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ARTÍCULOS ORIGINALES

DISCOVERY OF THE NOVEL AUXILIARY FACTOR FOR B-LACTAM RESISTANCE BLRA IN COMMUNITY-ACQUIRED METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most important human pathogens that cause a variety of infections in community and hospital settings. This organism displays high levels of resistance to β-lactam antibiotics and this principally occurs due to the production of β-lactamase and the penicillin binding protein PBP2a encoded by *mecA* gene. Furthermore, additional mechanisms, such as auxiliary factors, significantly contribute to β-lactam resistance. However, the function of most of these factors is still unknown.

Recently, in our laboratory, the novel auxiliary factor gene *blrA* was initially identified by transposon mutagenesis in the community-acquired MRSA strain JE2. Next, the transposon mutation was transduced to the community-acquired MRSA strain MW2, resulting in the formation of a transposon mutant for *blrA (blrA::ermB)*, denoted as MW2A. Initially, our results suggested that MW2A is more susceptible to oxacillin than the MW2 wild-type. Then, we performed the Minimum Inhibitory Concentration (MIC) method for the β-lactam antibiotics: oxacillin, cefoxitin, cephradine, ceftazidime, cefaclor and imipenem. MW2A exhibited a two-fold reduction for all tested β-lactam antibiotics compared to the MRSA wild-type strain MW2. Additionally, no phenotypical changes were observed after performing PBP2a latex agglutination test and phage spot assay. Finally, using DeepTMHMM we show that BlrA is likely to act intracellularly, which is typical of auxiliary factors.

In this study, we show that BlrA is clearly involved in β-lactam resistance, but does not affect the expression of the gene *mecA* or the structure of the wall teichoic acids. Furthermore, we propose that that BlrA acts as an auxiliary factor and is activated in response to antimicrobial stress.

Key words: *auxiliary factors, Staphylococcus aureus, antibiotic resistance, β-lactam antibiotic.*

1. INTRODUCTION

S. aureus is both a commensal and a pathogenic organism. This organism colonises the nasal nares of nearly 30% of the human population (1). The principal site of colonisation is the nose; however, other studies suggest that other parts of the body may be colonised as well (2). *S. aureus* causes a variety of suppurative infections and toxin-mediated infections (3). *S. aureus* has become a real threat in the community, hospital settings and livestock due to the fact that this organism has acquired resistance to many antibiotics by different mechanisms, including vancomycin as in the case of vancomycin insensitive *S. aureus* or vancomycin resistant *S. aureus* (4).

S. aureus as other gram-positive bacteria has a very thick cell wall, which is mainly composed of many layers of peptidoglycan (PG). PG is a polymer that mainly consists of two alternating sugars: N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues, which are linked via β-(1-4) glycosidic bonds (5). A tetrapeptide side chain is attached to the carboxyl group of the N-acetylmuramic acid residue and is composed of four alternating D- and L-amino acids: L-alanine, D-iso-glutamic acid, L-lysin and D-alanine (5). The PG of *S. aureus* contains a pentaglycine interbridge, which crosslinks different tetrapeptides by connecting the carboxyl group of the terminal D-alanine to the amino group of L-lysine thus forming a mesh-like interconnected network (6).

For long time to the present, the diseases caused by *S. aureus* have been treated using β-lactam antibiotics. The most important structure shared by all the antibiotics that belong to this group is the β-lactam ring, which is crucial for their activity (7). β-lactam antibiotics have a similar structure to the D-alanyl-D-alanine end of the peptidoglycan (8). Therefore, β-lactam antibiotics competitively inhibit the enzymes involved in the process of transpeptidation, which involves the tetrapeptide crosslink formation (8, 9).

Methicillin-resistant *S. aureus* (MRSA) are strains of *S. aureus* that have developed intrinsic resistance to β-lactam antibiotics such as methicillin, oxacillin or nafcillin, cephalosporins and carbapenems (10). Infections caused by MRSA were first detected in healthcare facilities (healthcare acquired/associated (HA) MRSA). HA-MRSA is considered one of the major nosocomial pathogens in healthcare settings (11). Nevertheless, cases of MRSA in the community (community acquired/associated (CA) MRSA) and from livestock (livestock acquired/associated (LA) MRSA) have been reported in the last decades (12). Recently, it was found that some CA-MRSA isolates are becoming more resistant to several non β-lactam antibiotics as well such as tetracycline, erythromycin or levofloxacin (13).

All MRSA strains possess two main resistance determinants: *blaZ* and *mecA* (14, 15). The product of the gene *blaZ* is a β-lactamase, which provides resistance to penicillins only. The gene *mecA*, in turn, encodes an extra PBP which has low affinity to penicillins and is referred to as penicillin binding protein 2a (PBP2a). This last enzyme is able to perform the cross-linking of the cell wall even in the presence of β-lactam antibiotics and is the principal mechanism of resistance to β-lactam antibiotics (16). The gene *mecA* is localised in a specific mobile genetic element known as staphylococcal chromosome cassette (SCC*mec*), which is absent in methicillin-susceptible Staphylococcus aureus (MSSA) (17).

Different studies using mutants produced by transposon mutagenesis show that the expression of PBP2a is necessary for the resistance of MRSA to β-lactam antibiotics, but not sufficient. Therefore, apart from *mecA* and *blaZ*, many other genes involved in resistance to β-lactam antibiotics have been identified and named such as fem genes (factors essential for methicillin resistance) and aux genes (auxiliary genes). Both of these factors are not related to *mecA* gene (18). Although the mechanisms of resistance to β-lactam and other group of antibiotics employed by S. aureus have been studied for many years, there are still many unclear mechanisms. The aim of the present study is to identify phenotypic changes in CA-MRSA *S. aureus* strain MW2 bearing a mutation in the putative auxiliary gene *blrA* (NRS_1669), encoding the protein BlrA (WP_000080029).

2. METHODOLOGY

Bacterial strains and growth conditions

All bacterial strains and phages used in this study are listed in Table 1. Bacterial strains were cultivated on Columbia Blood Agar (CBA) and Tryptic Soya Broth (TSB) (Thermo Fisher Scientific) at 37°C. For the cultivation of the MW2A mutant and the complemented mutant S. aureus c-MW2A media were supplemented with erythromycin and chloramphenicol, respectively.

Table 1: *List of bacterial strains and phages used in this study.*

Bacterial strain or phage	Genotype	Source
S. aureus strain JE2	Wild type	(19)
JE2A	blrA::ermB	(19)
S. aureus strain MW2	Wild type	This study
MW2A	blrA::ermB	This study
Podophage ϕ68	-	This study

S. aureus genomic and plasmid DNA isolation

The genomic DNA was extracted from S. aureus with the aid of a FastPrep®-24 instrument (MP Biomedicals, Santa Ana, CA, USA), using the default settings. The concentration of the isolated DNA was measured using the Nanodrop machine (Thermo Fisher Scientific, USA).

The plasmid was extracted from the E. coli DC10B using the miniprep plasmid purification kit (Qiagen) according to manufacturer's instructions.

DNA amplification

The primers used for DNA amplification were designed based on the sequence data of the Nebraska transposon mutant in order to detect

the erythromycin resistance gene marker *ermB* of the transposon, located within the *blrA* mutant allele. For this purpose, we used the primers listed on Table 2.

Table 2: *List of primers used in this study*

Primer	Sequence
blrA-J2-705up	5' TTGTTCTTTAGGTCTTTCCA 3'
blrA-J2-705dn	5' ATGTCGCAAAAAGTGTTGGT 3'

The amplification of fragments was performed in 25 µL volumes with Premix Taq™ DNA Polymerase (Bioline, Australia), 1 µL of each 20 µM primer set (forward and reverse) and 100 ng of genomic DNA. Each fragment was afterwards amplified in a thermocycler (Gene Amp PCR System 9700) using conventional Hot Start PCR. For the detection of *ermB* the following conditions were applied: 95°C for 1 min, 25-35 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 1 min, with a final extension at 72°C for 10 min. For the detection of the *blrA* mutant allele, the following conditions were applied: 95°C for 1 min, 25-35 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 50 s, with a final extension at 72°C for 10 min. The DNA fragments were visualised with the aid of agarose gel electrophoresis. The band size was verified using the 1 Kb plus DNA Ladder Thermo Scientific™.

BSAC Standardised Disc Diffusion Method

The bacterial suspension was adjusted in order to achieve a turbidity equivalent to a 0.5 McFarland standard. After this, the suspension was spread evenly using sterile swab over the entire surface of commercially prepared Mueller Hinton agar plates. Afterwards, within 15 min, with the aid of a disc dispenser (Oxoid, Basingstoke, Hampshire, UK), the antibiotic discs were firmly applied to the surface of the plate. The required content of the antibiotic discs was determined according to BSAC guidelines. The plates were incubated at 37°C overnight and the zone of inhibition around the disc was observed the following day.

Minimum inhibitory concentration (MIC) determination by broth microdilution method

Antibiotic stock solutions were prepared from antibiotic powders to obtain a concentration of 10 mg/L. All the antibiotics were sterilised with the aid of 0.22-µm membrane filters. All β-lactam antibiotics were two-fold serially diluted starting from a concentration of 512 mg/L. Finally, 0.1 mL of each antibiotic dilution was transferred to wells of a 96-well microplate.

The bacterial suspension was adjusted to an optical density of 0.1 at 578 nm (OD_{578}). Then, 5 µL of the bacterial suspension was added to each well of the 96-well microplate. The microplate was incubated overnight. Minimum inhibitory concentration (MIC) is defined as the lowest antibiotic concentration that completely inhibits the growth of the studied organism [4]. Therefore, the MIC was established by comparing the wells containing the tested organism with the negative (no growth) and positive controls (growth). The well with no visible growth (cloudy solution or a dot at the bottom) was defined as the MIC.

Penicillin-binding protein (PBP2a) latex agglutination test

This agglutination test was performed using the Oxoid™ PBP2' Latex Agglutination Test Kit and following manufacturer's instructions. Agglutination is observed with the test organisms if they produce PBP2a, but not with test organisms that lack PBP2a or the Control Latex within three minutes.

Phage spot assay

A single colony of the studied organism was taken from the stock plates and suspended in TSB containing erythromycin, chloramphenicol or no antibiotic depending on the organism. The broth was then incubated overnight using a shaking incubator at 37°C and 200 rpm. On the following day, the broth suspension of the organism was diluted and adjusted to an optical density of 0.1 at 578 nm (OD_{578}) using a spectrophotometer (Thermo Scientific™ GENESYS 10S UV-Vis) to measure the OD. Then, 100 µL from the suspension were taken and thoroughly mixed with 5 mL of pre-warmed soft agar. Then, the pre-warmed soft agar containing the suspension was poured onto CBA plates. Once the soft agar had set, 5 -10 µL of a suspension containing the podophage ϕ68 were applied to the overlay and the plates were aerobically incubated at 37°C for 24 hours.

3. RESULTS

Previously, in our laboratory, the function of genes required for β-lactam resistance was studied with the aid of transposon mutagenesis, whereby a transposon mutant library was constructed based on *S. aureus* strain JE2 and mutants with reduced oxacillin resistance were isolated from this library (unpublished data). The transposon used for the generation of the mutant library contains an erythromycin resistance (*ermB*) cassette, which enables the selection of the mutant organism on selective media containing erythromycin (19). Thereby, we identified a novel gene involved in β-lactam resistance with a size of 705 bp. This gene was designated as *blrA* since the product of this gene shares an amino acid sequence similarity of 41.33% with the protein BlrA (WP_041210465) from *Aeromonas jandaei*. An *S. aureus* strain JE2 transposon mutant with the *blrA* gene disrupted (JE2A) exhibited a reduced β-lactam resistance when compared to wild type *S. aureus* strain JE2 (unpublished data). Next, this mutation was transferred from this mutant strain to *S. aureus* strain MW2 (MW2A) using phage ϕ11-mediated transduction. The transposon disruption of *blrA* in MW2A was verified with the aid of a PCR reaction (Fig. 1).

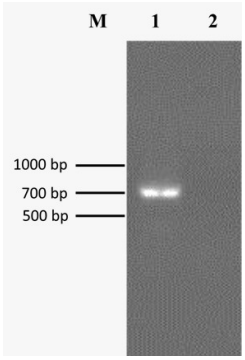

Figure 1: *Agarose gel electrophoresis analysis of PCR products amplified from genomic DNA of MW2 and MW2A using blrA-specific primers. M – 1 kb plus DNA Ladder; 1 – PCR amplification of the wild-type gene blrA from MW2, which has a size of 705 bp; 2 – absence of PCR amplification due to transposon disruption of blrA in MW2A.*

The transposon mutant MW2A was resistant to β-lactam antibiotics

Our preliminary results using disc diffusion test showed that MW2A is more susceptible to oxacillin than MW2 (Fig. 2). Next, we performed the MICs of the MW2 wild type and the *blrA* transposon mutant MW2A to the β-lactam antibiotics listed In Table 3. The *blrA* transposon mutant MW2A exhibited a two-fold reduction for all β-lactam antibiotics used in this study (Table 3).

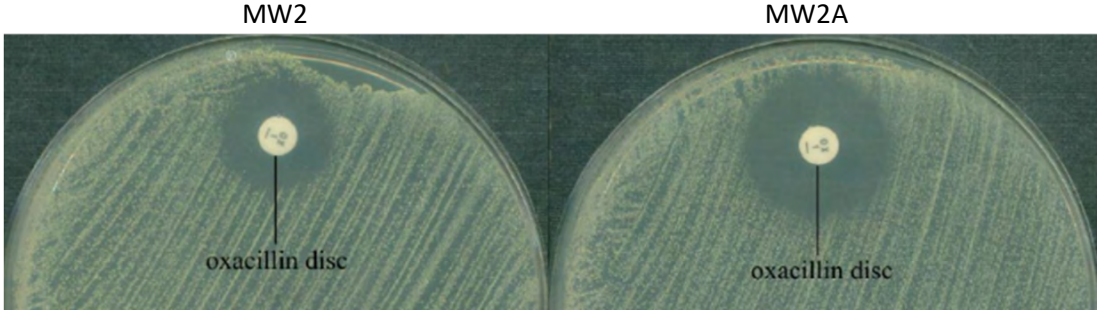

Figure 2: *Disc diffusion test using an oxacillin disc. The inhibition zones on the left and right have a diameter of 19 mm and 25 mm, respectively. This suggests that MW2A is more susceptible to oxacillin than MW2.*

Table 3: *MICs for MW2 and MW2A.*

	MIC[1] (µg/mL)					
	Oxacillin	**Cefoxitin**	**Cephradine**	**Ceftazidime**	**Cefaclor**	**Imipenem**
MW2	32	32	128	128	64	1
MW2A	16	16	64	64	32	0.5

[1] Values are means of three biological replicates.

Detection of PBP2a expression in the mutant MW2A

Some auxiliary factors are crucial during cell wall synthesis or may be involved in the regulation of the activity of the gene *mecA* (20). Therefore, we proposed that the mutation *blrA* may somehow affect the expression of *mecA* or the cell wall assembly. In order to prove this, PBP2a agglutination latex test was performed. As shown

in Fig. 3, agglutination was observed in both the wild type *S. aureus* MW2 and the mutant MW2A.

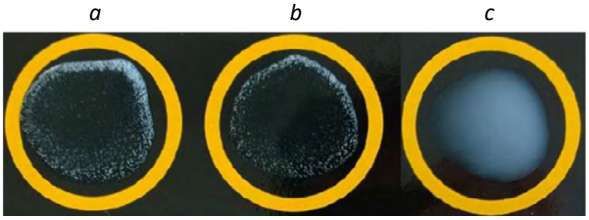

Figure 3: *PBP2a agglutination latex test; a: MW2; b: MW2A; c: negative control. The figure shows agglutination for both MW2 and MW2A, suggesting that both strains produce the penicillin-binding protein PBP2A.*

Detection of possible structural changes in the cell wall of the mutant MW2A

Considering also the possibility that BlrA may be crucial for the regulation of the expression of other genes that are involved in the synthesis of specific components of the cell wall, including WTAs, we decided to prove if the mutation in this auxiliary gene affects the expression of genes required for the synthesis of components of WTAs. It is also known that WTAs serve as phage receptor and, in consequence, any mutation in auxiliary genes may prevent phage adsorption (21). Therefore, phage spot assay was performed. As shown in Fig. 4, plaques were observed in both organisms.

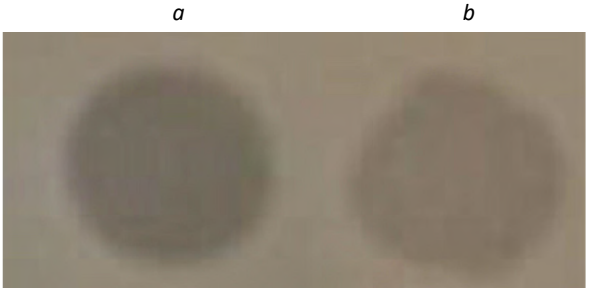

Figure 4: *Phage spot assay; a: MW2; b: MW2A. The figure shows a clear spot on lawns produced by MW2 and MW2A, demonstrating that phage is able to infect both strains.*

Prediction of transmembrane helices in BlrA.

Since many auxiliary factors for β-lactam resistance module the cell response to antimicrobial stress either directly, if they are membrane-bound, or indirectly, if they are intracellular, we used the DeepTMHMM (Transmembrane Helices; Hidden Markov Model) software (22) to determine whether BlrA is attached to membrane or not. As shown in Fig. 5, BlrA is not attached to a membrane and is more likely an intracellular protein.

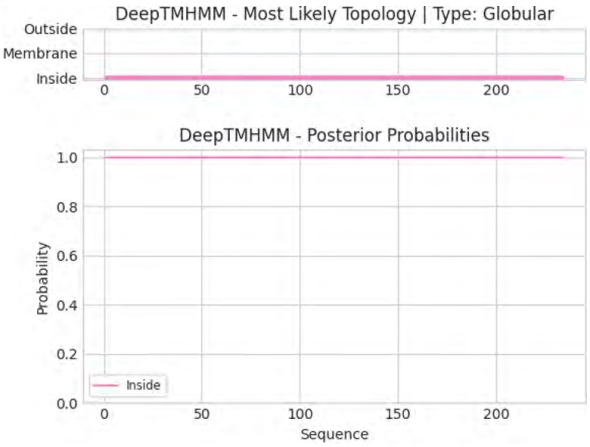

Figure 5: *DeepTMHMM protein topology prediction of BlrA. This figure shows that BlrA is a non-bound to membrane protein, which is more likely located inside the cell.*

4. DISCUSSION

BlrA was previously described as a β-lactam response regulator transcription factor in *Aeromonas hydrophila*. This protein belongs to the family of phosphorylation-dependent response regulators and activates three β-lactamases (23). Therefore, we proposed that BlrA is also involved in β-lactam resistance in MRSA strains. In this study, we observed that the mutation of the novel gene *blrA* in *S. aureus* strain MW2 decreased the resistance of this organism to the β-lactam antibiotics used in this study.

PBP2a is the most important protein involved in β-lactam resistance because it participates in the process of synthesis of the cell wall even in the presence of β-lactam antibiotics (16). It is known that some auxiliary factors may regulate the activity of PBP2a. For instance, these factors may exert a posttranscriptional effect on PBP2a, probably, by mediating the export or folding of this protein (24). In this study, we observed positive agglutination in both MRSA MW2 wild type and the mutant MW2A during the PBP2a latex agglutination test. This suggests that the mutation has no effect in the expression of the gene *mecA* since the mutant also produces PBP2a. Hence, the mechanism of action of the gene *blrA* is, like most of auxiliary genes (25), *mecA*-independent and this may confirm our previous supposition that the product of the gene *blrA* is crucial for the regulation of other genes required for β-lactam resistance.

BlastP analysis reveals that BlrA shares a 42.17% identity with PhoB. The latter protein

is an intracellular response regulator that was previously linked to the regulation of antimicrobial resistance (26). Following a DeepTMHMM analysis (Fig. 5), we predicted that BlrA is not bound to membrane similar to PhoB. Therefore, BlrA in S. aureus is very likely to act as an intracellular response regulator and regulates genes involved in β-lactam resistance in response to antimicrobial stress. Furthermore, we can speculate that BlrA is part of an unknown regulon like PhoB. Further studies will reveal which genes are regulated by BlrA and the regulon that is controlled by this protein.

There are some reports suggesting that mutations in auxiliary genes may affect the synthesis of components of the cell wall like, for example, WTAs, which are the most abundant components of the cell wall (21). In this study, we observed absolutely no difference between the wild type and the mutant in terms of phage plaque formation, suggesting that the mutation did not reduce the affinity of the phage ϕ68 to the WTAs; therefore, the mutation does not affect the structure or assembly of WTAs.

The increasing amount of antibiotic-resistant strains is a concern for global health. It is estimated that by 2050 ten million people worldwide will die due to antibiotic resistance (27). Understanding the mechanism of action of auxiliary factors will provide a valuable tool to combat the rise of antibiotic resistance. Currently, alternative strategies, such as phage therapy and combination of antibiotics, are being considered in order to tackle antibiotic resistance (28, 29).

5. CONCLUSION

The present study demonstrates that the auxiliary factor BlrA increases the resistance of the CA-MRSA strain MW2 to β-lactam antibiotics. This auxiliary factor does not affect the expression of *mecA* and does not affect the structure of the bacterial cell wall. However, it more likely acts intracellularly and regulates the expression of β-lactamases and thus increases resistance to β-lactam antibiotics.

Future studies will be required to confirm the function of BlrA. This involves the creation of a clean *blrA* knock-out. Next, in order to verify if the phenotype is due to the *blrA* mutation, it is important to create a complement mutant. This is usually performed using the plasmid pRB474 as described in previous studies (30). The complement mutant should exhibit a similar phenotype as the wild-type strain. Additionally, the protein BlrA can be expressed and purified to study its specific activity.

6. CONFLICT OF INTEREST

The author declares no conflict of interest

7. LIMITATIONS OF LIABILITY

The author takes complete responsibility for the information presented in this original scientific article.

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