The Journal of Phytopharmacology 2018; 7(3): 298-304
Online at: www.phytopharmajournal.com

Research Article

ISSN 2320-480X
JPHYTO 2018; 7(3): 298-304
May- June
Received: 30-05-2018
Accepted: 22-06-2018

Luke G. Huggins
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Kathryn D. Robinson
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Kyra P. Lasko
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Lauren B. Clower
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Avery J. Gookin
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Dustin E. Segraves
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

James C. Gainer
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Grant P. Basagic
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Kelly R. Machuca
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Jacobo Rendon
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA

Correspondence:
Luke G. Huggins
Department of Biology and Environmental Science, West Virginia Wesleyan College, Buckhannon, WV, USA
Email: huggins[at]wvwc.edu

Screening for community-acquired strains of methicillin-resistant *Staphylococcus aureus* susceptible to extracts of *Centaurea nigrescens*

Luke G. Huggins*, Kathryn D. Robinson, Kyra P. Lasko, Lauren B. Clower, Avery J. Gookin, Dustin E. Segraves, James C. Gainer, Grant P. Basagic, Kelly R. Machuca, Jacobo Rendon

ABSTRACT

The rates of infection by community-acquired multi-drug resistant *Staphylococcus aureus* have risen dramatically over fifteen years in the United States. Community-acquired multi-drug resistant *Staphylococcus aureus* is responsible for rapidly progressive diseases, including necrotizing pneumonia, severe sepsis, and necrotizing fasciitis. Consequently, novel antibacterial strategies are needed to combat the rising antibiotic resistance seen in community-acquired multi-drug resistant strains. We have screened the Nebraska Transposon Mutant Library for MRSA strains that are either susceptible or resistant to methanol extracts of *Centaurea nigrescens* leaves and flowers. 10 strains containing mutations affecting transporter proteins were identified as having either significant resistance or susceptibility to Centaurea extract. Insertions in two different drug efflux transporter families have been identified. The EmrB/QacA drug resistance transporter subfamily is a multi-drug efflux pump responsible for the export of toxic molecules from bacteria and yeast. The ABC transporters are involved in drug import and export. These results confirm the effectiveness of the screen as a means for identifying drug-resistance genes affected by the *C. nigrescens* methanolic extract and suggest a role for drug efflux proteins in the resistance of *S. aureus* community-acquired multi-drug resistant *Staphylococcus aureus* to antibacterial plant metabolites.

Keywords: *Centaurea*, Antibacterial, MRSA, *Staphylococcus aureus*, Screen.

INTRODUCTION

The rates of infection by community-acquired multi-drug resistant *Staphylococcus aureus* (CA-MRSA) have risen dramatically since the introduction of methicillin [1-3]. CA-MRSA infections differ significantly from hospital-acquired infections. While primarily associated with skin and soft tissue infections in previously healthy individuals within a localized community [4-6], CA-MRSA may occasionally cause invasive and severe infections including necrotizing pneumonia, necrotizing fasciitis, pyomyositis, osteomyelitis, and sepsis [7, 8].

The value of folk medicine and herbal remedies as sources for medicinal lead compounds has been recognized worldwide [9]. These sources have led, in part, to the discovery of important medicines, such as digitalis [10] and paclitaxel [11, 12]. In addition, other drugs are modifications of plant-derived compounds [13]. We have previously examined the antibacterial and cytotoxic properties of extracts from the red mangrove (*R. mangle*). Our results have shown that extracts of the red mangrove propagule, a viviparous seed with primitive root development, inhibited bacterial growth [14].

Knapweeds (Centaurea spp.) have been used in Anatolian folk medicine to treat a variety of ailments [15]. Of the approximately 500 species of Centaurea, several have been shown to produce novel secondary metabolites [16] and have antibacterial activity versus *S. aureus* [17]. Antibacterial compounds are important lead compounds for drug discovery, but few studies have looked at the effects of these compounds on CA-MRSA strains [18]. The tyrol knapweed, *Centaurea nigrescens*, is found locally in West Virginia and throughout the United States, but little is known about the complement of bioactive compounds it produces. We have examined the effect of *C. nigrescens* methanolic extract (CnME) on the growth of the USA300 CA-MRSA strain [19].

To understand the mechanism of action and potential molecular targets of the active compounds produced by *C. nigrescens*, we screened the Nebraska Transposon Mutant Library (NTML, BEI Resources. Manassas, VA, USA) versus CnME. The parental USA300 CA-MRSA strain was modified by the Center for Staphylococcal Research (CSR) at the University of Nebraska Medical Center to

include a mariner-based transposon, *bursa aurealis* [20-22]. Over 1,900 disruption mutants were generated, and the transposon insertion sites of each were mapped to specific, non-essential genes in *S. aureus*. We have screened the NTML using Kirby-Bauer disc diffusion assays [23] to identify mutant strains that exhibit either increased susceptibility or increased resistance to CnME with the goal of identifying mutant strains that are preferentially affected.

As a result of this screen, we have identified a group of drug-transporters that when mutagenized show differential growth in the presence of CnME. Over 100 ABC transporter subunits have been identified in the NTML with another 50 transport proteins from other classes. We have been able to show that 10 transporters are preferentially affected by CnME. This group of transporters are potential targets of attack to inhibit the growth of CA-MRSA.

METHODS

C. nigrescens was collected at sites LJS20 and PH33, in and around Buckhannon, WV. Plant material was air-dried, and stored at -20 ºC until extraction. 100 g of leaves, buds, and flowers from *C. nigrescens* were homogenized in 2 L methanol at 25 ºC until well blended. The resulting extract was filtered to remove insoluble material, and was concentrated by rotary evaporation at 45 ºC. The dried *C. nigrescens* methanol extract (CnME) was resuspended at 50 mg/ml in methanol. Samples were aliquoted and stored at -80 ºC.

The CnME was initially screened for antibacterial activity versus the parental CA-MRSA strain USA300 via a Kirby-Bauer disc diffusion assay [23]. USA300 CA-MRSA was maintained on Luria Bertani agar (Thermo Fisher Scientific, Waltham, MA, USA) and grown overnight in Luria Bertani broth at 37 °C. 25 µl (1.25 mg) of CnME in was spotted on 6 mm filter paper discs and allowed to air dry. Discs were incubated in triplicate on Mueller-Hinton agar (Thermo Fisher Scientific, Waltham, MA, USA) streaked to create a uniform lawn from overnight cultures of USA300. Inoculated plates were incubated 18 hours at 37 °C. The zone of inhibition around the filter discs were determined for each treatment. A methanol vehicle and 10 µg streptomycin (Thermo Fisher, Waltham, MA, USA) control discs were included with each treatment.

CnME extracts that showed inhibition of USA300 growth in the Kirby-Bauer assay were used to screen the NTML. All strains were assayed in triplicate versus CnME and vehicle as described above. The mean zone of inhibition was determined. A KB score for each strain was determined by subtracting the diameter of the mean zone of inhibition of the USA300 parental strain from the mean zone of inhibition of the transposon-mutated strains.

The minimal inhibitory concentration (MIC) of the CnME was determined by resazurin assay [24]. A two-fold dilution series of CnME versus the methanol vehicle was constructed across a 96-well plate beginning with either 1.0 mg CnME in 20 µl methanol, 20 µl of methanol, or 20 µl of 5 mg/ml streptomycin as a positive control. In addition, a 2-fold dilution series of CnME in Mueller-Hinton broth (MHB) (Thermo Fisher Sceintific, Waltham, MA, USA) was used to background-correct each dilution. Each well received 30 µL of USA300 diluted to 0.5 McFarland units in Mueller-Hinton broth, and contained 1.8 µg resazurin. Wells were adjusted to a final volume of 100 µl with MHB. The 96-well plates were incubated at 37 °C for 24 hours. The reduction of resazurin to resorufin was determined by subtracting the absorbance at 600 nm of resazurin from absorbance at 570 nm of resorufin for each dilution at 3 and 24 hours post inoculation. The absorbance values for the CnME blank were subtracted from the CnME assay values at each dilution to determine the corrected A570-600 values resulting from the CnME treatment. The methanol vehicle control was compared to the corrected CnME values at each dilution using a T-test to determine if the dilutions demonstrated a significantly different bacteriostatic activity ($p \geq 0.01$). The MIC was defined as the most dilute concentration that showed a significant inhibitory activity versus the vehicle control.

The effect of CnME on USA300 growth was determined by incubating 100 µl on USA300 overnight culture in 2 ml MHB at 37 °C for 24 hours. Optical density was measured at 600 nm every 30 minutes for 7.5 hours, and then again at 24 hours. USA300 was treated in triplicate with 2xMIC (0.6 µg/mL CnME), 1xMIC (0.3 µg/mL CnME), methanol vehicle control, and MHB negative control.

RESULTS

The CnME extract had an average diameter of inhibition of 8.7 mm when assayed versus the parental USA300 strain. Strains within the NTML were assayed and compared daily to the zone of inhibition of the parental USA300 strain. A KB score was determined for each mutant strain by subtracting the mean zone of inhibition diameter (mm) of the parental USA300 strain (n=3) from the mean zone of inhibition diameter (mm) for the mutant strain (n=3). The average KB score for the 1912 assayed strains in the NTML was 0.1 +/- 1.1 (mean +/- 1 SD). Strains with KB scores >2 standard deviations away from the mean were selected for further analysis. 66 strains were found to have KB scores >2SD (2.3), and 18 strains were found to have KB scores <2SD (-2.0).

The mutated strains with KB scores >2SD (2.3), are more susceptible to CnME, and we expect to find genes that encode proteins that under normal conditions either export or detoxify compounds in their environment. When mutated, the gene products from these strains result in decreased resistance to CnME. Of the 66 strains with KB scores >2.3, 9 strains have disruptions in known transport proteins, with an additional 25 unknown, hypothetical proteins (Table 1). In addition, we should see mutant lines with disrupted housekeeping genes in this group. Any mutant that is, in general, less healthy should be less resistant to CnME. As predicted, EmrB/QacA subfamily drug resistance transporter [25], 5 ABC transport proteins [26], and pbp4 penicillin-binding protein 4 [27] were identified.

Table 1: USA300 Strains with KB scores two standard deviations greater than the mean.

Strain	Gene	Description	KB Score
NE377		conserved hypothetical protein	4.3
NE1876	panC	pantoate--beta-alanine ligase	4.0
NE1063		chorismate-binding domain-containing protein	3.7
NE1221		chaperone protein HchA	3.7
NE1252	phnC	phosphonate ABC transporter ATP-binding protein	3.7
NE1829		2-oxoisovalerate dehydrogenase, E1 component, beta subunit	3.7
NE182		conserved hypothetical protein	3.7
NE1113	ureG	urease accessory protein UreG	3.5
NE1117		hypothetical protein	3.5
NE1507		hypothetical protein	3.5
NE378		putative membrane protein	3.3
NE376		putative membrane protein	3.3
NE923		hypothetical protein	3.3
NE1798	dut	dUTP diphosphatase	3.2
NE186	fnbA	fibronectin binding protein A	3.0
NE1008	sdaAB	L-serine dehydratase, iron-sulfur-dependent, beta subunit	3.0
NE12		drug resistance transporter, EmrB/QacA subfamily	3.0
NE248		conserved hypothetical protein	3.0
NE345		membrane protein	3.0
NE1569		superantigen-like protein	3.0
NE1578		isochorismate synthase family protein	3.0
NE1790		ABC transporter ATP-binding protein	3.0
NE1218		TENA/THI-4 family protein	3.0
NE1219		putative ATP-dependent Clp proteinase	3.0
NE1508		D-lactate dehydrogenase	3.0
NE760		transcriptional regulator, Fur family	2.8
NE1107	cinA	competence/damage-inducible protein cinA	2.8
NE1567	dapB	dihydrodipicolinate reductase	2.8
NE1022	fmt	fmt protein	2.8
NE575	cap5G	capsular polysaccharide biosynthesis protein cap5G	2.7
NE375	ear	Ear protein	2.7
NE567	epiG	lantibiotic epidermin immunity protein F	2.7
NE566	icd	isocitrate dehydrogenase, NADP-dependent	2.7
NE681	kdpB	K+-transporting ATPase, B subunit	2.7
NE679	pbp4	penicillin-binding protein 4	2.7
NE184		conserved hypothetical protein	3.0
NE569		conserved hypothetical protein	3.0
NE1398		conserved hypothetical protein	3.0
NE1112		hypothetical protein	3.0
NE845		hypothetical protein	3.0
NE1877		hypothetical protein	3.0
NE183		glycerate dehydrogenase-like protein	2.7
NE388		sodium-dependent transporter	2.7
NE767		putative chromosome partioning protein, ParB family	2.7
NE759		5'-nucleotidase, lipoprotein e(P4) family	2.7
NE1036		putative AMP-binding enzyme	2.7
NE1822		ABC transporter permease	2.7
NE1204		antibiotic transport-associated protein-like protein	2.7
NE1116	nsaS	nisin susceptibility-associated sensor histidine kinase	2.7

NE1314	oppC	oligopeptide ABC transporter permease	2.7
NE181		conserved hypothetical phage protein	2.7
NE758		conserved hypothetical protein	2.7
NE568		conserved hypothetical protein	2.7
NE1375		conserved hypothetical protein	2.7
NE1106		hypothetical protein	2.7
NE1111		hypothetical protein	2.7
NE1771		hypothetical protein	2.7
NE1681		hypothetical protein	2.7
NE1631		hypothetical protein	2.7
NE1505		hypothetical protein	2.7
NE683	oppB	oligopeptide ABC transporter, permease protein	2.5
NE1118	dapD	tetrahydrodipicolinate acetyltransferase	2.5
NE929	mtlF	PTS system, mannitol specific IIBC component	2.5
NE1042		hypothetical protein	2.5
NE1722		hypothetical protein	2.5
NE1208		hypothetical protein	2.5

The strains with KB scores <2SD (-2.0) are less susceptible to the CnME. Within this group, we expect to find mutations in gene products that intensify the effects of CnME. Drug-import proteins, and enzymes that increase the toxicity of compounds should show up here. 18 strains were identified with KB scores <-2.0. In this set, we identified 1 transporter, and 6 unknown, hypothetical proteins (Table 2). The opp-2F oligopeptide permease [28], ATP-binding protein, sirA [29], and ABC transporter iron compound-binding protein were identified.

Table 2: USA300 Strains with KB scores two standard deviations less than the mean.

Strain	Gene	Description	KB Score
NE1701	dapA	dihydrodipicolinate synthase	-4.0
NE1894		hypothetical protein	-2.7
NE1699		hypothetical protein	-2.3
NE1609	opp-2F	oligopeptide permease, ATP-binding protein	-2.3
NE1384		hypothetical protein	-2.2
NE1201		hypothetical protein	-2.2
NE1545	moeB	molybdopterin biosynthesis protein B	-2.0
NE1831		YibE/F-like protein	-2.0
NE1767	sirA	ABC transporter iron compound-binding protein SirA	-2.0
NE1885	tgt	queuine tRNA-ribosyltransferase	-2.0
NE1694	typA	GTP-binding protein	-2.0
NE1695		branched-chain α-keto acid dehydrogenase subunit E2	-2.0
NE1891	gpmI	phosphoglyceromutase	-2.0
NE1610	lpdA	dihydrolipoamide dehydrogenase	-2.0
NE1802		aldo/keto reductase family oxidoreductase	-2.0
NE1440		replication initiation factor family protein	-2.0
NE1581		hypothetical protein	-2.0
NE1886		hypothetical protein	-2.0

We have determined the minimal inhibitory concentration of CnME to be 0.34 mg/ml with an IC_{50} of 2.5 mg/ml using resazurin assay [24] (Figure 1). The growth of USA300 was inhibited at both 1xMIC and 2xMIC in liquid culture through 8 hours. By 24 hours, the growth of USA300 at 1xMIC returned to control levels while the 2xMIC treatment only recovered to ~80% of the control levels (Figure 2). These results suggest that the CnME extract is not bacteriocidal, but may be mildly bacteriostatic at concentrations tested.

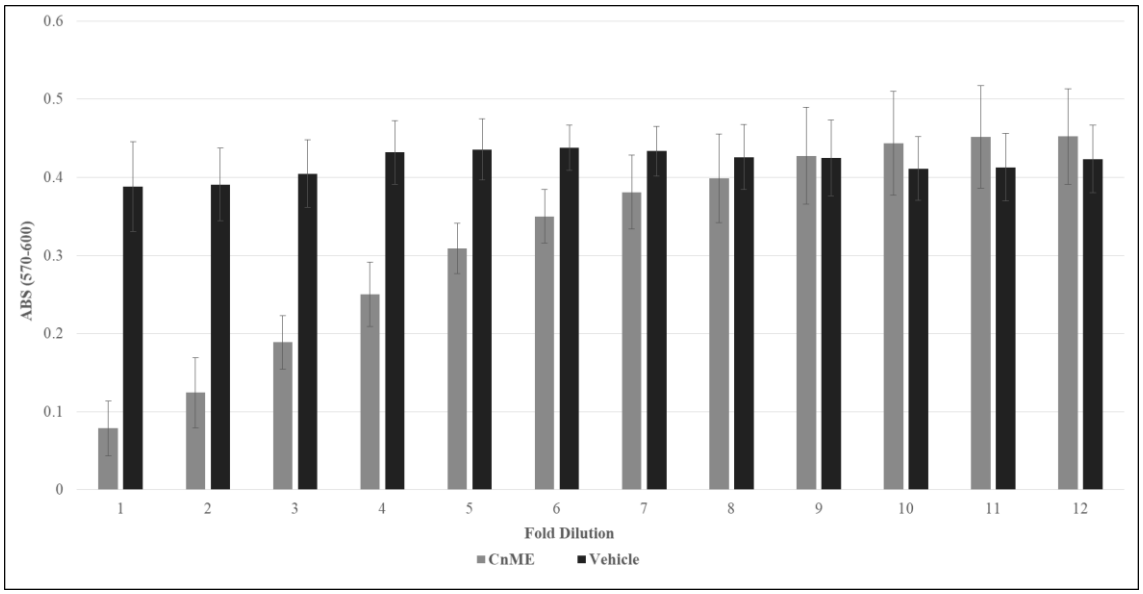

Figure 1: CnME significantly decreased the rate of resazurin reduction by USA300. Cell viability was assayed by resazurin reduction to resorufin and was tracked by $A_{570\text{-}600}$. The baseline-corrected $A_{570\text{-}600}$ of treatments was determined at 3 hours. A T test was used to compare the methanol vehicle control and CnME at each dilution ($p \geq 0.01$). The MIC was defined as the lowest concentration that exhibited a significant difference between the vehicle control and CnME. The CnME significantly inhibited the growth of USA300 at 32-fold dilution=0.34 mg CnME/ml.

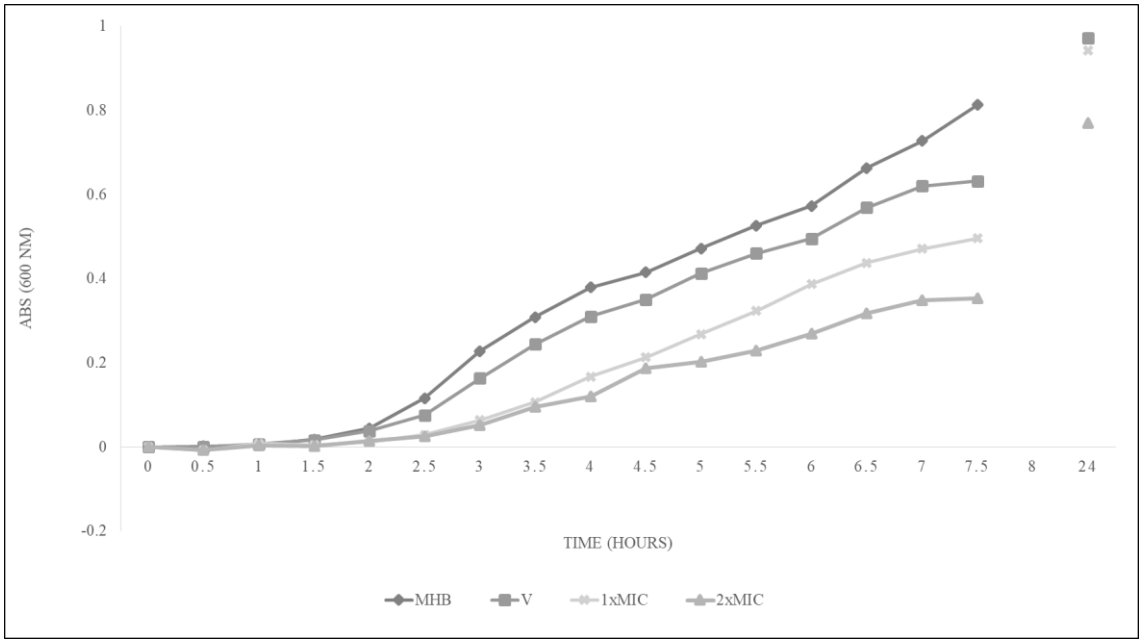

Figure 2: Mean cell growth of USA300 in MHB measured by optical density at 600 nm every 30 minutes for 7.5 hours, and then again at 24 hours. USA300 was treated in triplicate with 2xMIC, 1xMIC, methanol vehicle control (V), and MHB negative control (NC).

DISCUSSION

Methanol extracts of tyrol knapweed, *C. nigrescens*, leaves, buds and flowers have been shown to inhibit the growth of CA-MRSA USA300. In this study, we examined 1912 mutant USA300 strains from the Nebraska Transposon Mutant Library. 84 strains with KB scores >2SD divergent from the mean KB score of 0.1 were selected for further analysis. A Kirby Bauer assay [23] is inherently biased because of the 6 mm filter disc that is used for delivery of test compounds. The lower boundary for bacterial growth is reached when the test strains contact the disc. The upper boundary is not limited. As a consequence, more strains with positive KB scores are expected. Of the 88 strains, 66 strains with positive >2SD divergent from the mean were obtained versus only 18 strains with negative KB scores.

Strains with decreased resistance to CnME included a range of non-essential genes with housekeeping functions including the dehydrogenases IpdA and strain NE1695 that are associated with the citric acid cycle [30, 31]. The DapA gene plays a role in lysine biosynthesis [32] while the moeB gene appears to encode a molybdoterin synthatase sulfurylase potentially involved in nitrate reduction [33]. More interesting are the two transporters identified in this group. Opp-F2 is a transporter involved in oligopeptide uptake and may be important for growth and survival during infections [28]. The sirA gene product is an iron siderophore uptake protein [29]. These two transport proteins could be targets aiding in the entry of select toxins within the CnME. Disruption of either sirA or Opp-F2 resulted in decreased sensitivity possibly due to the loss of entry into the bacterium.

Within the group of strains with increased susceptibility to CnME, four proteins that are associated with pathogenicity or antibiotic resistance mechanisms were identified. Penicillin-binding protein 4, a nonessential enzyme with both transpeptidase and carboxypeptidase activities that functions in cell wall biosynthesis, is associated with low-level beta-lactam antibiotic resistance [27, 34]. The cap5G protein plays a role in cell wall lipopolysaccharide synthesis [35]. The Ear protein, (*Escherichia coli* ampicillin resistance) is an exoprotein, predicted to be a superantigen and plays roles in antibiotic resistance and virulence [36]. Finally, the fibronectin binding protein 4 appears to play a role in attachment and colonization of host tissues and can be upregulated by antibiotics [37].

A group of transporters were identified that, when disrupted, increase the sensitivity of USA300 to CnME. The EmrB/QacA protein is used to pump out quatemary amine compounds and basic dyes. This multidrug efflux systems works primarily with amphiphilic molecules with positive charges [25]. In addition a group of ATP-dependent ATP-Binding Cassette (ABC) transporters were identified (Table 1). The ABC transporter family export toxic compounds from the inner leaflet of the cytoplasmic membrane to the aqueous extracellular medium (Putman *et al*., 2000). The epiG protein, a component of the EpiFEG ABC transporter has been shown to increase antibiotic resistance by the expulsion of lantibiotic peptide antibiotics into the surrounding medium [38].

By identifying mutant strains preferentially affected by tyrol knapweed CnME, it is possible to identify bacterial mechanisms of resistance. In addition to previously identified genes involved in antibiotic resistance, we have identified 10 transport proteins that could be targets for either toxin entry or export. Of the 153 known transporter gene mutants present in the NTML, we have already seen 9 mutagenized strains that have increased susceptibility to CnME, and 1 strain that has decreased resistance. The transporters identified, provide a fertile area for exploration and targeting of novel antibacterial compounds. By blocking the export of potential toxins, the inhibitory effects can be intensified and prolonged.

CONCLUSION

30 USA300 CA-MRSA strains containing mutations affecting transporter proteins were identified as having either increased resistance or susceptibility to methanolic extracts of *C. nigrescens*. Two different drug efflux transporter families have been identified. The EmrB/QacA drug resistance transporter subfamily is a multi-drug efflux pump responsible for the export of toxic molecules from bacteria and yeast. The ABC transporters are involved in drug import and export. These results confirm the effectiveness of the screen as a means for identifying drug-resistance genes affected by the *C. nigrescens* methanolic extract and suggest a role for drug efflux proteins in the resistance of *S. aureus* community-acquired multi-drug resistant *Staphylococcus aureus* to antibacterial plant metabolites.

Acknowledgement

Supported by NIH Grant P20GM103434 to the West Virginia IDeA Network for Biomedical Research Excellence and a Colonel Lee B. Ledford Grant from the Appalachian College Association to KDR.

REFERENCES

1. Barber M. Methicillin-resistant staphylococci. J. Clin. Pathol. 1961; 14:385-393.
2. Benner EJ, Kayser FH. Growing clinical significance of methcillin-resistant Staphylococcus aureus. Lancet 1968; 2:741-744.
3. Fridkin SK, Hageman JC, Morrison M, Sanza LT, Como-Sabetti K, Jernigan JA, *et al*. Active Bacterial Core Surveillance Program of the Emerging Infections Program Network Methicillin-resistant Staphylococcus aureus disease in three communities. N. Engl. J. Med. 2005; 352:1436-1444.
4. Millar BC, Prendergast BD, Moore JE. Community-associated MRSA (CA-MRSA): an emerging pathogen in infective endocarditis. J. Antimicrob. Chemother. 2008; 61:1-7.
5. Bassetti M, Nicco E, Mikulska M. Why is community-associated MRSA spreading across the world and how will it change clinical practice? Int. J. Antimicrob. Agents 2009; 34 Suppl 1:15.
6. DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. Community-associated methicillin-resistant Staphylococcus aureus. Lancet 2010; 375:1557-1568.
7. David MZ, Daum RS. Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic. Clin. Microbiol. Rev. 2010; 23:616-687.
8. Karampela I, Poulakou G, Dimopoulos G. Community acquired methicillin resistant Staphylococcus aureus pneumonia: an update for the emergency and intensive care physician. Minerva Anestesiol. 2012; 78:930-940.
9. Cragg GM, Grothaus PG, Newman DJ. New horizons for old drugs and drug leads. J. Nat. Prod. 2014; 77:703-723.
10. Wasserstrom JA, Aistrup GL. Digitalis: new actions for an old drug. Am. J. Physiol. Heart Circ. Physiol. 2005; 289:1781.
11. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J. Am. Chem. Soc. 1971; 93:2325-2327.
12. White MJ, Boyd JM, Horswill AR, Nauseef WM. Phosphatidylinositol-specific phospholipase C contributes to survival of Staphylococcus aureus USA300 in human blood and neutrophils. Infect. Immun. 2014; 82:1559-1571.
13. Potterat O, Hamburger M. Drug discovery and development with plant-derived compounds. Prog. Drug Res. 2008; 65:118.
14. Hicks M, Bailey MA, Thiagarajan TR, Troyer TL, Huggins LG. Antibacterial and Cytotoxic Effects of Red Mangrove (Rhizophora mangle, L. Rhizophoraceae) Fruit Extract. European Journal of Scientific Research. 2011; 63:339-346.
15. Zengin G, Aktumsek A, Guler GO, Cakmak YS, Kan Y. Composition of essential oil and antioxidant capacity of Centaurea drabifolia Sm. subsp. detonsa (Bornm.) Wagenitz, endemic to Turkey. Nat. Prod. Res. 2012; 26:1-10.
16. Broz AK, Broeckling CD, He J, Dai X, Zhao PX, Vivanco JM. A first step in understanding an invasive weed through its genes: an EST analysis of invasive Centaurea maculosa. BMC Plant. Biol. 2007; 7:25.
17. Politeo O, Skocibusic M, Carev I, Burcul F, Jerkovic I, Sarolic M, Milos M. Phytochemical profiles of volatile constituents from Centaurea ragusina leaves and flowers and their antimicrobial effects. Nat. Prod. Commun. 2012; 7:1087-1090.
18. Bach SM, Fortuna MA, Attarian R, de Trimarco JT, Catalan CA, Av-Gay Y, Bach H. Antibacterial and cytotoxic activities of the sesquiterpene lactones cnicin and onopordopicrin. Nat. Prod. Commun. 2011; 6:163-166.
19. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant Staphylococcus aureus isolates from the United States: establishing a national database. J. Clin. Microbiol. 2003; 41:5113-5120.
20. Bae T, Banger AK, Wallace A, Glass EM, Aslund F, Schneewind O, *et*

al. Staphylococcus aureus virulence genes identified by bursa aurealis mutagenesis and nematode killing. Proc. Natl. Acad. Sci. U. S. A. 2004; 101:12312-12317.
21. Bae T, Glass EM, Schneewind O, Missiakas D. Generating a collection of insertion mutations in the Staphylococcus aureus genome using bursa aurealis. Methods Mol. Biol. 2008; 416:103-116.
22. Bose JL, Fey PD, Bayles KW. Genetic tools to enhance the study of gene function and regulation in Staphylococcus aureus. Appl. Environ. Microbiol. 2013; 79:2218-2224.
23. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 1966; 45:493-496.
24. Sarker SD, Nahar L, Kumarasamy Y. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods 2007; 42:321-324.
25. Nikaido H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 1994; 264:382-388.
26. Tanaka KJ, Song S, Mason K, Pinkett HW. Selective substrate uptake: The role of ATP-binding cassette (ABC) importers in pathogenesis. Biochim. Biophys. Acta 2018; 1860:868-877.
27. Hamilton SM, Alexander JAN, Choo EJ, Basuino L, da Costa TM, Severin A, *et al*. High-Level Resistance of Staphylococcus aureus to beta-Lactam Antibiotics Mediated by Penicillin-Binding Protein 4 (PBP4). Antimicrob. Agents Chemother. 2017; 61:16. Print 2017 Jun.
28. Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, *et al*. Whole genome sequencing of methicillin-resistant Staphylococcus aureus. Lancet 2001; 357:1225-1240.
29. Heinrichs JH, Gatlin LE, Kunsch C, Choi GH, Hanson MS. Identification and characterization of SirA, an iron-regulated protein from Staphylococcus aureus. J. Bacteriol. 1999; 181:1436-1443.
30. Carothers DJ, Pons G, Patel MS. Dihydrolipoamide dehydrogenase: functional similarities and divergent evolution of the pyridine nucleotide-disulfide oxidoreductases. Arch. Biochem. Biophys. 1989; 268:409-425.
31. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, *et al*. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus. Lancet 2006; 367:731-739.
32. Girish TS, Sharma E, Gopal B. Structural and functional characterization of Staphylococcus aureus dihydrodipicolinate synthase. FEBS Lett. 2008; 582:2923-2930.
33. Neubauer H, Pantel I, Gotz F. Characterization of moeB--part of the molybdenum cofactor biosynthesis gene cluster in Staphylococcus carnosus. FEMS Microbiol. Lett. 1998; 164:55-62.
34. Leski TA, Tomasz A. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. J. Bacteriol. 2005; 187:1815-1824.
35. Sau S, Bhasin N, Wann ER, Lee JC, Foster TJ, Lee CY. The Staphylococcus aureus allelic genetic loci for serotype 5 and 8 capsule expression contain the type-specific genes flanked by common genes. Microbiology 1997; 143(Pt 7):2395-2405.
36. Singh VK, Ring RP, Aswani V, Stemper ME, Kislow J, Ye Z, *et al*. Phylogenetic distribution and expression of a penicillin-binding protein homologue, Ear and its significance in virulence of Staphylococcus aureus. J. Med. Microbiol. 2017; 66:1811-1821.
37. Renzoni A, Francois P, Li D, Kelley WL, Lew DP, Vaudaux P, Schrenzel J. Modulation of fibronectin adhesins and other virulence factors in a teicoplanin-resistant derivative of methicillin-resistant Staphylococcus aureus. Antimicrob. Agents Chemother. 2004; 48:2958-2965.
38. Otto M, Peschel A, Gotz F. Producer self-protection against the lantibiotic epidermin by the ABC transporter EpiFEG of Staphylococcus epidermidis Tu3298. FEMS Microbiol. Lett. 1998; 166:203-211.

HOW TO CITE THIS ARTICLE
Huggins LG, Robinson KD, Lasko KP, Clower LB, Gookin AJ, Segraves DE, Gainer JC, Basagic GP, Machuca KR, Rendon J. Screening for community-acquired strains of methicillin-resistant *Staphylococcus aureus* susceptible to extracts of *Centaurea nigrescens*. J Phytopharmacol 2018; 7(3):298-304.