrifugation (35 min, 100,000 × g). Samples from all fractions were loaded onto a 10% SDS-PAGE gel. Western analysis using an anti-His tag antibody (Zymed-Invitrogen) showed that a protein with the predicted molecular weight of MgtA was produced and exclusively present in the membrane fraction. The protein was purified from the solubilized membrane fraction using a 5-ml His-Trap high-performance (HP) column (GE Healthcare), and 0.8 mg was used for the generation of a polyclonal antibody using the Speedy program of Eurogentec.

Fractionation of S. pneumoniae cells. The fractionation of S. pneumoniae was performed essentially as described previously (56). S. pneumoniae was grown to the desired OD660 in GM17, after which the bacteria were spun down for 10 min at 7,000 rpm. The supernatant was precipitated using trichloroacetic acid (TCA), the cell pellet was taken up in 1/20 volume of lysis buffer (100 mM Tris-HCl, pH 8.0, 20% sucrose, 20 mM MgCl2), to which fresh lysozyme (5 mg/ml), mutanolysin (200 U/ml), and EDTA-free protease inhibitor (Roche) were added, and incubated for 30 min at 37°C. The proteoplasms were spun down at 3,000 × g for 10 min at 4°C, resuspended in 0.5 ml sucrose buffer (20% sucrose, 10 mM Tris-HCl, pH 8.0), and disrupted by the addition of 9.5 ml 100 mM Tris-HCl, with EDTA-free protease inhibitor and 1 mM EDTA; undisrupted protoplasts were removed by centrifugation (4,000 × g for 10 min at 4°C). The membrane and cytosolic fractions were separated by centrifugation at 100,000 × g for 30 min at 4°C. Samples were taken up in loading buffer, incubated for 5 min at 95°C, and used for SDS-PAGE and Western blot analysis.

SDS-PAGE, Western blotting, and analysis of Western blot signals. The presence of various proteins was detected by Western blot analysis. Protein fractions were separated by SDS-PAGE (using precast NuPAGE gels from Invitrogen) and then semidry blotted (1.25 h at 100 mA per gel) onto a nitrocellulose membrane (Protran; Schleicher and Schuell). Detection of antibodies was performed with fluorescent IgG secondary antibodies (IRDye 800CW goat anti-rabbit from LiCor Biosciences) in combination with the Odyssey infrared imaging system (LiCor Biosciences). In the Western case, the exposure time at 800 nm was recorded. Signals on the Western blots were quantified as follows: non-y-transformed images with nonsaturated signals, as determined with the plot profile function of ImageJ (http://rsb.info.nih.gov/ij), were analyzed using the gel function of the same program. Comparisons of the amounts of signal for each type of

<table>
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<th>Primer</th>
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*a* Restriction sites are underlined.

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RESULTS

Identification of the main Mg$^{2+}$ transporter in S. pneumoniae under in vitro growth conditions. Comparison of the Salmonella enterica serovar Typhimurium MgtA amino acid sequence, which has been demonstrated to be a functional Mg$^{2+}$ transporter (24, 50), with the genomes of S. pneumoniae strains R6, D39, and TIGR4 (25, 35, 54) using BLAST via the Comprehensive Microbial Resource (CMR) website (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi) showed that open reading frame (ORF) SPR1410 (SPD1383, SP1551) had the highest similarity (30% identity). The ORF is predicted to encode a cation transporter and, like other MgtA homologues, contains a classical cation transporter domain, a P-type ATPase domain, a haloacid dehalogenase-like hydrolase domain (14), and an MgtA domain. The ORF seems to be the first gene of a putative small operon consisting of SPR1410 and SPR1409 (putatively encoding a glutathione S-transferase family protein) and is preceded by a putative promoter region. Interestingly, in TIGR4, the protein has been reported to be a Ca$^{2+}$ exporter and shown to have a role in virulence (48). To determine whether SPR1410 indeed encodes a Mg$^{2+}$ transporter, the gene was deleted from the genome of strain R6. We compared the levels of growth of the parent strain and the mutant in GM17 broth containing 1.1 $\times$ 0.01 mM Mg$^{2+}$ and 0.21 $\times$ 0.087 mM Ca$^{2+}$. The growth of the mutant in GM17 was poor and did not reach the same OD as the wild type. Addition of MgCl$_2$ (5 mM) to the medium (Fig. 1A) restored growth to nearly normal levels. The counterion had no effect, as the addition of MgSO$_4$ restored growth to the same extent (Fig. 1A). Addition of 10 times more MgCl$_2$ did not further improve growth (data not shown), nor did the addition of MnCl$_2$ (5 mM) or CaCl$_2$ (5 mM). In fact, both cations seemed to inhibit the residual growth of the mutant (Fig. 1A). Addition of other divalent cations (such as Zn$^{2+}$, Co$^{2+}$, Cu$^{2+}$, and Cd$^{2+}$) did not restore growth either (data not shown), indicating that the effect was specific for Mg$^{2+}$. The residual growth of the mutant indicates that there are other (lower-affinity)

Microarray data accession number. The microarray data were submitted to the GEO database under accession number GSE26154.
transports present in *S. pneumoniae* that mediate magnesium uptake. These data indicate that SPR1401 encodes a Mg\(^{2+}\) transporter, and the ORF was designated *mgtA*.

**MgtA is the main Mg\(^{2+}\) importer in *S. pneumoniae* when the Mg\(^{2+}\) concentration is low.** Interestingly, SPR1401 has been reported to be essential in both strain TIGR4 and strain R6 (51, 55). Strain R6 contains a mutation in *dltA* (33) which might lead to an increased binding of cations to the cell wall. In addition, GM17 contains 1.1 mM Mg\(^{2+}\), which is probably why residual growth in GM17 was observed. Consequently, growth experiments performed in GM17 are likely to be an underestimation of the role of the transporter in *S. pneumoniae* Mg\(^{2+}\) homeostasis. On the other hand, the capsule of *S. pneumoniae* D39 is a negatively charged polymer that could act as a Mg\(^{2+}\) reservoir for the bacteria. Thus, the performance of the experiments in the unencapsulated R6 background could inadvertently have caused an overestimation. Therefore, the effect of the mutation was studied in strain D39 using our recently developed chemically defined medium (CDM) (28), which allows for the variation of the amount of Mg\(^{2+}\) and contains 0.16 ± 0.04 mM Ca\(^{2+}\). Initial growth experiments showed that D39 grew well and reproducibly starting at 500 μM MgCl\(_2\); no growth occurred at 100 μM or 250 μM MgCl\(_2\) (data not shown). Consequently, the growth of D39 and that of the SPD1383 mutant were compared in CDM containing 500 μM MgCl\(_2\). The gene was nearly essential under these conditions, as no growth was observed during 9 h and only after 24 h was some growth observed (Fig. 1B). The addition of 10 mM MgCl\(_2\) (Fig. 1B) restored growth to approximately 80% of wild-type levels with regard to both maximum OD and growth rate (wild-type average \(\mu_{\text{max}}\), 1.19 ± 0.042; mutant plus MgCl\(_2\), average \(\mu_{\text{max}}\), 1.43 ± 0.025). The maximum number of CFU of the mutant grown in CDM with magnesium reached 50 to 80% of that of the wild type. In conjunction with this, when the strains were first grown in CDM containing 10 mM MgCl\(_2\) and subsequently shifted to CDM with 500 μM MgCl\(_2\), the growth of the wild type continued normally, whereas the mutant stopped growing. When the mutant was shifted to medium with 10 mM MgCl\(_2\), growth continued, demonstrating that there was no effect of the procedure itself (data not shown). This indicates that the mutant does not have an extended lag phase but is indeed unable to grow under low-Mg\(^{2+}\) conditions. Subsequently, the gene was cloned into the pNZS048 vector (9) (pNZmgtA) and used for complementation analysis. The growth of the mutant and of the mutant with the empty pNZS048 vector in CDM was impaired (Fig. 1C) and could be restored by the addition of MgCl\(_2\) (data not shown). In contrast, the growth of the mutant in medium containing 500 μM MgCl\(_2\) was restored to near wild-type levels when the plasmid containing the *mgtA* gene was introduced (wild-type average \(\mu_{\text{max}}\), 1.08 ± 0.036; mutant plus MgCl\(_2\), average \(\mu_{\text{max}}\), 0.99 ± 0.1) (Fig. 1C). Thus, the observed phenotype is due solely to the deletion of *mgtA*. This clearly indicates that ORFs SPR1410/SPD1383 (which are 100% homologous) are *mgtA* homologues that encode the main Mg\(^{2+}\) importer of *S. pneumoniae* under the growth conditions tested.

A homologue of this gene in TIGR4 has been designated a Ca\(^{2+}\) efflux protein (48), and the residual growth of our mutant was also inhibited by the addition of excess Ca\(^{2+}\) to the medium (Fig. 1A and B). The growth of the mutant in CDM with 10 mM MgCl\(_2\) was also inhibited by the addition of 5 mM CaCl\(_2\) (Fig. 1B). However, prolonged incubation of the mutant (>20 h) resulted in some growth of the mutant in CDM and in CDM when both extra MgCl\(_2\) and extra CaCl\(_2\) were added (Fig. 1B). These data suggest either that Ca\(^{2+}\) inhibited transporters that take up Mg\(^{2+}\) in the absence of MgtA or that the protein is a Mg\(^{2+}\)/Ca\(^{2+}\) antiporter. Several cations are known to inhibit Mg\(^{2+}\) transporters, such as Mn\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), and Ca\(^{2+}\) (24, 37). Thus, the effect of addition of these cations was tested when the mutant was grown in 10 mM MgCl\(_2\). These experiments showed that addition of Zn\(^{2+}\) and Mn\(^{2+}\) had an inhibitory effect on growth but that addition of Ni\(^{2+}\) and Co\(^{2+}\) did not (Fig. 1D). The growth of the wild type was not significantly affected by any of these cation concentrations (data not shown), which are just below the MICs reported for D39 (29). Thus, either MgtA is a Mg\(^{2+}\)/cation antiporter or the growth of the mutant at high Mg\(^{2+}\) concentrations is mediated by residual uptake systems that are inhibited by these cations.

**The growth defect of the mutant in blood can be restored by the addition of magnesium.** MgtA is needed for growth under the low-Mg\(^{2+}\) conditions of the CDM, and the mutant has a marked growth disadvantage compared to the wild type at concentrations that might reflect those in the human mucosa. However, blood and serum are thought to contain relatively high (free) Mg\(^{2+}\) concentrations (~1 mM), and the protein might not be needed while the bacteria are causing invasive disease. Compared to the wild type, the mutant was unable to proliferate in blood, as observed before (48), and the number of CFU steadily declined over 24 h (Fig. 2). Interestingly, although the addition of extra Ca\(^{2+}\) had a detrimental effect, addition of Mg\(^{2+}\) to the blood restored the ability of the mutant to survive and proliferate in blood (Fig. 2). Thus, even under the supposedly Mg\(^{2+}\)-rich conditions of blood, the mutant is unable to proliferate, which indicates that the protein is
essential for survival in the host because the mutant has a
growth defect. In contrast to the situation in CDM, addition of Mg$^{2+}$ in the context of the relatively high Ca$^{2+}$ (~1 mM)
concentrations in blood restored the ability of the mutant to
survive and to grow to some extent.

**MgtA is a membrane protein.** Analysis of the amino acid
sequence of MgtA with localization and topology prediction
programs using the website TOPCONS (http://topcons.cbr.su
.se/) (2) indicated that the protein resides in the membrane
and contains 10 transmembrane helices, as is the case for other
P-type ATPases. The mgtA gene, including sequence encoding
a C-terminal His$_6$ tag, was cloned and expressed in *L. lactis*
using the NICE system (34), and polyclonal antibodies against
the purified protein were generated. Interestingly, the His-
tagged protein was detected only in the membrane fraction of
*L. lactis* and not in the cytoplasm (data not shown). Western
blotting was performed using both D39 and the mgtA mutant
and showed the presence of specific anti-MgtA antibodies (Fig.
3A). Subsequently, both D39 and D39 Δeps were subjected to
fractionation and Western analysis. This clearly showed that
MgtA is associated with the membrane irrespective of the
presence of capsule (Fig. 3B and data not shown). To control
for correct separation of the various fractions, the same Western
blots were also probed using cross-reacting antibodies against
*Staphylococcus aureus* TrxA, a confirmed, conserved
cytoplasmic protein in Gram-positive bacteria (32, 41), which
was associated predominantly with the cytoplasm fraction (data not shown). Thus, MgtA is a membrane protein and is
expressed under normal growth conditions. Considerable ge-
etic variation in *S. pneumoniae* strains has been reported;
however, in all *S. pneumoniae* strains tested, a protein of the
correct size that cross-reacts with the MgtA antisera is pres-
ent (Fig. 3C). Thus, the MgtA protein is conserved and ex-
pressed under normal growth conditions in several *S. pneu-
moniae* strains.

**MgtA is conserved in pathogenic streptococcal species.**
Analysis of the sequenced genomes of several *Streptococcus*
showed that they contain proteins that are highly homologous to
MgtA. Alignment of these sequences with ClustalW (http://
www.ebi.ac.uk/Tools/msa/clustalw2/) (5) indicates that al-
though they are highly similar, all homologues fall into distinct
groups (see the guide tree in Fig. 3D). Therefore, several clinical isolates of streptococcal species were grown under
standard conditions, as was the generally recognized as safe
(GRAS) organism *L. lactis* and some *Staphylococcus aureus*
and *Staphylococcus epidermidis* strains. Interestingly, no cross-
reactive band of the correct height was observed in the staph-
ynylococcal strains or in the *L. lactis* strain (data not shown), but
in all streptococcal species, a cross-reacting band of the ex-
pected size was observed (Fig. 3E). Thus, the MgtA protein, or
at least its key epitopes, appears to be conserved in pathogenic
streptococcal species and seems to be expressed under normal
growth conditions.

**Expression of mgtA does not respond to environmental
Mg$^{2+}$.** In *Salmonella* and *E. coli*, MgtA is important for the
uptake of Mg$^{2+}$ under limiting conditions and its expression is
controlled by the amount of available magnesium (reviewed in
references 18 and 42). To investigate whether this is also the
case in *S. pneumoniae*, a lacZ fusion was generated with the
promoter of SPD1383 in the chromosome of strain D39 using the
pPP2 vector (21). As expected based on the Western blots,
the gene was expressed in GM17. Interestingly, transcription
did not change upon addition of extra Mg$^{2+}$ or Ca$^{2+}$ (Table 3).
This indicated that in GM17, the transporter capacity of the
protein is sufficient for *S. pneumoniae* to maintain a stable level
of intracellular Mg$^{2+}$ and other cations and does not necessi-
tate changes in transcription. Levels of expression in CDM and
CDM with extra Mg$^{2+}$ were similar (Table 3). Addition of extra Ca$^{2+}$ to the CDM induced expression slightly, to approx-
imately 2-fold, which was statistically significant ($P < 0.05$,
Student’s $t$ test) (Table 3). However, the amounts of MgtA
protein under all conditions were similar, and the maximum
fold change in protein levels from levels with no cation addi-
tion was 1.1 times (Fig. 3E). This suggests that in *S. pneu-
moniae*, the amount of MgtA protein is not controlled by the
Mg$^{2+}$ or Ca$^{2+}$ concentration in the environment.

**Deletion of mgtA leads to an increased release of pneumo-
lysin.** Magnesium transporters of many pathogenic bacteria
are involved in the interaction with host cells. Therefore, ad-
hesion to and invasion of HBMEC (52) by the mutant and the
wild type were studied. In an unencapsulated background, an
average 8-fold difference in adhesion between the wild type
and the mutant was observed (data not shown). During the
course of these studies, we observed that, when added in compar-
able numbers, the mutant was more toxic to the cells after
2 h than the wild type. This was the case with both unencap-
sulated (data not shown) and encapsulated (Fig. 4A) strains.
The mutant did not grow in the tissue culture medium (data not shown); thus, it seemed unlikely that increased numbers of
the mutant accounted for the observed toxicity. *S. pneumoniae*
produces a cholesterol-dependent toxin, pneumolysin, which
causes, among other things, pore formation in the membranes
of eukaryotic cells (39). The ply gene was deleted in the mgtA
mutant, and coincubation of this strain with the HBMEC did
not result in any observable toxicity (Fig. 4A), indicating that
the observed toxic effects were indeed due to pneumolysin.
The mutant was able to grow, albeit more slowly than the wild
type, in GM17 (Fig. 1A). Thus, to investigate whether the mgtA
mutant indeed produced or released more pneumolysin, bac-
teria were grown in this medium and the amounts of Ply in the
bacterial cells and the supernatant were determined. The mu-
tant released approximately 4 times more pneumolysin into the
medium in both the early exponential and stationary growth
phases than the wild type (Fig. 4B). Interestingly, the ratio of
extracellular to intracellular pneumolysin was approximately
2-fold higher in the mutant, indicating that the effect is not
simply due to an increase in expression. Transcriptome analysis
(see Table S1 in the supplemental material) indicated that
although the expression of only a few genes was changed more
than 2-fold, the expression of many genes was slightly but
significantly changed in the mutant; many ribosomal genes and
genes involved in amino acid metabolism were downregulated,
clearly indicating a growth defect. Furthermore, deletion of
mgtA had some polar effects on the expression of spd1382
(sp1550) (see Table S1 in the supplemental material). However,
the observed growth defects were due solely to deletion of
spd1383 (Fig. 1C). The expression of ply was not affected. The
mechanism behind the release of pneumolysin in *S. pneu-
moniae* is unclear, and the protein lacks any known secretion
FIG. 3. MgtA is a membrane protein, part of the core genome of *S. pneumoniae*, and conserved in pathogenic *Streptococcus* species. (A) Generation of polyclonal antibodies against MgtA. Western blot analysis of total protein samples of D39 and D39 ΔmgtA with antiserum raised in rabbits inoculated with purified MgtA-His protein. A specific band of around 100 kDa is present in the wild type (wt) but not in the mutant (ΔmgtA). (B) MgtA is a membrane protein. Western blot analysis using the anti-MgtA serum of the supernatant (sup), cytoplasm (cyto), and membrane (memb) fractions of strain D39 grown in GM17. A specific band of around 100 kDa is present in the membrane fraction. (C) MgtA is expressed in various *S. pneumoniae* strains. Western blot analysis using the anti-MgtA antiserum of total protein samples of strains D39, G54, 23F, 670-6B, and TIGR4. (D) Guide tree of Clustal W analysis of the amino acid sequences of MgtA homologues from sequenced *S. pneumoniae* strains and other (pathogenic) streptococcal species. (E) MgtA key epitopes are conserved, and the protein seems to be expressed in various pathogenic streptococcal species. Western blot analysis using the anti-MgtA antiserum of total protein of strain D39, two *Streptococcus mitis* strains, one *Streptococcus sanguis* strain, three *S. pyogenes* strains, and three *Streptococcus agalactiae* clinical isolates. (F) Production of MgtA in D39 grown in CDM and GM17 with the addition of various concentrations of Mg²⁺ and Ca²⁺. "--", no addition; +Mg, with 10 mM MgCl₂ added; +Ca, with 5 mM CaCl₂ added.
signals. Lysis is thought to play a role, especially in stationary phase. To further investigate the mechanism behind the observed increase in pneumolysin, we used the amount of TrxA in the supernatant as a marker for lysis. In exponential and stationary phase, there was an increase in the amount of TrxA in the supernatant of the mutant, which was similar to the increase of Ply (Fig. 4B). Thus, it seems that the increased pneumolysin release is due to a higher rate of lysis in the mutant. The transcriptome analysis showed that the expression of two autolysin-encoding genes, \textit{lytA} and \textit{lytC}, had not changed and that expression of \textit{lytB} was slightly decreased in the mutant (see Table S1 in the supplemental material), indicating that the increased lysis in the mutant is not directly caused by an increase in autolysin gene expression. Thus, deletion of MgtA leads to increased lysis and the release of the cytoplasmic content, including pneumolysin, into the environment.

**DISCUSSION**

Magnesium is an essential factor for all life, including bacteria. Several transporters are known to be involved in Mg\textsuperscript{2+} uptake, and the \textit{S. pneumoniae} genome contains several homologues of these proteins. We identified SPR1410/SPD1383 as an \textit{mgtA} homologue and demonstrated that it is the main Mg\textsuperscript{2+} transporter under \textit{in vitro} growth conditions. Interestingly, the same Mg\textsuperscript{2+}-dependent growth was observed when the gene was deleted in an unencapsulated TIGR4 strain (data not shown). As far as we are aware, this is the first description of a protein involved in Mg\textsuperscript{2+} uptake in streptococci. The published genomes of \textit{S. pneumoniae} contain several other homologues of Mg\textsuperscript{2+} uptake systems, among which are a CorA homologue and several other P-type ATPases; one or more of these are probably responsible for the restoration of the normal growth of the mutant upon the addition of an excess of MgCl\textsubscript{2}.

MgtA appears specific for Mg\textsuperscript{2+}, as the addition of other ions did not restore growth. The observed growth defects \textit{in vitro} strongly suggest that the \textit{mgtA} mutant is attenuated in all aspects of disease because the free Mg\textsuperscript{2+} concentration in various tissues is not enough to support the (robust) growth of the mutant. The TIGR4 homologue of SPR1410/SPD1383, which is SP1551, has been characterized as a Ca\textsuperscript{2+} efflux protein important for survival in the host. In this study, normal growth of the mutant in complex media after overnight incubation and growth inhibition in blood and with high levels of Ca\textsuperscript{2+} were reported; no effect was detected by the addition of

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<th>Condition</th>
<th>Avg (\beta)-galactosidase activity of D39 (±SD) in \textsuperscript{a}</th>
<th>CDM</th>
<th>GM17</th>
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<tr>
<td>No addition</td>
<td>42 (±3)</td>
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<td>10 mM MgCl\textsubscript{2}</td>
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<td>5 mM CaCl\textsubscript{2}</td>
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<td>41 (±13)</td>
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\textsuperscript{a} Results are the averages of results from two experiments with two independent transformants ± the standard deviations. * \(P < 0.05\) by Student’s \(t\) test.

![FIG. 4. Deletion of \textit{mgtA} leads to an increase in pneumolysin release into the environment. (A) The \textit{mgtA} mutant has an increased toxicity to HBMEC. Confluent HBMEC layers were incubated with approximately 1 \times 10\textsuperscript{7} CFU of D39, D39 \textit{ΔmgtA}, and D39 \textit{ΔmgtA Δply}. The pictures are taken after 2 h of incubation; at the start of the experiment, the cells in each well looked similar (data not shown). Incubation with D39 \textit{ΔmgtA} led to increased toxicity compared to that of the wild type, as is evidenced by the shrinking and rounding of the HBMEC and the disruption of the monolayer. This was not observed in the D39 \textit{ΔmgtA Δply} mutant. (B) Increased pneumolysin release in the medium by the mutant. The D39 (W) and D39 \textit{ΔmgtA} (M) strains were grown in GM17 and supernatant (sup), and cells were harvested at the exponential and stationary phases. Samples were subjected to Western blot analysis using anti-Ply and a cross-reactive \textit{S. aureus} anti-TrxA antiserum, and signals on the Western blot were quantified using ImageJ.](image-url)
Mg$^{2+}$ (48). In our study, the growth of the mutant was impaired even under low-Ca$^{2+}$ conditions. The slight but significant downregulation of many ribosomal and metabolism genes observed in the transcriptome analysis reinforces the observation that the mutant has a growth defect. However, the addition of Mg$^{2+}$ was able to rescue the mutant even in the presence of elevated Ca$^{2+}$ levels (Fig. 1D and 2). Several cations had an inhibitory effect on the growth of the mutant under high-Mg$^{2+}$ conditions, indicating either that the protein is also involved in the transport of these cations or that the residual uptake of Mg$^{2+}$ by other transporters is inhibited by these cations. Genetic differences between the *S. pneumoniae* strains used might account for these dissimilar findings, as has been reported before for other genes (4, 40). An alternative explanation might be that deletion of mgtA induces the transcription and/or activity of another transporter with a high affinity for calcium, which causes the observed phenotype. However, a more likely hypothesis that unites all reported findings is that the SPR1410/SPD1383/SP1551 protein is a Ca$^{2+}$/Mg$^{2+}$ antiporter. This hypothesis had been proposed before for the *Salmonella* MgtB protein (38) but was thought to be incorrect because although Ca$^{2+}$ was able to inhibit the growth of a strain containing only MgtB (24), addition of Ca$^{2+}$ did not inhibit the transport of $^{25}$Mg$^{2+}$ by either MgtA or MgtB (50). The observation that, in *S. pneumoniae*, addition of Ca$^{2+}$ severely affects the restoration of growth of the mutant in the presence of high levels of Mg$^{2+}$ suggests a function as an antiporter.

Expression of the gene did not alter significantly upon a 20-fold change in Mg$^{2+}$ and increased only 2-fold upon a 25-fold change in Ca$^{2+}$ concentration in CDM (Table 3). Furthermore, this did not result in a corresponding 2-fold change in the amount of protein (Fig. 3E), suggesting that protein levels might be fairly constitutive. The expression also did not seem to be induced by Zn$^{2+}$ stress (29). In Gram-negative bacteria, the response to Mg$^{2+}$ is under complex regulatory control, which is mediated by, among other things, the two-component system PhoPQ, an important virulence determinant (18). Little is known about the (regulation of) Mg$^{2+}$ homeostasis in Gram-positive bacteria. In *Streptococcus pyogenes*, the CovRS (CsrRS) two-component system responds to Mg$^{2+}$ and is also an important virulence determinant (19, 20). However, the published genomes of *S. pneumoniae* do not seem to contain a homologue of either PhoPQ or CovRS. In *S. pneumoniae*, the TCS04 two-component system has been reported to respond to a metal ion, Mn$^{2+}$, but it does not regulate mgtA (40). Interestingly, in *Streptococcus mutans*, the CiaRH two-component system seems to be autoregulated by Ca$^{2+}$ (23). The *S. pneumoniae* CiaRH homologue has been extensively studied, but mgtA does not seem to be part of its regulon (22). Both MgtA and MgtE have been reported to be under the control of riboswitches (7, 8). The SPR1410/SPD1383/SP1551 gene seems to have a 5′ untranslated region (UTR), and analysis using the BLISS tool (http://bliss.biology.yale.edu/) (6) indicated that it might contain a riboswitch that is dissimilar to the one controlling MgtE. Thus, at present, if or how mgtA is regulated remains unclear, but it seems likely that its regulator will have a large impact on the virulence of this bacterium.

Notably, deletion of mgtA led to increased lysis of the bac-

terium and a subsequent increased release of pneumolysin, leading to cell toxicity. The transcriptome analysis did not indicate that this increased lysis was due to an increase in the expression of the autolysin genes. However, the activities of the autolysins might be altered in the mutant, or the structural integrity of the cell wall might be weakened due to smaller amounts of Mg$^{2+}$.

The protein could be detected in all *S. pneumoniae* strains tested, and analysis of the genome sequences showed that homologues were present in each *S. pneumoniae* strain sequenced so far, which underlines the idea that the gene is indeed part of the proposed core genome of *S. pneumoniae* (44). In addition, the MgtA proteins of other (pathogenic) streptococcal species are highly homologous (Fig. 3D), and the antiserum recognized the key epitopes of the MgtA protein in a protein of similar size produced during normal growth conditions in several pathogenic streptococcal strains, suggesting that the function of this protein is conserved in these species as well.

The data presented in this study and by others (48) suggest that inhibition of MgtA through antimicrobials in *S. pneumoniae* severely impairs the ability of the bacteria to proliferate in the human host and to cause serious invasive disease. Together with its location in the membrane and its large degree of homology among various *S. pneumoniae* strains and pathogenic streptococcal species, the protein has all the characteristics of a promising candidate for incorporation into protein vaccines and/or of a target for the development of antimicrobial drugs. However, it also seems to play a role in the prevention of pneumococcal lysis and the subsequent release of the cytoplasmic content, including pneumolysin, which could create adverse effects in the host when the protein is inhibited in wild-type strains during infection. This illustrates the facts that proteins that are conserved, conditionally essential for pathogens, and membrane accessible might be ideal candidates for new antibacterial therapies but that possible adverse side effects of their deletion/inhibition need to be carefully investigated.

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