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Successful Application of Site-directed Mutagenesis Polymerase Chain Reaction to Mutate TMS 11 of the Staphylococcal Multidrug Efflux Protein QacA

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Abstract

Staphylococcus aureus is a major problem in both the clinical setting and within the community. *S. aureus* can quickly develop resistance to a wide range of antibiotics through a number of different mechanisms, of which, using transporters located in the cell membrane to pump antibiotics out of the cell is the most serious concern. In staphylococcal species, QacA, one such important transporter, is encoded by *qacA*. QacA is 55kD in size and has 14 transmembrane segments (TMS) (TMS 1-TMS 14). This research describes the mutation process of the amino acid residues in TMS 11 of QacA using site-direct PCR. In this research, 15 primers were successfully designed for site-directed mutagenesis PCR. The site-mutagenesis PCR was successfully conducted to create 15 *qacA* mutants. These mutants will be used in further functional research of QacA.

Keywords

S.aureus, gene *qacA*, protein QacA, site-directed PCR

Introduction

Staphylococcus aureus is a major problem in both the clinical setting and within the community (Kumar *et al.*, 2020). It is an opportunistic pathogen that can cause a range of diseases with high mortality rates including septicemia, pneumonia, and toxic shock syndrome (Bassetti *et al.*, 2018). *S. aureus* has the ability to quickly acquire and spread antibiotic resistance genes through horizontal gene transfer on mobile genetic elements such as plasmids. This has resulted in many strains having high levels of resistance, limiting the

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treatment of infections to just a few options (Vogel *et al.*, 2016).

S. aureus can develop resistance to a wide range of antibiotics through a number of different mechanisms. These mechanisms include enzyme inactivation of the antibiotic, alteration of the antibiotic target, alteration of metabolic pathways, inhibition of drug uptake via altered permeability of the cell wall, and increased efflux of the antibiotics out of the cell (Radestock & Forrest, 2011). Resistance via efflux pumps is of particular concern in comparison to the other known mechanisms due to just one transport protein, namely QacA, being able to extrude multiple, unrelated classes of antimicrobial compounds from the cell (Costa *et al.*, 2013). Efflux is performed by active transporters localized in the cytoplasmic membrane of all classes of cells and requires a chemical energy source to pump out unwanted compounds (Nikaido, 2009). *S. aureus* has a wide range of efflux transporters including TetA(K), which is the efflux pump that confers resistance to tetracycline, as well as the permeases NorA, NorB, and QacA/B that are all involved in mediating resistance to a diverse spectrum of functional and structural substrates (Nikaido, 2009).

In staphylococcal species, QacA, one such important transporter, is encoded by *qacA* genes that have almost identical sequences and were first described in the plasmids pSK1 and pSK23, respectively. They are members of the DHA 2 family of MFS transporters and mediate drug resistance via the PMF (Pao *et al.*, 1998; Saier Jr *et al.*, 1999; Brown & Skurray, 2001). QacA is a 514 amino acid, membrane-bound protein with a predicted size of 55kDa and 14 TMS (Tennet *et al.*, 1989; Paulsen *et al.*, 1996). QacA mediates resistance to a broad range of antiseptic and disinfectant agents by extruding them out of the cell using a proton motive force generated from the transmembrane electrochemical proton gradient (Lin *et al.*, 2018).

The aim of this research was to apply site-directed mutagenesis PCR to mutate 15 codons in *qacA* encoding for 15 amino acids that are involved in TMS 11 of QacA. Each of the *qacA* mutants will then be used for the functional analysis of QacA in terms of antibiotic and antiseptic efflux pumps in further research.

Materials and methods

Bacterial strains and plasmids

The bacterial strain *E.coli* DH5α (Hanahan, 1983) was used as the host in this study. The plasmid pSK7201, which was called as WT plasmid, contained the *qacA* gene with a 3' histidine tag sequence encoding a C-terminal His-Tagged QacA protein and pBluescript (vector). The *qacA* gene encodes for the QacA protein belonging to *S. aureus*. The WT plasmid and pBuescript were used as the positive control and negative control, respectively, in every single assay.

Growth conditions

E. coli DH5α cells were grown at 37°C on solid LB agar overnight in a Laboro incubator or in LB broth with aeration for a duration of 16 to 24 hours (h). Culture media was supplemented with ampicillin (AMP_{100}) (100 μg mL^{-1}) for selection where appropriate. All bacterial strains were kept in 30% glycerol at -80^{o}C.

Plasmid DNA manipulation techniques

Isolation of plasmid DNA for screening and sequencing

Plasmid DNA was isolated using the Isolate II Bioline plasmid mini kit (Bioline, Australia) following the manufacturer's instructions. The vector (pBluescript), WT plasmid, and constructed plasmids were used to transform competent *E. coli* DH5α cells. The transformants were then grown overnight. Single, well-isolated colonies were used to inoculate each 10mL of LB broth with AMP_{100} for selection. The broths were incubated in a gyratory shaker at 220 revolutions per min (rpm) at 37°C overnight. A 10-mL volume of overnight culture was centrifuged in an Avanti J-26XPI centrifuge (Beckman Coulter, USA) (4,500rpm/5min/4°C) to pellet the bacterial cells. Plasmid DNA was isolated from the collected cells by following the kit instructions precisely. The concentration of purified plasmid DNA was then measured using

a NanoDrop-1000 Spectrophotometer (Thermo Scientific, USA) and the sample stored at -20°C.

Agarose gel electrophoresis of DNA

DNA fragments between 1-10kb obtained from PCR amplification or plasmid purification were visualised on a 1% agarose gel following standard molecular biology methods (Sambrook and Russell, 2001). Agarose (1%) was dissolved in 0.5 x tris-acetate EDTA (TAE) buffer. A 5-µL volume of Bioline HyperLadder™ I (1 kb DNA ladder) was used as a molecular weight marker to estimate the DNA molecular weight and had the following fragment sizes: 10000, 8000, 6000, 5000, 4000, 3000, 2500, 2000, 1500, 1000, 800, 600, 400, and 200bp. DNA samples were diluted in bioline 5x sample loading buffer to give a 1x final concentration before being loaded onto the agarose gel. The gels were electrophoresed in TAE buffer at 200 volts until the loading buffer dye had reached approximately 2cm from the end of the gel. Gels were stained in Gel-red (Biotium Inc, USA) for approximately 10min. The stained agarose gels were visualised and photographed using a DigiDoc™UV imager (Bio-Rad, USA).

Restriction endonuclease digestion

DNA restriction endonucleases were obtained from New England Biolabs and used according to the manufacturer's instructions. Each DNA digestion reaction typically contained 1000ng of plasmid DNA with the appropriate restriction enzyme and buffers to reach optimal enzymatic activity. Each digestion reaction was performed in a 0.5-mL microtube and incubated for 2h at 37°C in a Laboro incubator. Digested DNA was then visualised by gel electrophoresis.

DNA sequencing and analysis

The DNA used for sequencing was prepared and quantified. To sequence the entire *qacA* gene, each DNA sequencing reaction was comprised of 750ng of plasmid DNA and 1µL of the appropriate primer (reverse or forward primer) in a total volume of 12µL. DNA sequencing reactions were analysed at the Australian Genome Research Facility (AGRF, Australia). Nucleotide sequences were obtained, analysed, and stored using the CLC Sequence Viewer 7.0 and Sequencher™ 4.9 program (Gene Codes Corporation, USA). The sequences generated were then aligned with the previously determined *qacA* sequence (Genbank accession number GU565967.1) to confirm that the codons encoding cysteine at the correct position were present and to confirm that no other mutations were leading to codon changes.

Site-directed mutagenesis

Design of oligonucleotide primers

The oligonucleotide primers were designed using the Sequencher™ 4.9 program (Gene Codes Corporation, USA). One pair of oligonucleotide primers was designed for each desired cysteine substitution within TMS 11 of QacA. Where possible, primers were designed to introduce a restriction site into the newly synthesized DNA, which aided in the identification of the QacA mutants. The choice of the restriction site introduced was determined using Watcut (NCBI, 2005) and NEBcutter V2.0 (New England BioLabs). The length of each primer ranged between 32 to 53bp, including 12 to 22 nucleotides on each side of the nucleotide changes. The primers were synthesized by Sigma-Aldrich (Australia) and resuspended (overnight, 4°C) in sterilised milliQ H_2O (MQH_2O) to give a final concentration of 100mM, then diluted to a concentration of 10mM for use in PCR site-directed mutagenesis.

Site-directed mutagenesis PCR

PCR site-directed mutagenesis was performed using the appropriate oligonucleotide primers and the QuickChange™ PCR method (Stratagene, Australia). The template DNA used in each reaction was pSK7201. Each PCR reaction included 200ng of template DNA, 100ng each of the appropriate forward and reverse primers, 10µM of dNTPs, 1x Velocity (Bioline), and MQH_2O to give a final volume of 50µL in a 0.5-mL microtube. Amplification of the DNA was performed using a Multigene PCR thermal cycler (Labnet Int, USA) with the following reaction conditions: initial denaturation at 95°C for 5min, followed by 30 cycles consisting of denaturation at 95°C for 30s, annealing at between 50°C to 72°C (depending on the melting temperature of each primer) for

30s, and extension at 72°C for 6min. Each reaction finished with a single final extension stage at 72°C for 10min. The PCR products (5µL) were visualised by agarose gel electrophoresis to confirm DNA amplification. The PCR products were treated with the restriction enzyme *Dpn*I to digest the methylated template DNA.

Transformation of E. coli DH5α

Preparation of competent E. coli DH5α cells

The preparation of the competent *E. coli* DH5α cells was performed according to the methods described by Kahn *et al.* (1979). A 10-mL overnight (stationary phase) culture of *E. coli* DH5α cells was generated from inoculation of a single colony into LB followed by incubation with shaking at 200rpm at 37°C. The culture was diluted 1:20 in fresh LB broth and incubated as above to the exponential phase ($A_{650\ nm} = 0.6$). The cells were pelleted by centrifugation (4,500rpm/5min/4°C), then resuspended in cold 0.1M TfbI and left on ice for 5min. Cells were centrifuged (4,500rpm/5min/4°C) to harvest the cells, then carefully resuspended with cold 0.1M TfbII and left on ice for 15min. Aliquots (100µL) of competent cells were put into fresh microtubes and stored at -80°C.

Transformation of competent E. coli DH5α cells with plasmid DNA

The *E. coli* DH5α transformation procedure followed methods modified from Kahn *et al.* (1979). Aliquots of 100µL competent *E. coli* DH5α cells were thawed on ice prior to use. Depending on the plasmid DNA concentration, between 1µL to 50µL of plasmid DNA (equating to 50 to 100ng of DNA) were incubated with the *E. coli DH5α* competent cells on ice for 30min to allow the DNA to associate with the cell membrane. The cells were then heat-shocked by rapidly heating the mixture at 42°C for 45s, and then placed on ice for 2min to recover. For recovery, 1mL of LB broth was added to each transformation mix and the mixtures were incubated with shaking for 45min at 37°C. For selection of the transformants, aliquots of each transformation mix were plated onto LB agar media containing AMP_{100} and then the plates were incubated overnight at 37°C in a Laboro incubator. Single, well-isolated transformants were re-streaked and stored until use.

Results and discussion

Construction of site-directed cysteine mutants of QacA

Cysteine-scanning mutagenesis is an approach for identifying functionally important amino acids and allowing for topological analysis of membrane proteins (Frillingos *et al.*, 1998; Zhu & Casey, 2007). The procedure involves the construction of a set of mutants where selected amino acids are individually substituted for a cysteine using site-directed mutagenesis. Cysteine is often selected for site-directed mutagenesis to determine the location of an amino acid and relative importance, as cysteine has relatively neutral characteristics and the sulfhydryl groups can enable highly specific modification. Importantly, wild type (WT) QacA, which is the His-tagged QacA protein, used in this study, did not contain any cysteine residues (Reddy *et al.*, 2012).

Oligonucleotide primers specific for *qacA* (**Table 1**) were designed to incorporate three nucleotide substitutions (TGT or TGC) encoding cysteine to replace codons encoding each of the 15 amino acids in TMS 11. Each primer was between 32 and 53bp in length with 12-22 nucleotides on each side of the substituted nucleotides. In addition, to allow for the identification of mutants using restriction enzyme digestion, some primers had nucleotide changes introduced to generate silent restriction sites (chosen on the basis of the DNA sequence surrounding the substituted sites). If the primer incorporated a restriction site, this site contained at least one nucleotide in the codon encoding the cysteine substitution, which aided in identifying more accurately correct mutants.

Site-directed mutagenesis was conducted using the plasmid pSK7201 as the template DNA. This template contained the *qacA* gene under the control of a T7 promoter. Each site-directed mutagenesis reaction was treated with *Dpn*I to remove the original pSK7201 (methylated pSK7201), increasing the ratio of transformants containing the newly synthesized

plasmid that contained the correct mutation. A 5µL to 45µL volume of the *Dpn*I-digested plasmid DNA (depending on the quantity of the PCR amplification visualised on 1% agarose gel) was used to transform competent *E. coli* DH5*α* cells. The transformants were grown overnight at 37°C.

Confirmation of site-directed QacA mutants

Confirmed mutation by restriction enzyme

To identify the transformants containing the correct plasmid, the plasmids were extracted from their cells and digested with the appropriate restriction endonuclease. Digests were electrophoresed in a 1% agarose gel to separate the fragments of the digested plasmid DNA. DNA fragment sizes of the mutant plasmids were compared to the DNA fragment sizes generated by the digested template plasmid with the same restriction enzyme. The template DNA contained restriction sites for the enzymes *Dde*I, *Bsg*I, *Pvu*I, and *EcoR*I (**Figure 1**). Additionally, restriction sites for *Dde*I, *Bsg*I, and *Pvu*I were introduced into the primers used to construct the mutants with P344C, I349C, and M354C substitutions, respectively, which aided in identifying those mutants. These mutated plasmids were identified on the basis that they produced additional fragments when digested compared to the digested WT plasmid. For instance, the *Dde*I digestion of the P344C mutant DNA showed five fragments (1734bp, 1680bp, 540bp, 409bp, and 166bp) while the digestion of the WT plasmid generated only four fragments (3414bp, 540bp, 409bp, and 166bp). On the other hand, two restriction enzyme sites, *Nsi*I, which was incorporated into the primers designed for the G346C, G348C, and A351C mutants, and *Nde*I, which was designed for the G353C and M357C mutants, were not present in the template pSK7201. Thus, double digestion of *EcoR*I (present in the WT plasmid) with *Nsi*I or *Nde*I was investigated to distinguish these mutants from the WT plasmid. Indeed, double digestion of the G348C plasmid with *Nsi*I and *EcoR*I (**Figure 2**) generated two bands (3.4kb and 1.1kb) while only one band was observed after digestion of the WT plasmid (4.5kb). The remaining mutant plasmids (L343C, S345C, I347C, A350C, I352C, I356C, and Y358C) could not be screened in this way, as restriction sites could not be incorporated in the primers. Our experiment design agreed with the methods used in the study of Hassan *et al.* (2006). In summary, the construction of eight out of the fifteen mutated plasmids involved the addition of restriction sites into the oligonucleotide primers that were investigated to confirm the mutant plasmids. Up to ten transformants were screened by restriction analysis and two of the transformed mutant plasmids identified were chosen for sequencing.

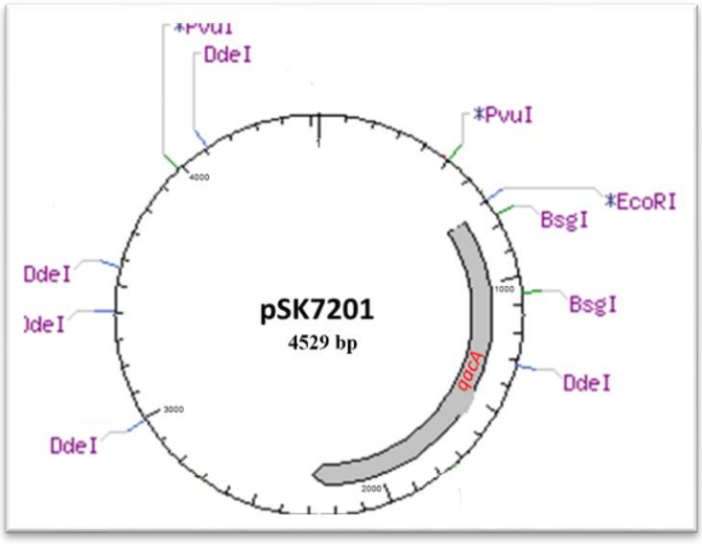

Figure 1. Diagram showing the restriction endonuclease sites in the pSK7201 that were used for the screening of constructed plasmids

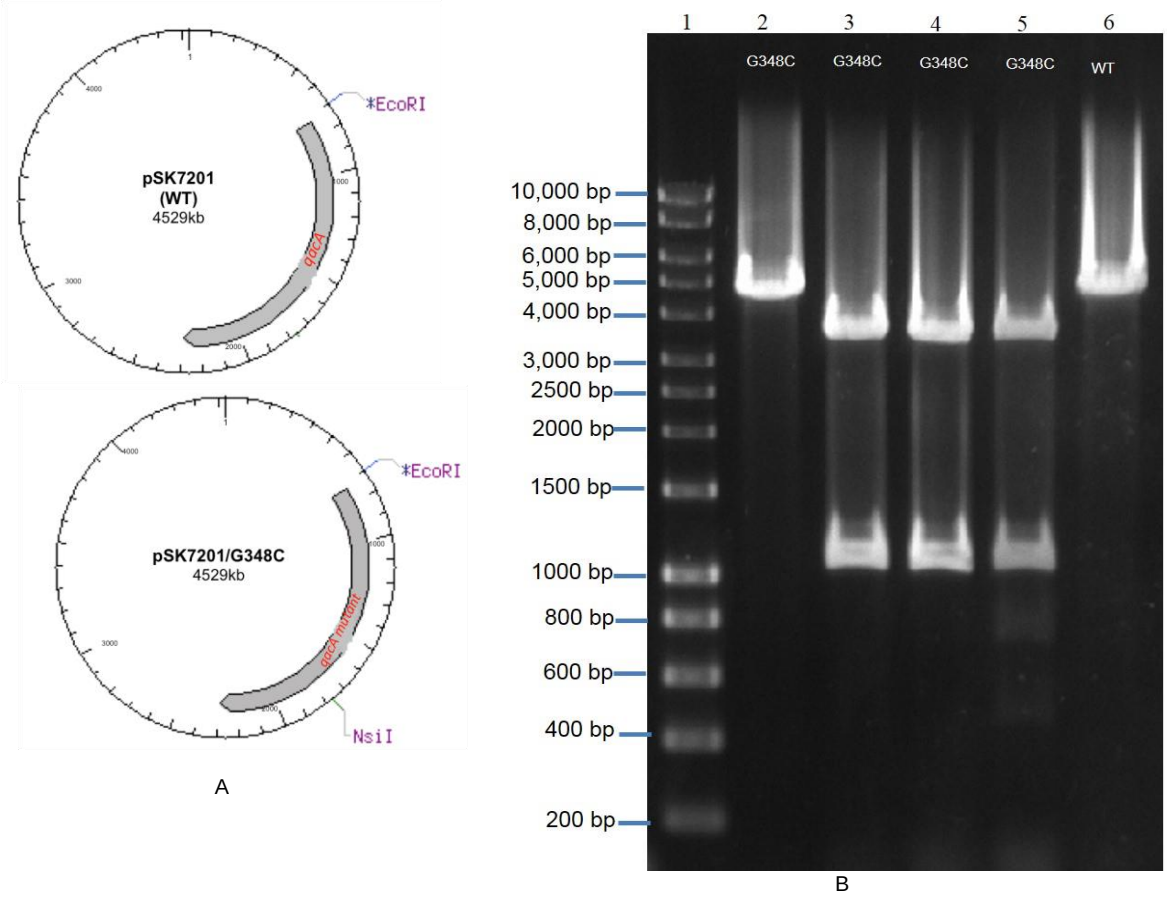

Figure 2. Restriction site analysis of the G348C QacA plasmid

The plasmid pSK7201 (4529bp) was used as the template for site-directed mutagenesis. The *qacA* gene is shown with the direction of transcription indicated by the grey arrow. Naturally occurring restriction sites for the enzymes *Dde*I, *Bsg*I, *Pvu*I, and *Eco*RI are shown. The plasmid pSK7201 does not contain *Nsi*I or *Nde*I restriction sites which were also used for plasmid identification.

The plasmid pSK7201 was used as the template to produce the mutant G348C by site-directed PCR using primers designed to incorporate a cysteine codon. (A) The PCR product was treated with *Dpn*I to digest the template DNA and then used to transform *E. coli* DH5α cells. Plasmid DNA was isolated from transformants then double digested with *Eco*RI and *Nsi*I to obtain two fragments or one fragment for the WT plasmid. (B) Digested plasmid DNA was electrophoresed on 1% (w/v) agarose gel. Lane 1 is the 1kb Bioline DNA HyperLadder^{TM} I with standard fragment sizes shown. Lane 2 is a non-mutated plasmid with one band, which is the same as the band in WT, lane 6. Lanes 3, 4, and 5 show plasmid digests with two DNA fragments (3.4kb and 1.1kb) due to the *Nsi*I site being successfully incorporated by the site-directed mutagenesis.

Confirmed mutation by sequencing

To confirm that the codons encoding the cysteine were incorporated into the *qacA* sequence at the correct position and that no other unexpected nucleotide substitutions had occurred, the whole *qacA* gene in each mutant plasmid was sequenced by AGRF. All returned *qacA* sequences were examined and aligned to the WT *qacA* gene (Genbank accession number GU565967.1) using the Sequencher^{TM} 4.9 program. A plasmid was identified with a cysteine codon inserted in the correct position for each of the 15 amino acid substitutions within TMS 11 as shown in **Figure 3**.

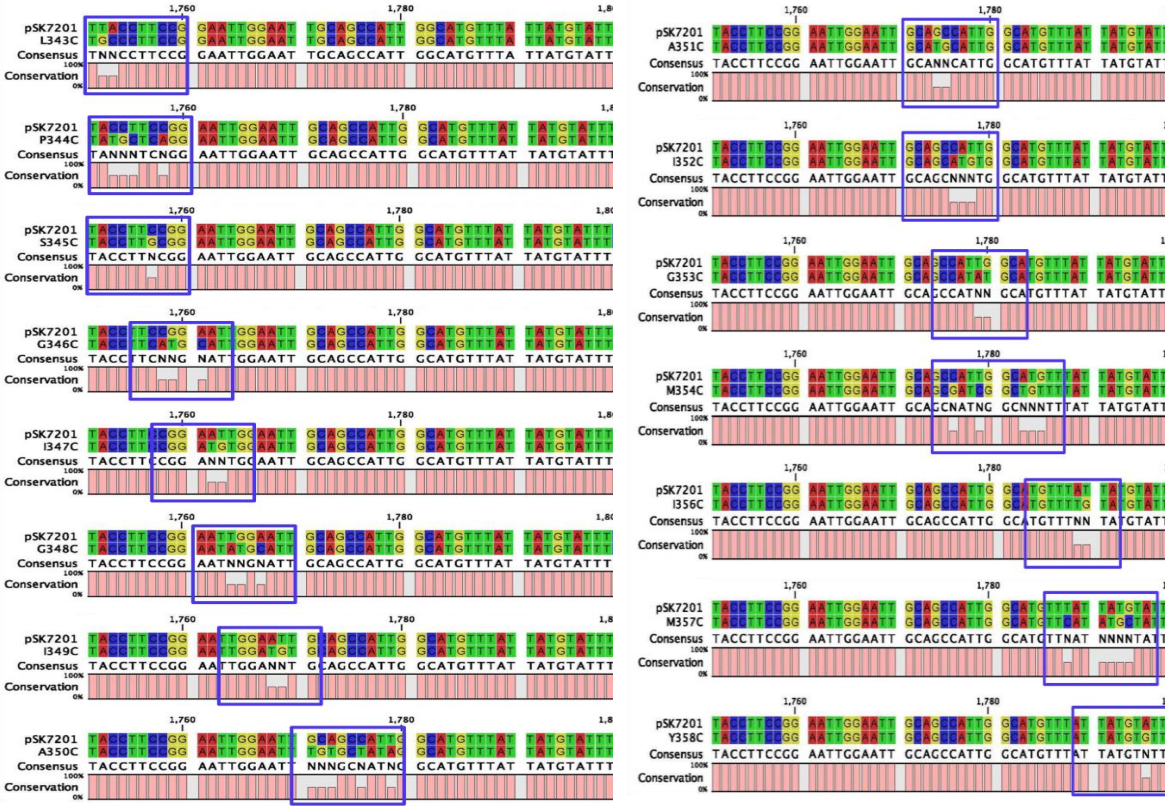

Figure 3. Sequencing analysis of the QacA mutant proteins

Sequencing was performed. The mutated region of the sequences (boxed in blue) generated from each mutated plasmid (lower sequence) was aligned with the equivalent region in the wild type *qacA* sequence within pSK7201 (top) (Genbank ID GU565967.1) using CLC sequence viewer 7. The bars below indicate the sequence conservation and identify the position of the cysteine codon and where a restriction site was incorporated. The entire *qacA* gene sequence of each mutant was determined and aligned to ensure no other unexpected mutations were present (not shown) using the Sequencher^{TM} 4.9 program (Gene Codes Corporation, USA).

Conclusions

The mutation process of the amino acids within TMS 11 of QacA was performed using cysteine-directed PCR mutagenesis. A total of 15 amino acids were successfully substituted (L343C, P344C, S345C, G346C, I347C, G348C, I349C, A350C, A351C, I352C, G353C, M354C, I356C, M347C, and Y358C) using site-directed mutagenesis PCR. The cysteine substitution for each amino acid within TMS 11 was confirmed in each plasmid using restriction enzyme analysis and sequencing of the mutant *qacA*. This research is one of the main steps for studying the function of TMS11 in the efflux function of the QacA protein in *S. aureus* resistance.

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