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Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH-1) Is Not Required For Optimal *In Vitro* Growth Of *Neisseria meningitidis* Serogroup B

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Abstract: The present study was aimed to investigate whether the GapA1, a so-called housekeeping enzyme, is required for maximal *in vitro* growth of *N meningitis*. To achieve the goals, a GapA1 knock-out mutant was created in *N. meningitidis* using an inverse PCR and allelic exchange methodology. Phenotypic analysis of GapA1-deficient mutant strains such as growth profiling experiments showed that the GapA1-deficient mutant grew at the same rate (in broth culture and on solid media) as the wild-type strain, suggesting that GapA1 is not required for maximal growth of *N. meningitidis* under the *in vitro* conditions tested. Visual inspection of colony morphology or bacterial cell morphology using light microscopy showed no difference between wild-type and GapA1 deficient mutant strains.In summary, despite being a central enzyme in the glycolytic cycle, GapA1 is not required for *in vitro* growth of *N. meningitidis* sero-group B suggesting its additional (moonlighting) roles in pathogenesis of meningococcal disease.

Keywords: *Neisseria meningitidis*, GapA1, Mutagenesis, Growth Kinetics, Glycolytic Cycle

1. INTRODUCTION

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a classical cytoplasmic glycolytic enzyme found in all organisms studied so far. (Kim and Dang, 2005). In addition to its well-established metabolic function, recent studies have demonstrated unexpected, non-glycolytic biological functions of GAPDH in physiological and pathological processes (Kim and Dang, 2005). Glycolytic enzymes, which along with other enzymes involved in central metabolism, are sometimes called 'housekeeping enzymes' are classical cytoplasmic proteins that are found in all cell types. These enzymes are known to be constitutively expressed and localised mainly to the cytoplasm of the cell. There is growing evidence that several so-called housekeeping enzymes, including fructose bisphosphate aldolase (FBA) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), are often localised to the surface of bacterial pathogens. Whilst their intracellular functions are well known, it is unclear whether they perform any additional functions on the bacterial surface.

The localisation of GAPDH on the surface of a pathogen was first described in the Gram-positive pathogen, *S. pyogenes* (Lottenberg *et al.,* 1992; Pancholi 2003), and was shown to bind several proteins including the membrane protein on pharyngeal cells (uPAR/CD87) (Jin *et al.,* 2005; Lottenberg *et al.,* 1992; Winram and Lottenberg, 1996), and its involvement in intracellular host cell signaling events (Pancholi and Fischetti, 1997). GAPDH was also demonstrated on the surface of other Gram-positive bacteria including staphylococci (Modun and Williams, 1999), *S. agalactiae* (Seifert *et al.,* 2003) and *Listeria monocytogenes* (Schaumburg *et al.,* 2004). In addition, GAPDH has been reported to be expressed on the surface of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli*. In these organisms, GAPDH has been demonstrated to bind to human plasminogen and fibrinogen, thus suggesting a possible role in pathogenesis (Egea *et al.,* 2007). Surface-localised GAPDH of *Mycoplasma genitalium*, plays role in adhesion to human mucin (Alvarez *et al.,* 2003) whereas in *Lactobacillus plantarum*, a human gastrointestinal tract pathogen, GAPDH was demonstrated to be involved in adherence to gastric mucin and Caco-2 cells (Kinoshita *et al.,* 2008; Ramiah *et al.,* 2008).

GAPDH has also been shown to be expressed on the cell surface of fungi, for example, the cell wall associated GAPDH of *Candida albicans* was shown to be involved in mediating adhesion to fibronectin, laminin and plasminogen (Gil-Navarro *et al.,* 1997; Gozalbo *et al.,* 1998; Jonathan *et al.,* 2003). Hence, GAPDH is a multi-facet protein present on the surface of several Gram-positive pathogens and fungi and may act as virulence factor (Egea *et al.,* 2007). The aim of this study was to characterize the role of GAPDH-1 in *N. meningitidis* serogroup B strain MC58.

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2. MATERIALS AND METHODS

2.1 Bacterial strains, growth condition, and media

Bacterial strains and plasmids used in this study are listed in (**Table 1).** *E. coli* strains were routinely grown at 37°C in LB broth or on LB agar. Where appropriate, antibiotics were used at the following concentrations: ampicillin (100 µg ml^{-1}), kanamycin (30 µg ml^{-1}). Meningococcal strains (Table 1) were grown on Brain Heart Infusion (BHI) agar and/or Brain Heart Infusion Broth or Muller-Hinton Agar (MHA) or Muller-Hinton Broth (MHB) or Dulbecco's Modified Eagle Medium (DMEM) supplemented with Vitox (Oxoid) at the concentration suggested by the manufacturer (Oxoid) or 2% Fetal calf serum [FCS] Invitrogen) or 10% Human serum and where appropriate kanamycin (50 µg ml-1) were used to select mutant strains and incubated in an atmosphere of 5% CO2 at 37°C with or without shaking. Where appropriate, blue/white selection of transformants in cloning experiments was achieved using IPTG (Roche) and X-gal at 0.5 mM and 80 µg ml-1, respectively. All liquid cultures were aerated by agitation at 200 revolutions per minute (rpm) in a shaking incubator.

2.2 DNA manipulation techniques

Chromosomal DNA of *N. meningitidis* was prepared by using a DNeasy Blood & Tissue kit (Qiagen) using the protocol for bacterial cells recommended by the manufacturer. The plasmid purification was achieved from 5 ml overnight cultures of appropriate *E. coli* strain using a QIAprep Spin Kit (Qiagen) according to the manufacturer's instructions. *Taq* polymerase, Restriction enzymes and T4 DNA ligase were purchased from Roche, UK. All enzymatic reactions were carried out according to the manufacturer's instructions. The concentration of purified plasmid and genomic DNA was quantified using a NanoDrop (ND-1000) spectrophotometer (Agilent Technologies). DNA sequencing was carried out at the School of Biomedical Sciences (University of Nottingham) on an ABI 377 automated DNA sequencer.

2.3 Polymerase chain reaction (PCR) and agarose gel analysis.

All PCR reactions were performed in a 25 µl final volume using sterile 0.2 ml thin-walled PCR tubes and cycled using a C1000 model Thermal Cycler (BIO-RAD). An agarose gel (0.8-1%) was prepared according to the standard protocol. DNA ladders 2-Log (New England BioLabs) and 0.5 kb (Fermentas) were run alongside the samples to enable analysis of DNA fragment size in the samples. Gels were viewed using an Uvitec gel documentation system.

2.4 Engineering constructs by Inverse PCR (i-PCR) for GapA1 mutagenesis.

A *ca*. 3-kb region of DNA encompassing GapA1 and 1-kb flanking DNA from either side of the gene, which contained a copy of neisserial uptake sequence, was amplified by PCR using the primer pair GapA1 (F) FL5'-CCAAACCCAATGCCGCGAATG-3' and GapA1 (R) FL 5'-GAGAACTGTCATGCGTA TTCC-3' and chromosomal DNA prepared from *N. meningitidis* strain MC58. The PCR was performed with the setting as: annealing at 48°C for 1 min, primer extension at 68°C for 3.5 min, and 94°C for 45s, with final incubation at 48°C for 1 min and 68°C for 10 min. The desired PCR product was Gel-purified using a Gel Extraction Kit (Qiagen) according to manufacturer's instructions. The PCR product was then cloned into the pGEM-T Easy vector (Promega, UK). Putative transformants were identified as white colonies on selective agar plates containing X-gal and IPTG. Recombinant clones were verified by restriction digestion analysis and PCR using the primer pair GapA1 (F) FL and GapA1 (R) FL.

The plasmid pSAT-6 (Table 1) was used as a template DNA in i-PCR using the primer pair GapA1_M1 (IF) 5'-GCGAGATCTGCAACAAACC GTC-3' and GapA1_M2 (IR) 5'-GCGAGATCTGGT TTGTTCCTTT GTTGAGGG-3' and chromosomal DNA of *N. meningitidis* strain MC58. The PCR setting were as follows: annealing at 48°C for 1 min, primer extension at 68°C for 5 min, and 94°C for 45 s. The i-PCR product was Gel-purified and digested with *Bgl*II for 1h at 37°C and then overnight at 25°C to achieve complete digestion. The *Bgl*II-digested i-PCR product was then self-ligated using T4 DNA ligase to generate pSAT-7 (Table-1). The plasmid pSAT-7 was purified and subsequently digested with *Bgl*II and ligated to 1.5-kb antibiotic cassette (encoding resistance to kanamycin). The resulting plasmid was designated pSAT-8.

2.5 Preparation of selectable marker.

The antibiotic cassette encoding resistance to kanamycin (1.5 kb) was obtained by restriction digestion of pJMK30 plasmid (Table 1) with *Bam*HI followed by preparative gel electrophoresis and gel extraction. The purified kanamycin resistance cassette (Kan^R) was then ligated to linearized pSAT-7 to yield pSAT-8 (Table 1).

2.6 Mutagenesis by natural transformation and allelic exchange in *N. meningitidis*.

N. meningitidis strain MC58 cells were grown into BHI broth supplemented with Vitox and incubated

overnight in 5% CO2 at 37°C. The following day, overnight broth cultures were diluted 1:20 in fresh BHI broth and grown to an optical density of 0.2. An aliquot of 0.2 ml of the culture was transferred to a sterilized 15-ml tube with conical bottom containing 1.5 ml of BHI agar supplemented with Vitox. After incubation for 5-6 hrs at 37°C in 5% CO_2 without shaking, 10 µl (*ca.* 1 µg) of the pSAT-8 (mutagenic plasmid DNA) was added to the culture tubes and incubation was continued for 16 h. The putative transformants were selected on BHI containing kanamycin 50 µg ml^{-1}. Kanamycin-resistant *N. meningitidis* colonies were obtained and further analyzed by PCR and immunoblotting of whole cell extracts of the putative mutants.

2.7 Determining *in vitro* growth characteristics.

Meningococcal growth characteristics in liquid cultures were conducted by re-suspending (1:100) overnight cultures of *N. meningitidis* in 10 ml of MH broth with and without human serum/FCS, and in DMEM with and without human serum/FCS. The following day, overnight broth cultures were diluted in fresh BHI or DMEM and adjusted to a starting OD_{600} of 0.06. The cultures were incubated with shaking at 200 rpm The OD_{600} reading was taken at hourly intervals for 8h and then at 24h h and experiments were performed in triplicate.

2.8 Preparation of whole cell proteins

Meningococcal cells were grown overnight in BHI supplemented with Vitox (Oxoid) at 37°C in a shaking incubator as described elsewhere. The cultures were harvested by centrifugation at 13000 × g for 10 min. The pelleted cells were re-suspended in sterile PBS and 5× SDS-PAGE sample buffer to achieve 1× final concentration of whole cell protein extracts. The whole cell lysate was briefly sonicated using a 10s pulse to obtain a homogenous suspension, boiled for 5 min and then mixed thoroughly before SDS-PAGE analysis.

2.9 SDS-PAGE and immunoblotting.

The recombinant proteins were separated by 10% SDS-PAGE using Mini- Protean III equipment (BIO-RAD) following the method of Laemmli (Laemmli, 1970). Proteins separated by SDS-PAGE were visualized by staining with Simply Blue SafeStain (Invitrogen) or PageBlueTMProtein Stain Solution (Fermentas) according to the manufacturer's instructions. Gels were scanned using a GS-800 calibrated densitometer (BIO-RAD). For Immunoblot analysis, the protein mixtures separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Amersham Biosciences, UK) in semi-dry blotting buffer (5.82 g Tris base, 2.93g Glycine, 3.75ml 10% SDS, 200 ml methanol) using a Trans-Blot SD semidry transfer cell (BIO-RAD) according to the manufacturer's instructions. The membranes were incubated in 1% Bovine serum albumin (BSA) (Sigma) for 30 min. The membranes were then probed with mouse anti-pentahistidine antibody (Qiagen) or rabbit primary antibody diluted 1:10,000 and 1:1000, respectively, in blocking buffer (5% [wt/vol] non fat dry milk, 0.1% [vol/vol] Tween 20 in 1 × phosphate-buffered saline [PBS]) and incubated overnight with shaking at 4°C. The following day, the membrane was washed three times 15 min each with PBS containing 0.05% Tween-20 (PBS-Tween), and incubated into secondary antibody (anti-mouseIgG conjugated to alkaline-phosphtase or anti-rabbit conjugated to alkaline phosphtase) (Sigma) at the concentration of 1:30,000 for 2 h at room temperature. After 2 h the membrane was washed thrice with PBS-Tween for 15 min each time. The membrane was then developed using the BCIP (5-bromo-4-chloro-3-indolylphosphate)–Nitro Blue Tetrazolium liquid substrate (Perkin Elmer TM). The membrane was finally washed with dH2O and a digital image of the membrane was taken using a GS-800 calibrated densitometer (BIO-RAD).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
JM109	endA1 recA1 gyrA96thi hsdR17(rK-rK-) relA1 supE44 Δ(lac-proAB) [F'traD36 proABlaqIqZΔM15]	Promega
TOP10F'	F'lacIqTn10(TetR) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galUgalKrpsLendA1 nupG	Invitrogen
N. meningitidis		
MC58	wild-type serogroup B strain	(Tettelin *et al.*, 2000)
MC58ΔgapA-1	GapA1 deletion and replacement with kanamycin cassette	This study
Plasmids		
pGEM-T Easy	Cloning vector	Promega
pJMK30	Source of kanamycin resistance cassette	(van Vliet *et al.*, 1998)
pSAT-6	pGEM-T Easy vector cloned with 3 kb insert of GapA1 plus flanking DNA	This study
pSAT-7	pGEM-T easy vector with 2 kb DNA flaking either side of GapAI gene amplified by PCR and self-ligated to construct a vector	
pSAT-8	pSAT7 cloned with kanamycin resistance cassette of 1.5 kb	This study

3. RESULTS AND DISCUSSION

3.1 Construction of mutagenic plasmids for GapA1 in *N. meningitidis*.

In order to create a GapA1 knock-out mutant in *N. meningitidis* strain MC58, a fragment of DNA encompassing GapA1 and *ca.*1-kb of DNA both upstream and downstream of the start codon of GapA1was amplified by PCR (**Fig.1A).** This amplicon was purified and ligated with pGEM-T Easy to yield pSAT-6. The plasmid pSAT-6 was then used to transform *E. coli* JM109 cells and the successful clones were identified by blue/while selection. Plasmid DNA was prepared from putative transformant clones and analysed by restriction digestion with *Sma*I. Two *Sma*I sites are present in the cloned DNA fragment, thus, as expected two bands of 3.0 and 3.3 kb were obtained **(Fig. 1B).** PCR analysis of pSAT-6 also confirmed the successful ligation **(Fig.1C).**

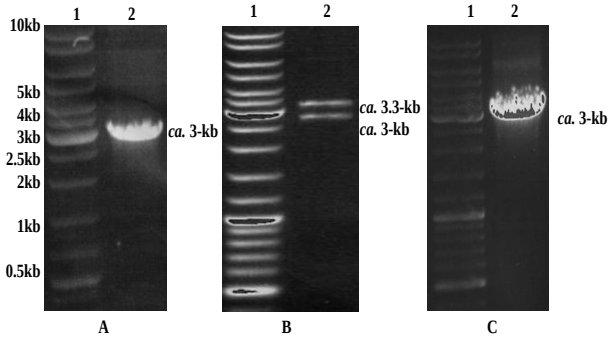


Fig. 1A Agarose gel showing PCR amplification of GapA1 plus flanking DNA. Lane 1, DNA markers, lane 2, PCR product consisting of GapA1 and flanking DNA. **Fig.1B** Agarose gel showing the restriction digestion of pSAT-6 with *Sma*I. Lane 1, DNA markers, lane 2, pSAT-6 plasmid DNA. As expected two bands (3 and 3.3-kb) were observed in lane 2 confirming the successful ligation of DNA fragment in pSAT-6 **Fig. 1C** Agarose gel showing the PCR amplification of GapA1 plus flanking DNA from the plasmid construct pSAT-6. Lane 1, DNA markers, lane 2, PCR product of GapA1 plus flanking DNA.

3.2 Mutagenesis of GapA1 by i-PCR

The plasmid pSAT-6 was employed as template DNA in inverse PCR to amplify a 5-kb band **(Fig. 2A)**. This PCR resulted in deletion of the GapA1and introduction of a unique *Bgl*II site to facilitate the introduction of selectable marker encoding resistance to kanamycin. The i-PCR product was digested with *Bgl*II and self-ligated generating a pSAT-7. The pSAT-7 plasmid was subsequently purified from a transformant clone and digested with *Bgl*II to facilitate the insertion of the Kan^R cassette (**Fig. 2B)**. The kanamycin resistance marker was ligated to the plasmid pSAT-7 digested with *Bgl*II to yield pSAT-8. Plasmid DNA was purified from putative pSAT-8 transformants and used to verify the cloning of Kan^R cassette by restriction digestion using *Sma*I. As expected, three DNA bands were obtained by this digestion confirming the successful cloning of the kanamycin resistance marker **(Fig. 2C).** The plasmid pSAT-8 was then used to mutate GapA1 in *N. meningitidis* strain MC58.

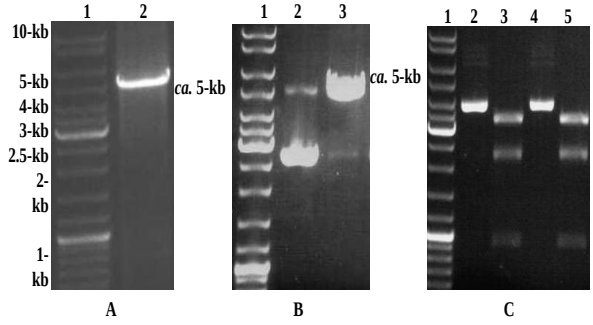


Fig. 2. Agarose gel analysis demonstrating (A) the successful amplification of inverse-PCR product for GapA1 mutagenesis, (B) the restriction digestion of pSAT-7 with *Bgl*II to enable the insertion of Kan^R cassette. Lane 1, DNA markers, lane 2, uncut pSAT-7, lane 3, pSAT-7 digested with *Bgl*II, lane 3 produced an expected 5-kb band, (C) the restriction digestion of pSAT-8 with *Sma*I for the presence of Kan^R cassette, lane 1, DNA markers, lanes 2 and 4, undigested pSAT-8, lanes 3 and 5, *Sma*I digested pSAT-8. As expected, three products were observed in digested samples confirming the ligation of Kan^R.

3.3 Verification of GapA1 mutagenesis

Initially, verification of GapA1 knock-out mutants was accomplished by PCR analysis. Chromosomal DNA was prepared from putative mutants and used to confirm deletion of GapA1 by amplifying a band corresponding to GapA1 As expected this PCR failed to produce a DNA band of the size for GapA1from the putative mutants, indicating that the GapA1has been successfully substituted with the kanamycin resistance marker, whereas the positive control of MC58-WT produced an expected *ca.* 1-kb band representing GapA1 **(Fig. 3A).** In addition, PCR analysis was performed to determine the orientation of the kanamycin resistance marker in the chromosome of putative mutants using the primers Kan_CTR 5'-GACAACGCAGACCGTTCCG-3' and GapA1 (R) FL. Where necessary, MC58 wild-type DNA was used as a control **(Fig. 3B)**. This PCR resulted in amplification of expected 1-kb band from the putative mutants cloned with the appropriately oriented Kan^R marker whereas the MC58-WT negative control did not produce a band. The GapA1knock-out mutants were designated MC58ΔGapA1.

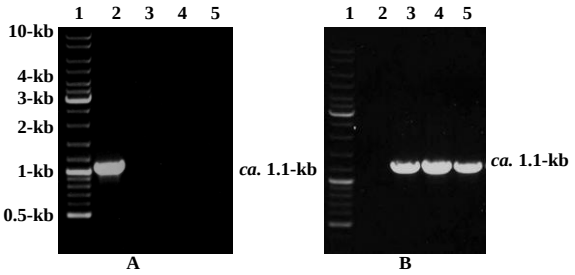


Fig. 3 Agarose gel analysis demonstrating (A) the verification of GapA1deletion. Lane 1, DNA markers, lane 2, MC58-WT DNA (positive control), lanes 3-5, putative GapA1 mutant DNA. Lane 2, shows the expected band corresponding to GapA1, which is absent in lanes 3-5, confirming the successful deletion of GapA1 in the mutant DNA.(B) the orientation and presence of Kan^R cassette by PCR using primers Kan-CTER and GapA1_(R) FL. Lane 1, DNA markers, lane 2, MC58-WT DNA (control), lanes 3-5, DNA from putative GapA1 mutant.

In order to confirm that GapA1is not expressed in the isogenic-null mutant strain, whole cell lysates of MC58-WT and MC58ΔGapA1 were resolved on SDS-PAGE followed by immunoblot analysis by probing the membranes with RαGAPDH-1 antiserum. A protein band of apparent molecular weight of *ca.* 37-kDa was detected in the lysate of the MC58-WT sample, which was absent from the lysate of MC58ΔGapA1 **(Fig. 4)**. This confirmed that the expression of GapA1has been abolished by the mutation. The RαGAPDH-1 also detected a number of additional protein bands, including one immediately above the GapA1band in lysates of both MC58-WT and MC58ΔGapA1, which were assumed to be non-specific due to the polyclonal nature of antiserum.

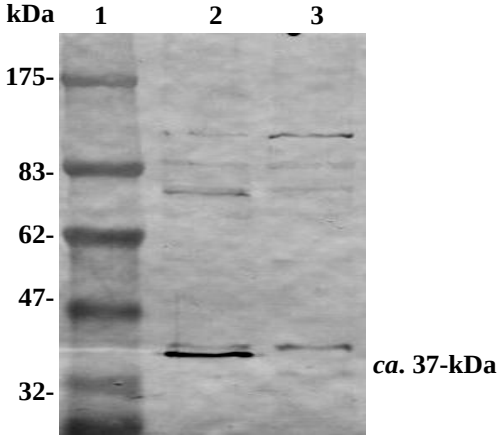


Fig. 4. Immunoblot analysis demonstrating the successful deletion of GapA1 in putative mutant strain, Lane 1, protein markers, lane 2, whole cell extracts from MC58-WT, and lane 3, whole cell extracts from MC58ΔGapA1.

3.4 GAPDH-1 is not required for maximal *in vitro* growth of *N. meningitidis*

Prior to carrying out phenotypic analysis of the GapA1 mutant, it was essential to demonstrate that mutation of the gene did not significantly affect the growth rate of *N. meningitidis*. Growth and colonial characteristics of mutant strains were assessed by visual inspection of colony morphology on agar plates and monitoring the rate of growth in liquid culture. The mutant strain demonstrated similar colony morphology and color to that of wild-type strain MC58. Growth rate was assessed by measurement of OD_{600} of samples that were removed from the culture at hourly intervals. The growth rate of the strain with deletion of GapA1 was not substantially different to that observed for wild-type MC58. Both strains were observed to grow to a similar OD_{600} by the final time point of eight and 24 h. These experiments were performed in triplicate. In order to further explore whether the addition of normal human serum may have any effect on the growth of wild-type and mutant strains, meningococcal growth was monitored in BHI or DMEM supplemented with 10% human serum. Again, no substantial differences between the growth rates of wild-type MC58 and GapA1mutant were observed **(Fig. 5)**. These findings are consistent with the findings of (Baart *et al.*,) who showed that the glycolytic pathway was non-functional in the meningococcus due to absence of one of the important enzymes in this pathway: phosphofructokinase (Baart *et al.*, 2007)

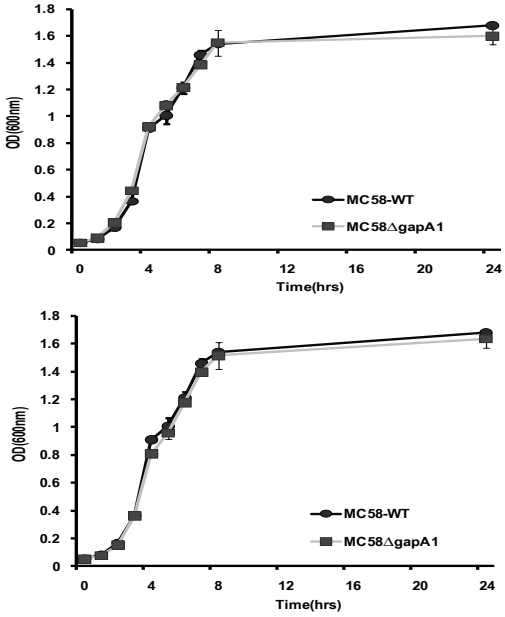


Fig. 5. Growth characteristics of meningococcal mutant strains compared to their wild-type parent strain. (A) *N. meningitidis* strain MC58-WT, MC58ΔGapA1, grown in BHI broth with 10% Human serum (B) *N. meningitidis* strain MC58-WT, MC58ΔGapA1, grown in DMEM supplemented with 10% human serum.

4. CONCLUSION

A number of reports have shown that the classical cytoplasmic house-keeping enzymes, including enolase, fructose-1, 6-bisphosphate aldolase and glyceraldehyde 3-phosphate dehydrogenase, are often transported to the surface of microbial pathogens, where they perform various non-biological functions, unconnected to their housekeeping roles (Kim & Dang, 2005). In summary, GapA1 isogenic mutant was generated in *N. meningitidis*. The growth rate of wild-type MC58 was shown to be unaffected by the mutation of GapA1 demonstrating that the product of the gene was not required for optimal growth of *N. meningitidis* under *in vitro* conditions tested. However presence of intact gene in the bacterium, suggests additional (moonlighting) roles in the pathogenesis of the disease.

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