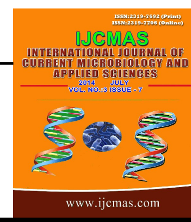

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 7 (2014) pp. 83-92**
http://www.ijcmas.com

Original Research Article

Characterization of a magnesium transport in *Campylobacter jejuni* and the role of CorA in magnesium uptake

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Keywords

Campylobacter, magnesium transport, CorA

A B S T R A C T

Magnesium (Mg^{2+}) is the most abundant divalent cation in prokaryotic that involved in many cellular functions. CorA (Cj0726C), the main magnesium transporter of *C. jejuni* was inactivated by allelic exchange to determine whether magnesium transport is vital for the growth of *C. jejuni.* The *corA* mutant did not grow without Mg^{2+} supplementation (20 mM), indicating that Mg^{2+} acquisition by *corA* is essential for *C. jejuni* growth *in vitro* and that it might play a key role in adaptation to low-Mg^{2+} conditions. In addition, this phenotype suggests that CorA is the primary Mg^{2+} transporter. CorA is likely to be important for colonisation and thus a CorA deficient strain may form the basis of possible vaccine strains of *C. jejuni*.

Introduction

Magnesium (Mg^{2+}) is the most abundant divalent cation in prokaryotic and eukaryotic cells. It is involved in many cellular functions including modulating signal transduction, energy metabolism and cell proliferation. Mg^{2+} is important in bacterial growth and is a cofactor in ATP-requiring enzymatic reactions (Reinhart, 1988). It is also directly involved in membrane stability (Nikaido andVaara, 1985).

There are three classes of magnesium transporter that have been characterized in bacteria and archaea, namely MgtE, CorA and MgtA/B (Kehres& Maguire, 2002).

Silver and colleagues first identified the genetic locus for the CorA transporter in *Escherichia coli* back in 1969 (Silver, 1969). CorA is now known to be an inner membrane protein that is present ubiquitously in prokaryotes and known to transport divalent metal ions. CorA is present in almost all bacteria and the archaea with the exception of only a few species, generally those with the smallest genomes, and in these the Mg^{2+} transporter system is replaced by MgtE (Kehres*et al.*, 1998). Because of its ubiquitous distribution, CorA is believed to be the primary Mg^{2+} transporter present in these two kingdoms.

However, multiple CorA-like sequences are present in some species (Smith & Maguire 1995; Kehres*et al*., 1998). A strain of *Salmonella enterica*serovarTyphimurium (*S*. Typhimurium), that lacks CorA, MgtA and MgtB and thus all Mg^{2+} transporters requires supplementation with 100 $mMMg^{2+}$ to grow (Hmiel*et al*., 1986, 1989; Snavely*et al*., 1989, 1991).

CorA has been demonstrated to be conserved among different strains of various Gram negative bacteria.In bacteria such as *H. pylori*, *S*. Typhimurium and *E. coli*, this protein has been extensively studied and in these bacteria, CorA is the major Mg^{2+} transporter and is also known to mediate the influx of Mg^{2+}, Co^{2+} and Ni^{2+} (Hmiel*et al*., 1986; Snavely*et al*., 1989). CorA was first cloned from *S*. Typhimurium in 1985 by Hmiel*et al*. (Hmiel*et al*., 1986). In *S*. Typhimurium, CorA is required for full virulence in a mouse model although other Mg^{2+} transport systems are also present (Papp-Wallace *et al*., 2008).

An analysis of the *C. jejuni*NCTC 11168 genome sequence showed it to encode a homolog of CorA (Cj0726c) but this transporter homologue has not been studied so far. Comparison of the NCTC 11168 sequence with that of 81-176 and NCTC 81116 confirmed the high degree of conservation of CorA within *C. jejuni.C. jejuni*NCTC 11168 CorA shares 99%, 99%, 48%, 31%, 30% and 30% identity with the amino acid sequences of *C. jejuni*81-176, *C. jejuni*81116, *H. pylori*, *E. coli*, *S*. Typhimurium and *B. subtilis*respectively (Table 1).

Materials and Methods

Bacterial strains, plasmid and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 2. *C. jejuni* NCTC 11168 and *E. coli* DH5α were obtained from the National Collection of Type Cultures (Colindale, London) and Life Technologies, UK respectively.

Bacterial culture preparation

All strains were stored in Microbank™ (Pro-Lab Diagnostics, UK) vials at -80°C until required. Campylobacters were grown routinely onto Mueller-Hinton agar (MHA, Oxoid) in microaerobic atmospheres generated using the Campygen (Oxoid) gas generating pack (5% O^2, 10% CO^2 and 85% N^2) at 37°C for 48 hours.For cloning experiments, *E. coli*DH5α was grown at 37°C in Luria–Bertani (LB) broth or on agar aerobically for 24 hours at 37°C. When required, antibiotic supplements were aseptically added to warm sterile media at the following concentrations; ampicillin 100 $\mu g\ ml^{-1}$ and kanamycin 50 $\mu g\ ml^{-1}$ for plasmid maintenance. All antibiotics were supplied by Sigma-Aldrich® (UK).

Construction of a *C. jejuni* Cj0726C (CorA)-deficient mutant

DNeasy® Blood & Tissue Kit (QIAGEN) was used to extract the genomic DNA from *C. jejuni* NCTC 11168 and the method was carried out as specified in the kit protocol.The *corA*gene (Cj0726C) was amplified from the genomic DNA by PCR using the forward primer 5’-GCCCAAGTATCCTTCCAGTTTG – 3’ and reverse primer 5’-CCCTAAGCGATAGAGCAAGTAGTGG – 3’. PCR reactions were performed using the Applied Biosystems 2720 thermal cycler which involved an initial denaturation period of 5 minutes at 95°C, 30 cycles of 45 seconds at 95°C to denature, 45 seconds at 55°C to anneal and 2 minutes at 72°C for extension with final extension period of 10 minutes at 72°C. The oligonucleotide

primers used for PCR were synthesized by Sigma-Aldrich, UK. The resulting 1331 bp PCR fragment was cloned into the TA cloning vector pGEM®-T Easy Vector System I (Promega, USA)and transformed into chemically competent *E. coli* DH5α.Cells containing the vector were selected using LB agar plates supplemented with 100 µg/ml ampicillin, X-gal and IPTG. White colonies were picked and a rapid plasmid preparation and PCR were carried out to screen all the colonies for the possession of the *corA*gene. The plasmid was designatedpEM1.The resulting plasmidwas digested with *BglII* and ligated to the 1600 bp kanamycin resistance gene cassette excised by *BamHI* digestion from pJMK30 (Baillon *et al*., 1999) and transformed into *E. coli* DH5αresulting in the suicide plasmid pEM2. pEM2 containing *corA* and a kanamycin resistance gene cassette was introduced into *C. jejuni* NCTC competent cells via electroporation at 2.5 kV, 25µF and 200Ω. The homologous recombination that resulted in a double cross-over event was verified by PCR and restriction digestion with *PstI* (Results not shown). Only cells, which had taken up the plasmid, were able to grow on MHA (+20 mM $MgCl_2$) supplemented with 50 µg/ml kanamycin as pEM2 contains a gene which encodes for kanamycin resistance, thus enabling selection of the *C. jejuni cor A* mutant. The resulting mutant was designated CJEM1.Putative mutants were selected and the chromosomal DNA was extracted from them using the Qiagen kit so that they could be rapidly screened by PCR to confirm the occurrence of allelic exchange by double homologous recombination.

Growth requirements of *C. jejuni cor A* mutant

The growth requirements of the *C.jejuni cor A* mutant was initially investigated by assessing growth on solid agar (MHA) to which 20 mM of magnesium chloride ($MgCl_2$) had or had not been added. The *C. jejuni cor A* mutant and wild type were streaked onto MH plates with and without 20mM $MgCl_2$ and the plates incubated for 48 hours at 37°C under microaerophilic conditions and a visual observation of colony growth made.

Growth assay of *C. jejuni*wild type and the *C. jejuni cor A mutant* in the presence or absence of magnesium

The growth curves of the *C. jejuni* wild types and corresponding *cor A* strains were determined. Both *C. jejun i*wild type strains and *C. jejuni cor A mutants* were grown on MHA plates for 48 hours prior to the start of the experiment on media with and without 20 mM $MgCl_2$ supplementation respectively. The cells were then harvested and grown in Mueller-Hinton broth with and without 20 mM $MgCl_2$ and were incubated microaerobicallyfor 24 hours. After 24 hours, MH broth (100 ml) containing 5 mM $MgCl_2$, 10 mM $MgCl_2$, 20 mM $MgCl_2$ and no added $MgCl_2$ was added to filtered tissue culture flasks. One ml aliquots of the *C. jejuni* wild type and *corA* strain cultures at OD600 0.6 were inoculated into the flasks and all flasks were incubated microaerobically at 37°C for approximately 5 days. The absorbance was determined at each sampling time. All experiments were done in triplicate.

Growth experiments using different cations other than Mg

Growth of the *cor A* mutant was assessed with cations other than Mg, to confirm that it was Mg and not the chloride anion that allowed the growth of the *cor A* mutant. The growth experiment was carried out using 20 mM $CaCl_2$, 20 mM $NaCl_2$, 20 mM $MgCl_2$ and only MH broth as a control. One ml of cells with the OD_{600} of 0.6 were added as in

previous experiments. All flasks were incubated microaerobically at 37°C. This experiment was performed in triplicate.

Results and Discussion

Transformation and PCR of CJEM1

Figure 1shows PCR products of the *C. jejuni cor A mutant* and that of the wild type. The wild type PCR product showed a 1331 bp product, a size which was similar to that when *corA*gene fragment was amplified initially, whereas the *C. jejuni cor A mutant* showed a 2931 bp product, which showed that the *corA*gene fragment had been inserted with the 1600 bp kanamycin fragment, thus confirming the genotype of the mutated strain (CJEM1). A diagrammatic presentation of CJEM1 is shown in Figure 2.

Growth of the *C. jejuni cor A mutant* on $MgCl_2$ plates

The mutant did not grow on MH plates without 20 mM $MgCl_2$ supplementation, but grew on the MH plates supplemented with $MgCl_2$, indicating that *corA*plays an important and major role in magnesium transport in *C. jejuni*(Fig.3).

Growth of the *C. jejuni cor A mutant*

The Mg^{2+} requirement caused by the *corA*mutation was further investigated by growth experiments using various concentrations of $MgCl_2$. The optical density (OD) reading of the *C. jejuni cor A mutant* when growing in media supplemented with 10 mM and 5 mM $MgCl_2$ was about 50% less than the absorbance reading of the wild type with and without the addition of $MgCl_2$ (Fig. 4), and thus after 56 hours of growth, $MgCl_2$ concentrations of less than 20 mM in MH broth do not restore full growth in the mutant. In addition, supplementation of MH broth with 20 mM $MgCl_2$ enabled the *cor A mutant* to grow in a manner identical to that of the wild type. In contrast, unlike the wild type, the *cor A mutant* did not grow in unsupplemented broth, thus further confirming the role of CorA in magnesium transport.

Survival and growth of *C. jejuni* and the *corA* mutant in media supplemented with alkaline earth cations other than $MgCl_2$

The survival and growth of *C. jejuni* and the *cor A mutant* was tested in the presence of other chloride compounds other than $MgCl_2$ to determine whether it was the magnesium ions or the chloride anion that were influencing the growth and survival of the *corA* mutant. As can be seen from Figure 5, there was no growth of the *C. jejuni corA* mutant when supplemented with other chloride compounds, namely calcium chloride ($CaCl_2$) and sodium chloride ($NaCl_2$). The near zero absorbance readings (OD_{600}) measured in the absence of $MgCl_2$ indicates that it must be the provision of magnesium ions alone that give rise to the growth of the *corA* mutant. However, when the mutant was treated with 20 mM $MgCl_2$, the growth was similar to that of the wild type. Two-tailed *t* test reported no statistical difference of the growth between the *corA* mutant supplemented with 20 mM $MgCl_2$ and the parental strain without any supplementation.

A molecular biological approach was used to generate a mutant deficient in CorA, the main magnesium transporter of *C. jejuni*. CorA is a 37-kDa integral membrane protein that forms the primary constitutive Mg^{2+} uptake system in many bacteria and some Archaea(Moncrief& Maguire, 1999). Initially, PCR was performed to extract a *corA*gene fragment from *C. jejuni* NCTC 11168.

Table.1 Conservation with *C. jejuni*NCTC 11168 CorA protein sequence

CorAorthologues	Overall % identity (Similarity)	Blast E value
*C. jejuni*81-176	99% (100%)	0
*C. jejuni*81116	99% (99%)	0
H. pylori 26695	48% (69%)	1e-105
E. coli K-12	31% (53%)	3e-47
S. Typhimurium LT2	30% (53%)	3e-46
*B. subtilis*168	30% (51%)	3e-10

Table.2 Bacterial strains and plasmids used in this study. amp^r, ampicillin resistance; kan^r, kanamycin resistance

Strain or Plasmid	**Characteristics**	**Source or reference**
Strain		
NCTC 11168	Genome Strain	National Collection of Type Cultures
CJEM1	11168: *corA*::kan^r	This study
E. coli DH5α	F^- φ80dLacZ ΔM15	Life Technologies, UK
Plasmid		
pGEM®-T Easy	Cloning vector amp^r	Promega,USA
Plasmid pJMK30	*C. coli* kan^r cassette in pUC19	Baillon*et al*., (1999)
Plasmid pEM1	1331-bp PCR product containing *corA*in *pGEM®-T Easy* cloned into *E. coli* DH5α	This study
Plasmid pEM2	Kan^r cassette cloned into a generated *BglII*site in the *corA*in pEM1, cloned into *E. coli* DH5α	This study

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Fig.1 Gel electrophoresis were run at 8V/cm for 1.5 hours using 1% agarose gel stained with GelRed^{TM} showing PCR products obtained from a wild type strain and a suspected *C. jejuni corA* mutant. PCR was carried out using primer Mut 1 and Mut 11. Lane 1 shows the *corA* mutant of *C. jejuni* with a size of 2931 bp. Lane 2 shows the *corA* gene fragment from the wild type strain showing a 1331 bp product. The 1600 difference in size between the PCR products from the *corA* mutant and the wild type confirmed the genotype mutated *C. jejuni corA* strain. Lane M showed a 1.0 kb DNA ladder marker.

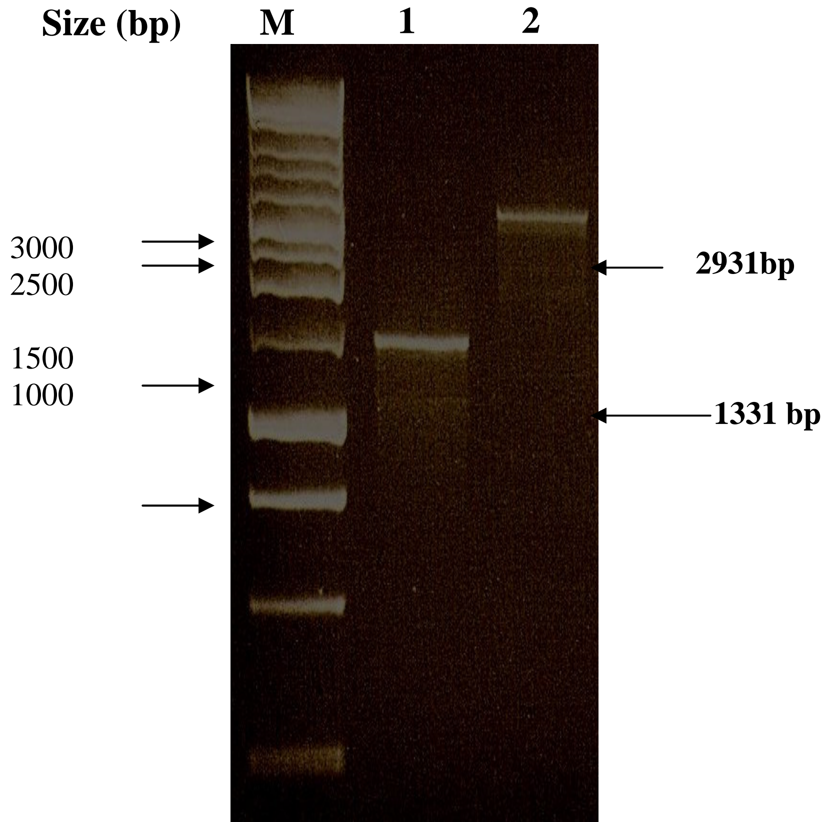


Fig.2 A diagrammatic presentation of the integrational plasmid pEM2 containing the truncated *corA*fragment and the kanamycin resistant marker

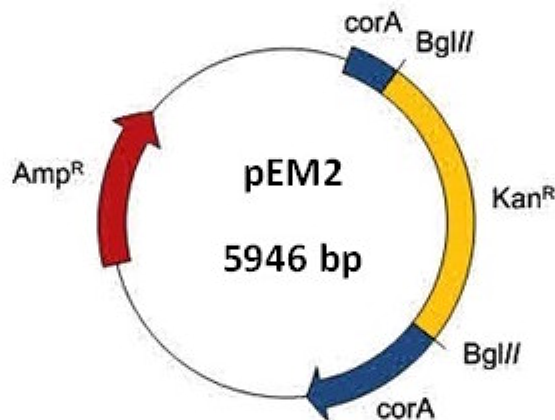


Fig.3 The *C. jejuni corA* mutant showing no growth when inoculated onto an MH plate without 20mM $MgCl_2$ (A) and growth of *C. jejuni corA* mutant when grown on a MH plate supplemented with 20 mM $MgCl_2$ (B).

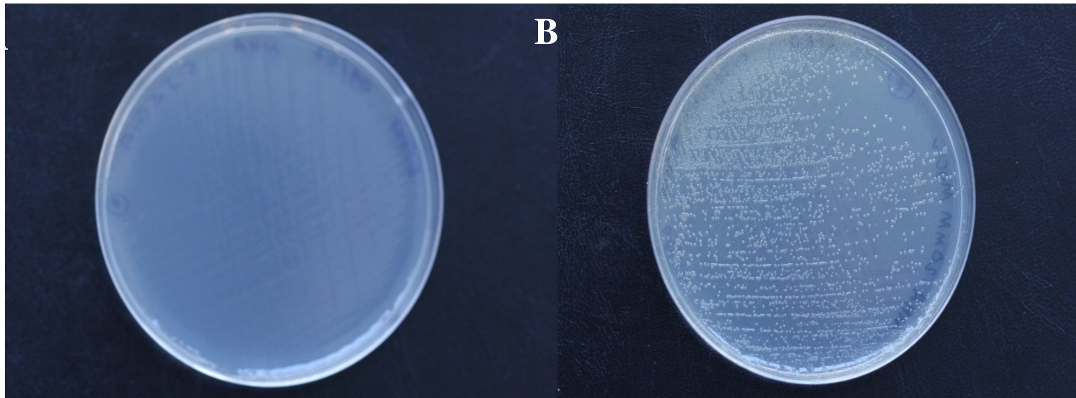


Fig.4 The growth of the *C. jejuni corA* mutant and wild type in MHB with and without the the addition of $MgCl_2$. *C. jejuni corA* mutant was incubated microaerophically with shaking at 150 rpm at 37°C with no additions of $MgCl_2$ (*), or to which 5 mM (•), 10 mM (+) and 20 mM (-) $MgCl_2$ was added to the broth. In addition, wild type *C. jejuni* growth was also assessed as a control with the addition of 5 mM (■), 10 mM (▲) and 20 mM (×) MgCl2 or no addition (♦) respectively. Growth was assessed at regular interval using optical density (OD600). Data shown are a mean of triplicates. Bars indicate one standard error of the mean.

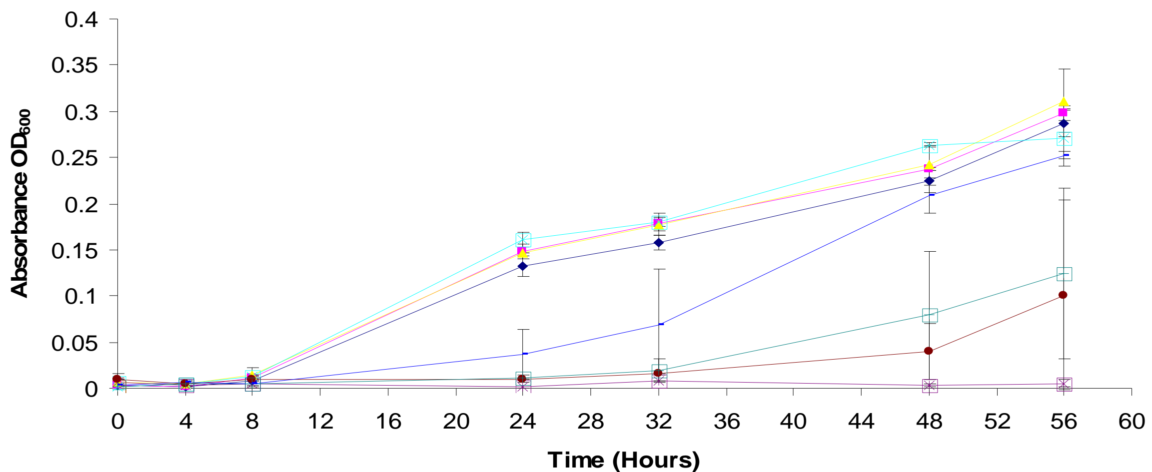
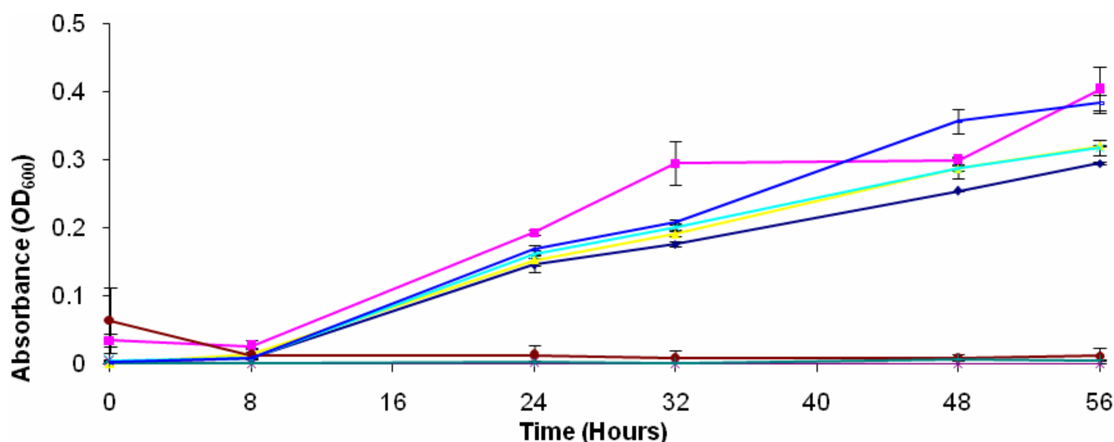


Fig.5 The growth of the *C. jejuni corA* mutant and the wild type in MHB with and without the addition of alkaline earth cations. *C. jejuni corA* mutant was incubated microaerophically, with shaking at 150 rpm at 37°C with no additions of $MgCl_2$ (x), or to which 20 mM of $CaCl_2$ (•), $NaCl_2$ (+) and $MgCl_2$ (-) was added to the broth respectively. In addition, wild type *C. jejuni* growth was also assessed as a control with no addition of $MgCl_2$ (♦), or to which 20 mM of $CaCl_2$ (■), $NaCl_2$ (▲) and $MgCl_2$ (×) were added to the broth respectively. Growth was assessed at regular intervals using optical density (OD_{600}). Data shown are a mean of triplicates. Bars indicate one standard error of the mean. Statistical evaluation was done using two-tailed Student's t test.



The purified PCR product was then ligated into the T-cloning vector, pGEM®T-Easy Vector System I (Promega) to generate pEM1. This plasmid was chemically transformed into *E. coli* DH5α and transformants selected on LB agar plates containing ampicillin.

Plasmid DNA, isolated from antibiotic resistant transformants, was found to contain a 1.3 kb insert, which corresponds to the size of the original *corA*PCR product. To construct *corA*-deficient mutants, the structural *C. jejuni corA*gene present in pEM1 was disrupted by the insertion of a 1.6 kb *BamHI*kanamycin-resistance cassette into the unique *BglII*site at position 252 within the *corA*gene. To achieve the inactivation of *corA*, the plasmid pEM2, which carried a mutated *corA*gene with an inserted kanamycin fragment, was introduced into *C. jejuni*NCTC 11168, by electroporation. During the selection process, MH agar was supplemented with 20 mM Mg^{2+} to avoid possible killing of *corA*mutants by Mg^{2+} depletion.

After PCR analysis revealed the correct insertion of the kanamycin cassette in the *corA*gene, the phenotype of this mutant was further investigated. To examine the phenotype of the kanamycin-resistant colonies, magnesium transport activity was assessed using the qualitative plate assay with and without Mg supplementation. The *C. jejuni cor A mutant* was grown on MH agar with and without 20 mM Mg^{2+} supplementation to assess whether the mutant could grow without magnesium supplementation.

The *C. jejuni cor A mutant* only grew in the supplemented agar media, providing evidence that CorA is the major magnesium transporter in *C. jejuni*. This also indicates that transformants exhibited a *corA*-negative phenotype consistent with allelic exchange resulting from a double crossover recombinational event. During the selection process, some of the transformants appeared to be the *corA*-positive transformants (data not shown) which is likely caused if integration of pEM2 had occurred via a single crossover which would regenerate an intact copy of the *corA*structural gene (Dickinson *et al*., 1995).

The inability of this strain to grow in media without Mg^{2+} supplementation demonstrates that CorA is essential for Mg^{2+} acquisition and most likely should be required for survival in low Mg^{2+} environments, such as the gut. Some 24-75% of the 300 mg Mg^{2+} consumed daily in the human gut is absorbed by the human intestine which suggests that a relatively low concentration of Mg^{2+} exists in the intestine (Kayne& Lee, 1993). Under this condition, it seems very unlikely that the *C. jejuni cor A mutant* would persist in the human or poultry gut for an extended period of time. The inability of the *cor A mutant* to grow within the gut in the absence of magnesium supplementation may provide the basis of potential future attenuated vaccine strains. The growth of the *cor A mutant* was further assessed using concentrations lower than 20 mM $MgCl_2$.

The growth assay revealed that even with the provision of 5 mM and 10 mM $MgCl_2$, the growth of the *cor A mutant* was still restricted and only the addition of 20 mM of Mg^{2+} could restore the growth to wild type levels. The complete growth deficiency in media without Mg^{2+}supplementation and the requirement for 20mM magnesium supplementation displayed by *corA*mutants indicates that *C. jejuni cor*A is essential for Mg^{2+} acquisition and is required for growth in low Mg^{2+} environments.

Acknowledgment

We thank the Ministry of Higher Education of Malaysia in collaboration with *UniversitiSains Malaysia*for funding this research.

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