Sindh Univ. Res. Jour. (Sci. Ser.) Vol.44 (3) 421-426 (2012)

SINDH UNIVERSITY RESEARCH JOURNAL (SCIENCE SERIES)

Generation Of Complementation Strain In *Cbba* Mutant Derivative Of *Neisseria Meningitidis*: A Molecular Approach Based At Allelic Exchange System

S. A. TUNIO*, N.J. OLDFIELD, K.G. WOOLDRIDGE. D.P.J. TURNER

Molecular Bacteriology and Immunology Group, Institute of Infection, Immunity and Inflammation, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, NG7 2RD, United Kingdom

Received 07th January 2012 and Revised 12th May 2012

Abstract: *N. meningitidis* remains an important cause of septicemia and meningitis and is associated with high morbidity and mortality. The bacterium is a naturally competent in uptake of foreign DNA and this unique feature has been exploited extensively for genetic manipulation in the laboratory. In this study, we present a molecular method for construction of gene complementation strain of *N. meningitidis* in *cbb*A-isogenic mutant strain. A complemented strain was created in *N. meningitidis cbb*A- isogenic-mutant using PCR based molecular approaches and allelic exchange methodology. A wild-copy of the *cbb*A gene together with its predicted promoter sequences were amplified by PCR and cloned between two meningococcal genes: NMB0102 and NMB0103 in complementation vector pSAT-11. The pSAT-11 was then used to reintroduce the single copy of wild-type *cbb*A at ectopic site in isogenic mutant strain. Complemented strain was shown to restore the expression of *cbb*A to the wild-type level at ectopic site. In growth profiling experiments both strains were shown to grow at the same rate suggesting that the gene has been returned back properly. In conclusion, the *cbb*A mutation in *N. meningitidis* MC58 was successfully complemented by using allelic exchange method and the newly constructed complemented mutant strain was demonstrated to restore the expression of the gene to the wild-type level by using western blot analysis.

Keywords: *N. meningitidis*, mutagenesis, constitutive expression, gene complementation.

1. INTRODUCTION

The genome sequences of *N. meningitidis* strain MC58 (serogroup B) (Tettelin *et al.*, 2000), strain Z2491 (serogroup A) (Parkhill *et al.*, 2000) and strain FAM18 (serogroup C) (Bentley *et al.*, 2007) have been published. In addition, the annotated genome sequence of obligate commensal species *N. lactamica* has also been made publicly available. The genome of *N. meningitidis* consists of about 2.1-2.2 million bases encoding approximately 2000 genes (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). The three sequenced meningococcal genomes differ from each other by approximately 9-10% and from the *N. gonorrhoeae* genome by roughly the same amount (Stephens *et al.*, 2007). The chromosome of *N. meningitidis* strain MC58 is almost 100 kb larger than the other two sequenced strains mainly due to large duplication of about 30 kb comprising 36 coding sequences (NMB1124-NMB1159 duplicated in NMB1162-NMB1197) and the acquisition of two additional islands of horizontally transferred DNA (IHT-B 17.1 kb and IHT-C 32.6kb). Both of these islands are absent in the genomes of strains Z2491 and FAM18 (Schoen *et al.*, 2007).

The completion of entire genome of the bacterium has facilitated significantly in deciphering the crucial information on pathogenesis of the disease and competent for transformation *in vivo* and *in vitro* which has aided a lot in generation of mutant strains as well as constructing complementation strains in isogenic mutants to verify the roles of mutated gene. Mutant strains created by homologous recombination and allelic exchange system have enabled functional studies of previously unrecognised and hypothetical genes in *N. meningitidis.* The uptake of foreign DNA during transformation process is facilitated by a 10-bp DNA sequences (5'-GCCGTCTGAA-3') known as DNA Uptake sequences (DUS) (Elkins *et al.*, 1991). The genome of the bacterium contains around 2000 copies of DUS (roughly 1% of 2.1-2.2 Mbp genome) scattered around the entire DNA. The transformation is a multistep process, which include DNA uptake, processing and chromosomal integration (van Dam & Bos, 2012). Meningococcal pilli has been shown to play crucial role in uptake of genus specific DNA (Fussenegger *et al.*, 1997). The detailed mechanism of DNA uptake in *N. meningitidis* is not well-known yet. However, it is thought that the mechanism appears to be similar to that of *N. gonorrhoeae*. In *N. gonorrhoeae*, following uptake of the plasmid DNA, it is linearized and then converted, in part, to a single stranded intermediate (Biswas *et al.*, 1986) while the subsequent steps are not yet fully known.

++ Corresponding author: E-mail: S. A. TUNIO sarfraztunio@hotmail.com Phone: +92- 222771198,
*Department of Microbiology, University of Sindh, Jamshoro, Pakistan

The *cbbA* gene encodes Fructose bisphosphate aldolase (FBA) in *Neisseria meningitidis*. According to information displayed at TIGR database (http://cmr.jcvi.org/tigrscripts/CMR/shared/GenePage.cgi?locus=NMB_1869), the FBA is annotated with locus tag NMB_1869 and gene symbol of *cbb*A. The *g*ene consists of 1065bp and encodes a protein of 354 amino acids.

The aim of the present study was to generate a complementation strain in *cbbA* isogenic mutants of *N. meningitidis* to validate the phenotype of the mutant strain and exclude the possibility of any secondary mutation and/or effect of the *cbbA* mutation on the adjacent genes in the same operon.

2. MATERIALS AND METHODS

2.1 Bacterial strains, growth condition, and media

E. coli JM109 competent cells (Promega, UK) were routinely grown at 37°C in LB broth or on LB agar. Where appropriate, antibiotics were added at the following concentrations: ampicillin (100 µg ml^{-1}), kanamycin (50 µg ml^{-1}). *N. meningitidis* MC58 WT and *cbbA* isogenic mutant strains were grown on Brain Heart Infusion (BHI) agar and/or Brain Heart Infusion Broth supplemented with Vitox (Oxoid) at the concentration suggested by the manufacturer (Oxoid) or 2% Fetal calf serum ([FCS] Invitrogen) and where appropriate kanamycin was added at the concentration of 50 µg ml^{-1} and erythromycin 5 µg ml^{-1} to select mutant and complemented strains and incubated in an atmosphere of 5% CO_2 at 37 °C with or without shaking. In cloning experiments, blue/white selection of transformants 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

Genomic DNA of *N. meningitidis* was extracted by using a DNeasy Blood & Tissue kit (Qiagen) according to manufacturer's instructions. The plasmid DNA from *E. coli* cells was purified using a QIAprep Spin Kit (Qiagen) according to the manufacturer's instructions. DNA manipulative enzymes such as *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. Purified plasmid and genomic DNA was quantified using a NanoDrop (ND-1000) spectrophotometer (Agilent Technologies). DNA sequencing was performed at the School of Biomedical Sciences (University of Nottingham) on an ABI 377 automated DNA sequencer. Unless otherwise stated, all PCR reactions were carried out in a 25 µl final volume and cycled using a C1000 model Thermal Cycler (BIO-RAD). An agarose gel (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.

2.3 Construction of pYHS25-based ectopic complementation vector

A DNA fragment of *ca.* 2.7-kb **(Fig 1A)** consisting of *opa* promoter and erythromycin resistance cassette flanked by two meningococcal genes (NMB0102 and NMB0103) was amplified by PCR using the primer pair CompCass_(F) 5'-ATGTGGCGGGTTTTGAGTGC-3' and Comp Cass_(R) 5'-GATTTTTCTTGCGGCGCGGC-3' and plasmid DNA pYHS25 (Winzer et al., 2002) . The PCR was performed as described in section 2.5 with the following modifications, annealing at 51°C for 1 min, primer extension at 68°C for 4 min, and 94°C for 45s. The gel purified PCR product was ligated to pGEM-T Easy vector (Promega). Screening for successful clones was performed on the basis of blue/white phenotype. Plasmid DNA from putative transformants was analysed by restriction digestion and DNA sequencing to confirm the successful cloning. The resulting plasmid was designated pSAT-11 **(Fig 1B).**

2.4 Engineering constructs for *cbbA* complementation

To complement the *cbbA* mutation, *cbbA,* together with its predicted promoter sequences, was amplified by PCR from genomic DNA of *N. meningitidis* strain MC58 using the primers FBA_COM (F)5'-CGC**GGATCC**ATGAGCTGTTTATGGTTTTTTGCTG-3'and FBA_COM (R) 5'-CGC**GGATCC**GGCATTTTGTTTACAGGCAACCTG-3' (Table 2) incorporating *Bam*HI restriction sites. The PCR conditions for the reaction were set as following: annealing at 55°C for 1 min, primer extension at 70°C for 2.5 min, and 94°C for 45s. The PCR product was then cloned into *Bam*HI-digested pSAT-11. Putative transformants were analysed by colony PCR, restriction digestion analysis and DNA sequencing to confirm in frame ligation. The resulting plasmid was designated as pSAT-12 **(Fig.1C)** and introduced in MC58Δ*cbbA* strain by natural transformation, introducing a single chromosomal copy of the complementing gene (*cbbA*) in the intergenic region between NMB0102 and NMB0103 ORFs, which are orientated in a tail-to-tail fashion. Insertion of the gene at this ectopic site was confirmed by PCR analysis with the primer pair NMB0102 (F) 5'- ATGTGGCGGGTTTTGAGTGC-3' and NMB0103 (R) 5'-TTTGGATTTTTCTTGCGGCGC-3' and western blot analysis for the expression of the gene using RαFBA antiserum.

2.5 Methodology of natural transformation of *N. meningitidis*

Natural transformation of *N. meningitidis* was performed as previously described (Hadi *et al.*, 2001) . Briefly *N. meningitidis* cells were grown into BHI broth supplemented with Vitox and incubated overnight at 37°C in 5% CO_2. The next day, overnight broth cultures were diluted 1:20 in fresh BHI broth and allowed to grow to an optical density of 0.2. An aliquot of 200 µl of the culture was transferred to a sterilized 15 ml tube with conical bottom containing 1.5 ml of BHI agar supplemented with Vitox. The culture was incubated for 5-6 hrs at 37^{o}C in 5% CO_2 without shaking, then *ca.* 1 µg of the pSAT-12 plasmid (complementation construct) was added to the culture tubes. The culture tubes were then incubated further for 16 h at the conditions described elsewhere. The putative transformants were selected on BHI containing kanamycin 50µg ml^{-1}erythromycin 5µg ml^{-1}. Single isolated colonies of Double resistant *N. meningitidis* (resistant of Kanamycin and erythromycin) were obtained and further analyzed by PCR and western blot analysis of whole cell extracts of the putative mutants.

2.6 SDS-PAGE and western blot analysis

The recombinant proteins were separated by 10-12% 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 SimplyBlue SafeStain (Invitrogen) according to the manufacturer's instructions. Gels were scanned using a GS-800 calibrated densitometer (BIO-RAD). Immunoblot analysis was performed essentially as described previously (Tunio *et al.*, 2010). Briefly, the proteins resolved by SDS-PAGE were transferred onto a nitrocellulose membrane (Amersham Biosciences, UK) using a Trans-Blot SD semidry transfer cell (BIO-RAD) according to the manufacturer's instructions. The membranes were then probed with mouse rabbit primary antibody diluted 1:1000 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 next 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 (PerkinElmer^{TM}). The membrane was finally washed with dH_2O and a digital image of the membrane was taken using a GS-800 calibrated densitometer (BIO-RAD).

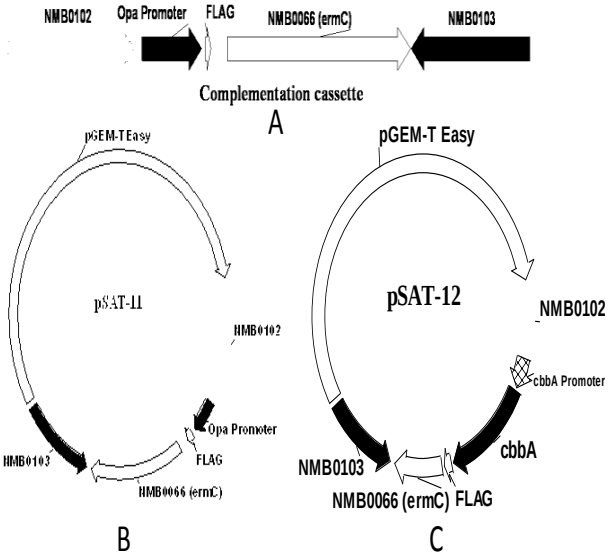

Fig.1 Schematic diagram representing (A) the gene organization of complementation cassette (B) and subsequent cloning of this cassette into the pGEM-T Easy vector to generate pSAT-11 (C) A map of the pSAT-12 constructed by cloning *cbbA* into pSAT-11 and used to complement *cbbA* mutation in isogenic-mutants. The plasmid contains ampicillin and erythromycin resistance genes and *cbbA* promoter for driving the expression of *cbbA* gene.

3. RESULTS AND DISCUSSION

3.1 Construction of pSAT-12 complementation vector

Complementation of mutant strains, in which a wild-type copy of the gene is reintroduced and expressed in the isogenic-mutant at an ectopic site, provides an important check that any observed phenotype can be ascribed to the loss of the mutated gene rather than to polar effects of the mutation on adjacent genes in the operon or additional undetected secondary mutations. In order to rule out polar effects on neighbouring genes during phenotypic analysis of the *cbbA* mutant strains, the *cbbA* mutations was complemented using an ectopic complementation vector pSAT-12 (Fig 1C) in which the gene of interest (*cbb*A) together with its predicted promoter sequences was cloned under upstream of the *ermy* gene (encoding resistance to erythromycin). These gene sequences are flanked by two meningococcal genes named NMB102 and NMB103, which are in a tail-to-tail configuration in both the complementation vector and the meningococcal genome. The construct facilitates insertion of genes of interest, under the control of the *cbbA* promoter, into the meningococcal genome at a site that is unlikely to effect the expression of any other genes. The complementation cassette ca. 2.7 kb was amplified by PCR from the pYHS25 **(Fig. 2A)** and ligated with pGEM-T Easy vector. The resulting ectopic complementation plasmid

was designated as pSAT-11 (Fig. 1B). The plasmid pSAT-11 was verified by restriction digestion with *Bam*HI and an expected band of a *ca*.6.5 kb corresponding to the linear pSAT-11was observed (Fig 2B). The pSAT-11 plasmid was further confirmed by DNA sequencing. This pYHS25-based newly constructed high copy number plasmid pSAT-11 was used as basis to clone complementing gene *cbbA* under its native promoter for driving the expression of complementing genes.

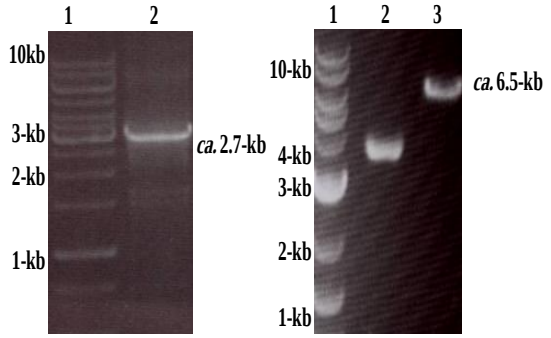

Fig. 2 Agarose gel analysis (A) showing the PCR product of complementation cassette Plasmid DNA was prepared from pYHS25 and used as template for amplification of a *ca.*2.7-kb DNA fragment corresponding to complementation cassette. Lane 1, DNA markers, lane 2, PCR product representing complementation cassette, (B) restriction digestion analysis of pSAT-11 with *Bam*HI showing the successful ligation of complementation cassette in pGEM-T Easy, lane 1, DNA markers, lane 2, uncut pSAT-11 DNA, and lane 3, *Bam*HI-digested linear pSAT-11 (*ca.*6.2 kb).

3.2 Construction of pSAT-11 based new complementation vector

In an attempt to identify promoter sequences upstream of *CBBA*, analysis of the organization of genes at the *cbbA* locus (NMB1869) was performed. This analysis demonstrated that *cbbA* is the first gene in its orientation, so must have its own promoter. The predicted *cbbA* gene (based on gene-finding algorithms used at the Comprehensive microbial resource [CMR] database) starts from an internal ATG start codon, meaning that the first 25 predicted amino acids in the ORF are not predicted to be part of the gene. This is in agreement with a visual inspection, which shows that there is a good ribososmal binding site (RBS) upstream of the internal ATG but not at the first ATG. The *cbbA* was amplified by PCR **(Fig 3)** and. from a position upstream of the predicted promoter to a position downstream of its stop codon and introduced into *Bam*HI-digested linearized pSAT-11. Putative transformants were selected on agar plates containing appropriate antibiotics and verified by restriction digestion analysis and subsequent sequencing of the putative clone to confirm the in-frame ligation. The resulting plasmid was designated pSAT-12 and used to complement *cbbA* mutation in isogenic mutant by natural transformation and allelic exchange.

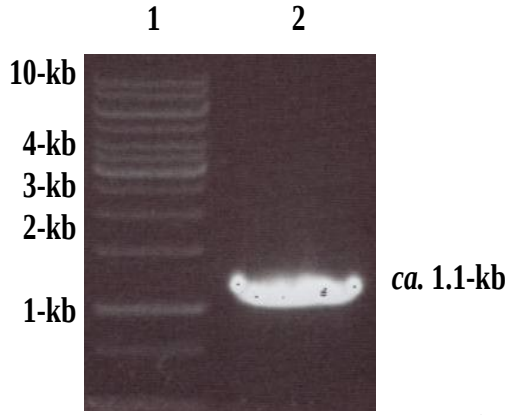

Fig. 3 Agarose gel analysis demonstrating the successful PCR amplification of *cbbA* from chromosomal DNA of *N. meningitidis.* Chromosomal DNA was prepared from MC58-WT and used to amplify the *cbbA* for cloning into pSAT-11 to generate pSAT-12. Lane 1, DNA markers, lane 2, PCR product of *cbbA* gene.

Prior to introduction of pSAT-12 into MC58Δ*cbbA* by natural transformation, *E. coli* JM109 harboring pSAT-12 were grown overnight and the culture pellet was used to determine the expression of cloned *cbbA* by western blot analysis using RαFBA antiserum. A number of clones were selected for analysis. Whole cell proteins extracts were resolved by SDS-PAGE and then transferred to a nitrocellulose membrane for western blot analysis with RαFBA antiserum. All transformant clones tested were shown to express full-length protein band with an apparent molecular mass similar to the expected size of FBA, which was absent in the pSAT-11 (negative control) suggesting that the FBA is constitutively expressed in *E. coli* from this plasmid without any induction **(Fig 4).**

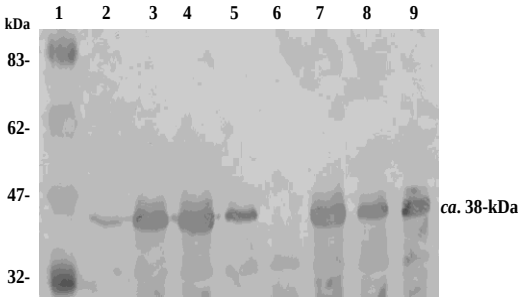

Fig. 4 Western blot analysis of whole cell lysates from *E. coli* harbouring pSAT-12. Whole cell lysates of *E. coli* were resolved by 10% SDS-PAGE followed by western blot analysis to determine the expression of FBA. Lane 1, DNA markers, lane 2, MC58-WT whole cell lysate, lanes 3-5, pSAT-12, 1-3 samples, respectively, lane 6, pSAT-11 (negative control), lanes 7-9, pSAT-12, 4-6 samples, respectively. As expected all pSAT-12 constructs represented a band equivalent to FBA.

The plasmid pSAT-12 was subsequently introduced into MC58Δ*cbbA* by natural transformation

and allelic exchange essentially as described previously (Hadi *et al.*, 2001), to introduce a single chromosomal copy of the wild-type *cbb*A gene *in trans* between NMB0102 and NMB0103, open reading frames, which are orientated in a head-to-head fashion. Transformants were selected on selective BHI agar plates for further verification. Putative transformants were sub-cultured and used for DNA extraction and preparation of whole cell extracts for confirmation by PCR and western blot analysis, respectively. The resulting complemented mutant strain was designated MC58Δ*cbbA cbbA*^{Ect}

3.3 Verification of MC58Δ*cbbA cbbA*^{Ect} complemented strain by PCR

Initial confirmation of the successful complementation of *cbb*A at ectopic site in isogenic-mutants was achieved by PCR analysis which resulted in amplification an expected band of *ca.* 1-kb representing *cbbA* from MC58-WT and complemented mutants, but absent in isogenic-mutant strain (Fig 5A) confirming the successful complementation of *cbbA* mutation in the isogenic mutant strain. Besides this, another PCR using the primers NMB0102 (F) 2 and NMB0103 (R) was performed to verify the insertion of complementation cassette at the proper location in the chromosome of complemented strains. Insertion of the complementation cassette at the proper location was identified by obtaining a *ca*. 4-kb band (consisting of complementation construct plus *cbbA*) in complemented mutant strains whereas WT and mutant strains were expected to produce a band of *ca.* 1.3 kb PCR product representing only NMB0102 and NM0103 genes. As expected the complemented strain produced an expected *ca*. 4-kb DNA band whereas MC58-WT and mutant strains produced 1.3 kb band, which further confirmed the insertion of *cbbA* at desired location in complemented strains **(Fig 5AB).**

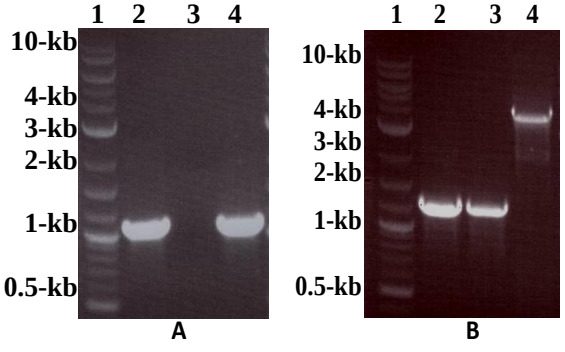

Fig. 5 (A) Agarose gel analysis demonstrating the amplification of *cbbA* from MC58-WT, cbbA-mutant and complemented mutant strain. Lanes 1, DNA markers, 2, MC58-WT, 3, MC58Δ*cbbA*, and 4, MC58Δ*cbbA cbbA*^{Ect} (B) Agarose gel analysis confirming the insertion of *cbbA* at desired location in complemented strains. Lanes 1, DNA markers, 2, MC58-WT 3, MC58Δ*cbbA*, and 4, MC58Δ*cbbA cbbA*^{Ect}.

3.4 Determining the expression of *cbbA* at ectopic site in MC58Δ*cbbA cbbA*^{Ect} strain

The whole cell extracts were prepared from *N. meningitidis*-MC58 WT, respective null-mutants and putative complemented strains and resolved on 12% SDS-PAGE followed by western blot analysis using RαFBA antiserum. The complemented strain MC58Δ*cbbA cbbA*^{Ect}, tested was shown to express a protein band corresponding to apparent molecular weight of *ca.* 38-kDa representing FBA **(Fig 6).**

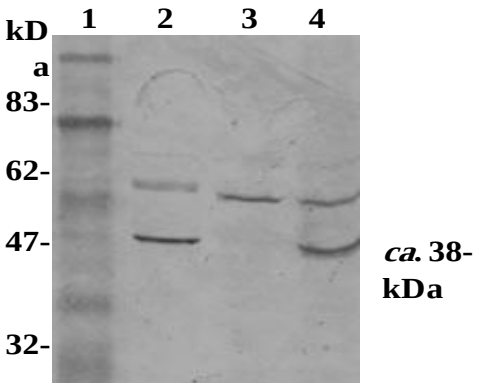

Fig. 6 Western blot analysis demonstrating the expression of *cbbA* in complemented mutant strains MC58Δ*cbbA cbbA*^{Ect}. Lane 1, protein marker, lane 2, MC58-WT, lane 3, MC58Δ*cbbA*, lane 4, MC58Δ*cbbA cbbA*^{Ect}.

4. CONCLUSION

The present study describes the detailed method of gene complementation in *N. meningitidis* strain MC58. We have previously shown that the *cbbA* mutant strain exhibit reduced capacity to adhere to human cells, this contributing to the pathogenesis of the disease. The mutant strains provide a valuable resource for studies of bacterial pathogenesis and vaccine development. However, in order to rule out the possibility of secondary mutations that may occur during the generation of mutant strains, the phenotypes observed for the mutations must be confirmed, The *cbbA* complementation was performed using a pYHS25-based ectopic complementation vector (pSAT-11) in which the expression of genes of interest is driven under the control of the *cbbA* native promoter. Moreover, the complementation vector facilitates insertion of gene of interest into the meningococcal genome at a site that is unlikely to effect the expression of any other gene. Successful complementation was confirmed by PCR analysis and western blot analysis using RαFBA antiserum. In summary, *cbb*A-isogenic mutant strain was successfully complemented with a wild-copy of *cbbA* gene and the expression of the gene in complemented mutant strain was shown to have been restored to wild-type parent strain level. The complemented strains can be employed to confirm the previously identified phenotype of *cbbA* mutant in various characterisation experiments.

REFERENCES:

Bentley, S. D., G. S. Vernikos, L. A. S Snyder,. and other authors (2007) Meningococcal Genetic Variation Mechanisms Viewed through Comparative Analysis of Serogroup C Strain FAM18. *PLoS Genet* 3, e23.

Biswas, G. D., K. L.Burnstein, and P. F.. Sparling, (1986) Linearization of donor DNA during plasmid transformation in Neisseria gonorrhoeae. *J Bacteriol* 168, 756-761.

Elkins, C., C. E.Thomas, H. S. Seifert, and P. F. Sparling, (1991) Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. *J Bacteriol* 173, 3911-3913.

Fussenegger, M., T. Rudel, R., Barten, R. Ryll, and T. F. Meyer, (1997) Transformation competence and type-4 pilus biogenesis in Neisseria gonorrhoeae - a review. *Gene* 192, 125-134.

Hadi, H. A., K. G. Wooldridge, K. Robinson, and D. A. A. Ala'Aldeen, (2001). Identification and characterization of App: an immunogenic autotransporter protein of Neisseria meningitidis. *Molecular Microbiology* 41, 611-623.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Parkhill, J., M. Achtman, K. D. James, and other authors (2000) Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature* 404, 502-506.

Schoen, C., B. Joseph, H. Claus, U. Vogel, and M. Frosch, (2007) Living in a changing environment: Insights into host adaptation in Neisseria meningitidis from comparative genomics. *International Journal of Medical Microbiology* 297, 601-613.

Stephens, D. S., B. Greenwood, and P. Brandtzaeg, (2007) Epidemic meningitis, meningococcaemia, and Neisseria meningitidis. *Lancet* 369, 2196-2210.

Tettelin, H., N. J. eacute, Saunders, and other authors (2000) Complete Genome Sequence of Neisseria meningitidis Serogroup B Strain MC58. *Science* 287, 1809-1815.

Tunio, S. A., N. J. Oldfield, A. Berry, D. A. Ala'Aldeen, K. G. Wooldridge, and D. P. Turner, (2010) The moonlighting protein fructose-1, 6-bisphosphate aldolase of Neisseria meningitidis: surface localization and role in host cell adhesion. *Mol Microbiol* 76, 605-615.

van Dam, V. and M. P. Bos, (2012). Generating knock-out and complementation strains of Neisseria meningitidis. *Methods Mol Biol* 799, 55-72.

Winzer, K., Y.H. Sun, A. Green, M. Delory, D. Blackley, K. R. Hardie, T. J. Baldwin, and C. M. Tang, (2002) Role of *Neisseria meningitidis* luxS in cell-to-cell signaling and bacteremic infection. *Infect Immun* 70, 2245-2248.