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Identification of a methicillin-resistant *Staphylococcus aureus* inhibitory compound isolated from *Serratia marcescens*

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Abstract

In this study, we identified an antimicrobial compound produced by the Gram-negative bacterium *Serratia marcescens*. Colonies of *S. marcescens* inhibited the growth of nine different methicillin-resistant *Staphylococcus aureus* (MRSA) isolates and several other tested Gram-positive bacterial species, but not Gram-negative bacteria. Genetic analysis revealed the requirement for the *swrW* gene which codes for a non-ribosomal peptide synthetase that generates the cyclodepsipeptide antibiotic serratamolide, also known as serrawettin W1. This is the first report describing the anti-MRSA properties of serratamolide.

Keywords

MRSA; Antibiotic; Aminolipid; Secondary metabolite; Non-ribosomal peptide synthetase; Nosocomial infection

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacterium, a major cause of hospital infections world-wide, and is responsible for more deaths per annum in the United States than HIV-AIDS (Klevens et al., 2007). Additionally, MRSA is a cause of blinding eye infections including keratitis and endophthalmitis (Blomquist, 2006). *S. aureus* has a propensity to acquire antibiotic resistance; therefore, the development of new antimicrobials is of importance. During the preparation of this manuscript, an elegant study was published, which showed that *Serratia marcescens* strain DB10, a non-pigmented strain isolated from *Drosophila*, has genes that produce the antibiotic althiomycin which is capable of inhibiting *S. aureus* growth (Gerc et al., 2012). The genus *Serratia* has been reported to produce antibiotics including a carbapenem, prodigiosin and serratamolide (Coulthurst et al., 2005; Gerber, 1975; Wasserman et al., 1962; Williamson et al., 2008). In this study, we show that *S. marcescens* strains inhibit the growth of MRSA isolates. The zone of clearing was increased by mutation of the *crp* or *hexS* transcription factors and decreased by mutation of the transcription factor *pigP*. Genetic analysis found that the *swrW* non-ribosomal peptide synthetase is required for this zone of clearing. Serratamolide, the biosynthetic product of SwrW, was found to be sufficient for the observed antimicrobial activity. Serratamolide is also known as serrawettin W1 and was isolated as a surface wetting agent involved in surface swarming motility (Matsuyama et al., 1989).

Serratamolide has also been shown to be cytotoxic to a human ocular cell line and mammalian erythrocytes (Shanks et al., 2012). This study highlights the potential of environmental organisms to produce potentially useful compounds to fight nosocomial infections.

2. Materials and methods

2.1. Strains and growth media

All bacteria were grown with LB (10 g tryptone, 5 g yeast extract, 5 g NaCl) (Bertani, 1951) or tryptic soya broth (TSB). For arabinose induction, 0.04% L-arabinose was added. Kanamycin was used at 50 µg/ml for experiments involving plasmids. *S. marcescens* ocular clinical isolate K904 and CMS376 laboratory strains were previously described (Kalivoda et al., 2010). *Escherichia coli* strain S17-1 *pir* was used for conjugation of plasmids (Miller and Mekalanos, 1988). Many strains were kindly donated by Niles Donegan, Ambrose Cheung, George O'Toole, and Michael Zegans of Dartmouth College, and Yohei Doi and Regis Kowalski of the University of Pittsburgh Medical School. Strains tested for susceptibility are listed in Table 1.

2.2. Transposon mutagenesis, mapping of mutations and plasmids

S. marcescens was mutated using mariner transposon delivery vector pSC189 and mutations were mapped as previously described (Chiang and Rubin, 2002) (Fender et al., 2012). The plasmids used in the study were reported elsewhere (Shanks et al., 2009, 2013, 2012), except for pMQ262 noted below. The L-arabinose-inducible prodigiosin strain was made using pMQ200 (Shanks et al., 2009) into which the *pigA* and part of the *pigB* open reading frame were cloned under transcriptional control of the *E. coli* *PBAD* promoter (pMQ262). pMQ262 is a suicide vector in *S. marcescens*, such that when introduced into *S. marcescens* by conjugation, it integrates at the *pigA-N* operon (verified by PCR and phenotype), placing *pigA-N* under control of the *PBAD* promoter. This recombination also leaves *pigA* and part of the *pigB* gene under control of the native promoter. When pMQ262 was introduced into *S. marcescens*, prodigiosin was not measurable without the addition of L-arabinose, whereas, addition of L-arabinose produces a dose-dependent increase in prodigiosin. L-arabinose does not induce prodigiosin production in the isogenic strain without pMQ262.

2.3. Growth inhibition analysis

S. marcescens and the test strains (Table 1) were grown overnight in LB or TSB medium depending upon the growth requirements of the test strain. The test strains were diluted 1:100 in LB or TSB medium and 100 µl was spread atop an LB or TSB agar or plate. *S. marcescens* (10 µl of overnight culture) was spotted onto the center of the plate. Plates were incubated for ~20 h at 30 °C except where noted otherwise.

3. Results

3.1. Pigmented strains of *S. marcescens* inhibit growth of MRSA

Previous studies with *S. marcescens* had shown that secondary metabolite production is inhibited at higher temperatures, typically tested at 37 °C, and is permissive at lower temperatures, typically tested at 25–30 °C (Bar-Ness et al., 1988; Blizzard and Peterson, 1963). When spotted onto lawns of MRSA isolates and incubated at 30 °C, *S. marcescens* clinical isolate K904 produced zones of growth inhibition, of 4–8 mm from K904 to the edge of the MRSA growth, in which all tested MRSA strains did not grow (Table 1). *S. marcescens* strain K904 produced larger zones of inhibition than laboratory strain CMS376, which generated almost no zone of clearing (Fig. 1A–B). When grown at 37 °C, no zone of inhibition was observed around colonies of strain K904 (Fig. 1A). Therefore, all subsequent

experiments with *S. marcescens* were performed at 30 °C since this was the temperature at which the inhibitory effect was observed. Each experiment was performed with multiple MRSA strains, yielding similar results; thus, the choice of strain used in each figure was based solely upon which set was most in focus.

3.2. Mutation of transcription factor genes *crp* and *hexS* led to elevated MRSA growth inhibition

To gain insight into the nature of the inhibitory molecule, defined mutant strains in the CMS376 background, known to affect the secretion of biologically active molecules, were assessed for their antimicrobial activity. Although CMS376 only slightly inhibited MRSA growth, isogenic *crp* and *hexS* mutants both produced larger inhibition zones (Fig. 1B). A *pigP* mutation in strain K904 (Shanks et al., 2013) produced a reduced zone compared to the parental strain (Fig. 1C). CRP, HexS, and PigP are transcription factors that have previously been shown to regulate production of the antimicrobial compounds prodigiosin and serratamolide (Fineran et al., 2005; Shanks et al., 2013, 2012; Tanikawa et al., 2006). CRP and HexS are negative regulators and PigP is a positive regulator of prodigiosin and serratamolide production (Fineran et al., 2005; Kalivoda et al., 2010; Shanks et al., 2013, 2012; Tanikawa et al., 2006). The findings that prodigiosin and serratamolide are produced at 30 °C, but not 37 °C (Bar-Ness et al., 1988; Blizzard and Peterson, 1963), are congruent with the observed MRSA inhibition zones. Based on these observations, we predicted that either prodigiosin or serratamolide or both are required for the *S. marcescens* anti-MRSA compound. Admittedly, factors such as althiomycin could also contribute to the inhibition zones (Gerc et al., 2012).

3.3. Serratamolide rather than prodigiosin is necessary for inhibition of MRSA growth

To test the hypotheses that prodigiosin and serratamolide inhibit MRSA growth, strains with mutations rendering them incapable of producing either prodigiosin or serratamolide production were tested for MRSA antimicrobial ability. We observed that a K904-derived strain harboring a mutation in the *pigD* gene, which is unable to make prodigiosin, still inhibited the growth of MRSA (Fig. 1D). Conversely, K904 with a transposon mutation in *swrW*, unable to produce serratamolide, did not inhibit MRSA growth (Fig. 1D). This transposon inserted at base pair 2390 out of 3393 in the *swrW* open reading frame (Fig. 1E, asterisk). To complement the *swrW* mutant defect, we used a genetic construct with the full-length *swrW* gene on a plasmid under the control of an arabinose-inducible promoter. The MRSA inhibitory zone was restored to the *swrW* mutant in an arabinose-dependent manner with *swrW* in trans (*pswrW*), but not with the empty vector (Fig. 2A). To further investigate the roles of prodigiosin and serratamolide, we used CMS376-based strains in which the chromosomal prodigiosin biosynthetic operon, *pigA-N*, or chromosomal serratamolide biosynthetic gene, *swrW*, was placed under tight inducible control of the *PBAD* promoter (Shanks et al., 2013). Induction of *swrW* but not *pigA-N* was sufficient to confer the MRSA inhibition zone upon CMS376 (Fig. 2B). As a negative control, arabinose did not allow CMS376 with an empty vector control to inhibit MRSA growth (Fig. 2B). Concurrent with the directed genetic approach, we used mariner-based transposon mutagenesis of the *crp* and *hexS* mutant strains, which make elevated MRSA inhibition zones, to find the gene(s) necessary for *S. marcescens* to inhibit MRSA growth. Transposon mutant strains that produced no MRSA inhibition zone were isolated. Two of these transposon insertion sites were mapped to different sites within the *swrW* gene at base pairs 1459 and 1989 in the 3933 base pair open reading frame (long arrows, Fig. 1E). Furthermore, previously identified mutant strains (Shanks et al., 2012) with transposon insertions in *swrW* at base pairs 76, 818, 828, 1384, 2582 and 3074 were all defective in inhibition of *S. aureus* growth (short arrows, Fig. 1E). Using the domain organization predicted by Li and colleagues (Li et al., 2005), this genetic analysis suggests that the predicted condensation (C), adenylation

(A), and thiolation (T) domains of SwrW are necessary for inhibition of MRSA growth (Fig. 1E). Consistently, purified serratamolide in DMSO (Shanks et al., 2012) spotted onto the MRSA lawns was sufficient to inhibit growth at 30 °C and 37 °C, whereas a DMSO control did not (Fig. 2C and data not shown).

3.4. Serratamolide was ineffective against gram-negative organisms

Dr. Wasserman and colleagues showed in 1962 that serratamolide was inhibitory to growth of tested Gram-positive organisms: MSSA, *Bacillus subtilis*, *Sarcinia lutea*, *Mycobacterium tuberculosis*, *M. avium* and one Gram-negative organism *Brucella bronchiseptica* (Wasserman et al., 1962). We used serratamolide-producing strain K904 and a Serratamolide-defective mutant strain (K904 *swrW*) to evaluate the effect of serratamolide on a wider range of organisms, including recent multidrug-resistant clinical isolates (Table 1). Of the four Gram-negative species (8 strains), all were able to grow proximal to K904 and K904 *swrW* mutant strains, indicating a tolerance or resistance to serratamolide. Conversely, of 19 tested Gram-positive species, only five were able to grow around *S. marcescens* K904 without growth inhibition. The remainder, including 8 MRSA strains, were unable to grow close to strain K904, but were able to grow in contact with or close to the K904 *swrW* mutant strain. These data suggest that serratamolide is inhibitory to a broad range of Gram-positive bacteria, with the noted exception of *Enterococcus* species.

4. Discussion

The data presented here identify serratamolide as an antimicrobial agent capable of impeding the growth of MRSA strains and other Gram-positive organisms, but does not eliminate the possibility that serratamolide facilitates the spread of other antimicrobial agents. Together with other studies, (Gerc et al., 2012; Williamson et al., 2008), this study implies that strains of the genetically tractable genera *Serratia* can be a source for antibiotics to combat deadly opportunistic infections as well as being an excellent model system to study bacteria–bacteria interactions. Because of the cytotoxic activity of Serratamolide, it is unlikely to be directly useful as a systemic antibiotic. However, the data from this study suggest that bacterial aminolipids may be a source for future antibiotics effective against MRSA and may play a role in microbial competition.

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Fig. 1. MRSA inhibition zone around *S. marcescens* strains. (A) Photographs of clinical *S. marcescens* isolate K904 spotted onto a lawn of MRSA strain MW2 and incubated at 30 °C or 37 °C. *S. marcescens* spot darkness here and in subsequent images reflects the ability of the strain to make prodigiosin pigment under the tested conditions. All subsequent experiments were incubated at 30 °C. (B) Photographs of CMS376 and mutant derivatives *crp* and *hexS* grown on MRSA strain NRS249. (C) Photographs of strain K904 with an empty vector and with a mutation in *pigP* on lawns of kanamycin-resistant MRSA strain N315. The empty vector (pMQ131) confers kanamycin resistance and is used to eliminate the theoretical phenotypic impact of kanamycin. This was done because the K904 *pigP* strain required kanamycin to maintain the *pigP* mutation. (D) Photographs of K904 and mutant derivatives *pigD* and *swrW* grown on MRSA strain NRS249. Representative images are shown from experiments repeated on three different days with similar results. (E) Scale map of the *swrW* gene with transposon insertion sites as upward pointing arrows (the asterisk and long arrows were insertion sites described in this study, and short arrows from a previous study). White boxes signify the extent of predicted protein domains. C = condensation domain, A = adenylation domain, TE = thioesterase domain, T = thiolation domain.

Fig. 2. Serratamolide rather than prodigiosin inhibits MRSA growth. (A) Photographs of *S. marcescens* strains K904 and an isogenic strain with a transposon mutation in the *swrW* gene, spotted onto lawns of MRSA strain N315 on LB agar plates without (-) or with (+) L-arabinose (4 mM). The empty vector (pMQ125) was unable to complement the *swrW* mutant defect, unlike the p*swrW* (pMQ367) plasmid. (B) Photographs of *S. marcescens* isolate CMS376 spotted onto a lawn of NRS249 strain on LB plates without (-) or with L-arabinose (+) (4 mM). The CMS376 contained either empty vector pMQ131, or plasmids that induced expression of the prodigiosin biosynthetic operon (*pigA*) or the serratamolide biosynthetic operon (*swrW*). (C) Photographs of lawns of MRSA strain MW2 treated with either 10 µl serratamolide (2.5 mg/ml in DMSO) (bottom) or 10 µl DMSO negative control (top) and incubated at 30 °C. Representative images are shown from experiments repeated on three different days with similar results.

Table 1Susceptibility of bacteria to *S. marcescens* strain K904 and K904 *swrW*.

Bacteria ^a	Strain	Source ^b	Zone around K904 ^c	Zone around K904 <i>swrW</i>
<i>Acinetobacter baumannii</i>	Acat285	Clinical isolate from Pittsburgh	–	–
<i>Acinetobacter baumannii</i>	Acat276	Clinical isolate from Pittsburgh	–	–
<i>Bacillus atrophaeus</i>	ATCC® 9372	Microbiologics	+	–
<i>Bacillus cereus</i>	ATCC® 11778	Microbiologics	+	–
<i>Bacillus pumilus</i>	ATCC® 14884	Microbiologics	+	–
<i>Bacillus subtilis</i>	ATCC® 11774	Microbiologics	+	–
<i>Enterococcus avium</i>	ATCC® 14025	Microbiologics	+	–
<i>Enterococcus durans</i>	ATCC® 11576	Microbiologics	–	–
<i>Enterococcus durans</i>	ATCC® 6056	Microbiologics	–	–
<i>Enterococcus faecalis</i>	PIC 522A	Presque Isle Culture Collection	–	–
<i>Enterococcus faecalis</i>	ATCC® 51229	ATCC	–	–
<i>Enterococcus faecalis</i>	ATCC® 51575	Microbiologics	–	–
<i>Enterococcus faecium</i>	ATCC® 6569	Microbiologics	–	–
<i>Enterococcus faecium</i>	ATCC® 6057	Microbiologics	–	–
<i>Enterococcus hirae</i>	ATCC® 8043	Microbiologics	–	–
<i>Enterococcus hirae</i>	ATCC® 10541	Microbiologics	–	–
<i>Escherichia coli</i>	YD438	Clinical isolate from Pittsburgh	–	–
<i>Escherichia coli</i>	YD429	Clinical isolate from Pittsburgh	–	–
<i>Klebsiella pneumonia</i>	AZ1032	Clinical isolate from Pittsburgh	–	–
<i>Klebsiella pneumonia</i>	AZ1093	Clinical isolate from Pittsburgh	–	–
<i>Listeria grayi</i>	ATCC® 19120	Microbiologics	–	–
<i>Listeria innocua</i>	ATCC® 33090	Microbiologics	+	–
<i>Listeria seeligeri</i>	ATCC® 35967	Microbiologics	+	+
<i>P. aeruginosa</i> UCBPP-PA14	SMC0232	Clinical isolate	–	–
<i>Pseudomonas aeruginosa</i>	F77124	Clinical isolate from Pittsburgh	–	–
<i>Staphylococcus epidermidis</i>	ATCC® 12228	ATCC	+	–
<i>Staphylococcus epidermidis</i>	ATCC® 51625	Microbiologics	+	–
<i>Staphylococcus aureus</i>	ATCC® BAA-1680	ATCC, USA300 MRSA	+	–
<i>Staphylococcus aureus</i>	ATCC® BAA-1681	ATCC, USA100 MRSA	+	–
<i>Staphylococcus aureus</i>	B1412	Clinical isolate from Pittsburgh, MSSA	+	–
<i>Staphylococcus aureus</i>	B1487	Clinical isolate from Pittsburgh, MRSA	+	–
<i>Staphylococcus aureus</i>	Col	NARSA strain NRS100, MRSA	+	–
<i>Staphylococcus aureus</i>	K950	Clinical isolate from Pittsburgh, MRSA	+	–
<i>Staphylococcus aureus</i>	MZ100	Laboratory strain, MSSA	+	–
<i>Staphylococcus aureus</i>	MW2	NARSA strain NRS123, USA MRSA	+	–
<i>Staphylococcus aureus</i>	N315	NARSA strain NRS70, MRSA	+	–
<i>Staphylococcus aureus</i>	NRS234	Clinical isolate from Pittsburgh, MRSA	+	–

Bacteria ^a	Strain	Source ^b	Zone around K904 ^c	Zone around K904 <i>swrW</i>
<i>Staphylococcus aureus</i>	SMC3256	Clinical isolate from Dartmouth Medical School, MRSA	+	–
<i>Streptococcus agalactiae</i>	ATCC® 13813	Microbiologics	+	–
<i>Streptococcus anginosus</i>	ATCC® 33397	Microbiologics	+	–
<i>Streptococcus equi subsp. zooepidemicus</i>	ATCC® 43079	Microbiologics	+	–
<i>Streptococcus gallolyticus</i>	ATCC® 49147	Microbiologics	–	–
<i>Streptococcus mutans</i>	ATCC® 25175	Microbiologics	+*	+

^aStrain of bacteria grown as a lawn on an LB or TSB agar plate.

^bMSSA indicates methicillin-sensitive *S. aureus*, MRSA indicate methicillin-resistant *S. aureus*.

^cZone of growth inhibition around *S. marcescens*.

“+” indicates a ≥ 2 mm inhibition zone. “–” indicates a <0.5 mm inhibition zone. “+*” indicates that the zone with K was notably larger than the zone around K904 *swrW*.