L-Ascorbyl 2, 6-Dipalmitate inhibits biofilm and virulence in methicillin-resistant Staphylococcus aureus and prevents triacylglyceride accumulation in Caenorhabditis elegan

Sivasamy Sethupathy[1+], Loganathan Vigneshwari[1+], Alaguvel Valliammai[1], Krishnaswamy Balamurugan[1] & Shunmugiah Karutha Pandian[1*]

[1]Department of Biotechnology, Alagappa University, Science Campus, Karaikudi 630 003, Tamil Nadu, India

*Correspondence and requests for materials should be addressed to SKP. (email:sk_pandian@rediffmail.com)

[+]These authors equally contributed to this work

Supplementary information

Methods

Assay for inhibition of initial bacterial attachment

The ability of ADP to inhibit the initial attachment of *S. aureus* MRSA on polystyrene surface was assessed using MTP assay[1]. Briefly, each 1 ml of TSBG was inoculated with overnight culture of *S. aureus* to an initial optical density (OD) of 0.05 at 600nm and supplemented with 10 to 100 µg mL^{-1} of ADP (with an increment of 10 µg mL) for 8 h at 37° C. Following incubation, spent medium containing non-adherent cells were removed and the wells were washed with distilled water and air dried. CV staining and quantification was done as mentioned previously.

Effect of plasma coating on antibiofilm activity of ADP

Twenty percentage of human plasma was prepared in 50 mM sodium bicarbonate and dispensed (1 mL) on to 24 well MTP and incubated at 4° C for 12 h. After incubation, 20% plasma solution was pipetted out and the wells were washed with sterile distilled water[2]. Biofilm formation of *S. aureus* MRSA on plasma coated surface in the presence and absence of 10 to 100 µg mL^{-1} of ADP (with an increment of 10 µg mL^{-1}) was assessed as mentioned previously.

Light microscopic observation of antibiofilm activity

MRSA was allowed to form the biofilm in the presence and absence of ADP (20, 40, 60, 80 and 100 µg mL^{-1}) on glass slides (0.5×0.5 cm) in 24-well MTP containing each 1 mL of TSBG for 24 h at 37° C. After incubation, biofilm formed on glass slides were washed with distilled water and stained with 0.4 % crystal violet (CV) staining solution for 5 min and excess CV was washed off using distilled water. Washed slides were visualised under light microscope at 400× magnification[3].

Confocal laser scanning electron microscopic analysis of antibiofilm activity

Control and ADP treated MRSA biofilm slides were prepared as mentioned previously and stained with 0.1 % (w/v) acridine orange for 1 min. Stained biofilm slides were rinsed with distilled water to wash off excess acridine orange and air-dried in dark. Then, control and treated MRSA biofilms were observed under CLSM (Carl Zeiss, Germany) equipped with Zen 2009 image processing software at 20 × magnification[3].

Autoaggregation assay

Effect of ADP treatment on the aggregation of MRSA was analysed as reported earlier[4]. Briefly**,** each 5 ml of TSBG was inoculated with overnight culture of *S. aureus* to an initial optical density (OD) of 0.05 at 600nm and supplemented without and with 20 to 100 µg mL^{-1} of ADP (with an increment of 20 µg mL^{-1}) for 24 h at 37° C with shaking at 160 rpm. After incubation, cells were harvested by centrifugation at 12, 000 rpm for 5 min and cells were washed thrice with PBS. Washed cells were pelleted and resuspended in 5 mL of PBS and transferred into sterile glass tubes and allowed to stand at room temperature for 20 h. Cell density of the top portions of PBS containing MRSA cells were measured after 10 and 20 h at OD_{600} nm. In addition, results were visually observed and photographed.

Hemolysin assay

Cell free culture supernatant (CFCS) of MRSA grown in the presence and absence of 10 to 100 µg mL^{-1} of ADP (with an increment of 10 µg mL) was collected by centrifugation and stored at -20° C. To assess the effect of ADP on hemolysin production, each 100 µL of CFCS from control and ADP treated MRSA cultures were mixed with 900 µL of PBS containing 2%

sheep red blood cells and incubated at 37° C for 1 h. Following incubation, the reaction mix was centrifuged at 12, 000 rpm for 10 min at 4° C and the supernatants containing free haemoglobin was read at 534 nm. The results were expressed as percentage of reduction in hemolysin production[5].

Lipase assay

CFCS of control and ADP treated MRSA was prepared as mentioned previously. To assess the effect of ADP on lipase production, each 100 µL of CFCS from control and ADP treated MRSA cultures were mixed with 900 µL of lipase substrate buffer containing 1:9 volume of 0.3% (w/v) p-nitro phenyl palmitate in isopropanol and 50 mM Na2PO4 buffer (pH 7.0) containing 0.2% (w/v) sodium deoxycholate and 0.1% (w/v) gummi arabicum and incubated at room temperature in dark for 1 h. Following incubation, lipase activity was terminated by adding equal volume of 1M Na_2CO_3 to the reaction mix and centrifuged at 12, 000 rpm for 10 min. The absorbance of the supernatant was measured at 410 nm and the results were expressed as percentage of lipase inhibition[6].

Autolysin assay

MRSA was allowed to grow in the presence and absence of 20 to 100 µg mL^{-1} of ADP (with an increment of 20 µg mL) at 37° C until the OD at 580 nm= 0.7. Then, the control and ADP treated cells were collected by centrifugation at 12, 000 rpm for 5 min and washed twice with ice cold sterile distilled water. Washed cells were pelleted and resuspended in autolysin buffer containing 0.05% (v/v) Triton X-100 in 0.05 M Tris–HCl, pH 7.0 to an initial OD of 1.0 ± 0.2 at 580 nm. Then, the cell suspensions were incubated at 30° C and the lysis of cells was monitored by measuring the OD at 580 nm in 30 min intervals[7].

Staphyloxanthin assay

MRSA was inoculated in 5 ml of TSBG supplemented with 10 to 100 µg mL^{-1} of ADP (with an increment of 10 µg mL^{-1}) and grown at 37° C for 24 h at 160 rpm. After incubation, cells were harvested by centrifugation at 10, 000 rpm for 5 min and the cell pellets were washed twice with PBS. Tubes containing MRSA control and ADP treated cell pellets were visually observed for staphyloxanthin inhibition and photographed. For qualitative analysis of

staphyloxanthin inhibition, cell pellets were resuspended in ethanol and incubated at 40° C for 20 min and centrifuged at 10, 000 rpm for 5 min. Ethanol extraction was repeated until the pellet becomes colourless. Then, the collected ethanolic extract was dried under vacuum and the crude staphyloxanthin concentrate was mixed with ethyl acetate/1.7 M aqueous sodium chloride (1: 1, v/v). The organic layer was saved and the ethyl acetate extraction was repeated until the aqueous layer becomes colourless. The collected ethyl acetate fraction was washed with distilled water and dried under vacuum. All the steps were carried out at room temperature and in the dark. The presence of carotenoids was measured using a quartz cuvette in multi-label reader (Spectramax M3, USA) at a suitable wavelength against ethyl acetate blank. Carotenoids such as 4, 4′-diapophytoene, 4, 4′-diaponeurosporene, 4, 4′-diaponeurosporenic acid and b-D-glucopyranosyl 1-O-(4,4′-diaponeurosporene-4-oate)-6-O-(12-methyltetradecanoate) [staphyloxanthin] were measured at 286, 435, 455 and 462 nm, respectively[7].

Effect of ADP on the colony morphology

To ascertain the effect of ADP on the colony morphology, 50 µL of MRSA culture with a turbidity of McFarland standard number 2 was spread on TSBG agar supplemented with and without 100 µg mL^{-1} of FDA. The plates were incubated at 37° C for 72 h and the colony morphology of control and ADP treated MRSA was visually observed and image acquisition was done using Gel Doc™ XR+ and processed with Image Lab™ software.

Whole-blood killing assay

The turbidity of MRSA control and ADP treated cultures were adjusted to a McFarland standard number 2. One volume of culture and 3 volume of freshly drawn blood (heparinized) were mixed and incubated at 37° C for 4 h with shaking at 160 rpm and enumerated for viable cells[8].

H_2O_2 sensitivity assay

MRSA control and ADP treated cultures were prepared as mentioned previously and their turbidity was adjusted to a McFarland standard number 2 with PBS containing 1 mM H_2O_2. Then, the cells were incubated at 37° C for 1 h with agitation at 160 rpm and enumerated for viable cells[8].

Singlet oxygen sensitivity assay

MRSA control and ADP treated cultures were prepared as mentioned previously and their turbidity was adjusted to a McFarland standard number 2. Methylene blue was added into 96 well plates containing 100 µL of diluted MRSA cells to a final concentration of 10 µg mL^{-1}. Then, exposed to light by precisely placing the plates 20 cm away from the 100 W light source for 60 min and enumerated for viable cells[8].

Supplementary table 1. List of primers used for Q-PCR analysis to study the effect of ADP on MRSA virulence and biofilm genes.

Genes	Forward primer	Reverse primer
saeS	5'-TGCCAATACCTTCATCGCTAA-3'	5'-CAATATCGAACGCCACTTGA-3'
aur	5'-CAAAAGAGTGATGCGGTCAA-3'	5'-AGGTGCATGAACACCATCAA-3'
crtM	5'-ATCCAGAACCACCCGTTTTT-3'	5'-GCGATGAAGGTATTGGCATT-3'
crtN	5'-GATGAAGCTTTGACGCAACA-3'	5'-TTCGCATGATACGTTTGCTC-3'
geh	5'-GTAGATTATGGCGCAGCACA-3'	5'-CCATGCGCTTTATGATAGGC-3'
agrC	5'-CATTCGCGTTGCATTTATTG-3'	5'-CCTAAACCACGACCTTCACC-3'
sarA	5'-CAAACAACCACAAGTTGTTAAAGC-3'	5'-TGTTTGCTTCAGTGATTCGTTT-3'
hld	5'-TAATTAAGGAAGGAGTGATTTCAATG-3'	5'-TTTTTAGTGAATTTGTTCACTGTGTC-3'
ebpS	5-CATCCAGAACCAATCGAAGAC-3	5- AGTTACATCATCATGTTTATCTTTTG-3
agrA	5′-TGATAATCCTTATGAGGTGCTT-3′	5′-CACTGTGACTCGTAACGAAAA-3′
gyrB	5'-GGTGCTGGGCAAATACAAGT-3'	5'-TCCCACACTAAATGGTGCAA-3'
icaA	5'-ACACTTGCTGGCGCAGTCAA-3'	5'-TCTGGAACCAACATCCAACA-3'
icaB	5'-ATGGTCAAGCCCAGACAGAG-3'	5'-AGTATTTTCAATGTTTAAAGCA-3'
sspB	5'-CCAGCAAATTGTTGTTGTGCTAG-3'	5'-AAGCCAAAGCCGATTCACACTC-3'
fnbA	5'-ATCAGCAGATGTAGCGGAAG-3'	5'-TTTAGTACCGCTCGTTGTCC-3'
fnbB	5'-AAGAAGCACCGAAAACTGTG-3'	5'-TCTCTGCAACTGCTGTAACG-3'
clfA	5'-ATTGGCGTGGCTTCAGTGCT-3'	5'-CGTTTCTTCCGTAGTTGCATTTG-3'
altA	5'-TGTCGAAGTATTTGCCGACTTCGC-3'	5'-TGGAATCCTGCACATCCAGGAAC-3'
hla	5'-CAACTGATAAAAAAGTAGGCTGGAAAGTGAT-3'	5'-CTGGTGAAAACCCTGAAGATAATAGAG-3'

Supplementary table 2. List of primers used for Q-PCR analysis to study the effect of ADP on *C. elegans*.

Genes	Primer sequences
acs-1 fp	5′-GAACGGGGAAAGAGTGGAAT-3′
acs-1 rp	5′-ATATCCAGCACGGAGACCAA-3′
atg-1 fp	5′-GACTGGGACAGTCGAAGCAT-3′
atg-1 rp	5′-CTGGCTCATTTCGATCAATC-3′
sams-1 fp	5′-GAGGGGCATCCAGACAAAAT-3′
sams-1 rp	5′-ACGACGGACGAGAACTTGAT-3′
fat-6 fp	5′-TCCCACTTGTCATCTTGTGC-3′
fat-6 rp	5′-GACGTTTTCGACTGGGGTAA-3′
akt-1 fp	5′-CCGGAGTATCTTGCACCAGA-3′
akt-1 rp	5′-GCCTTCCACACATCATCTCG-3′
eat-2 fp	5′-GCAAATTCCCCATGGTACAC-3′
eat-2 rp	5′-TCTCGTGCTCACTTTCCATG-3′
aak-2 fp	5′-TGCTTCACCATATGCTCTGC-3′
aak-2 rp	5′-ATTGCTGGGAGATGATCCAC-3′
daf-16 fp	5′-TGGAATTCAATCGTGTGGAA-3′
daf-16 rp	5′-CTGGAGGGCAGCATATTCAT-3′
daf-2 fp	5′-TCGAGCTCTTCCTACGGTGT-3′
daf-2 rp	5′-GACACGTGGTGGACAAGATG-3′
skn-1 fp	5′-CTCCATTCGGTAGAGGACCA-3′
skn-1 rp	5′-GAGAAATCGACAGTAGCGCC-3′
age-1 fp	5′-TTAGAGCTCCACGGCACTTT-3′
age-1 rp	5′-CGAAGAAAACGTGCTTGACA-3′
ins-7 fp	5′-AGGTCCAGCAGAACCAGAAG-3′
ins-7 rp	5′-GAAGTCGTCGGTGCATTCTT-3′
sgk-1 fp	5′-TCAGGCACAAGGAGACCAAA-3′
sgk-1 rp	5′-GTGAAACAAGGAAAGGGTGT-3′
egl-8 fp	5′-CGTATCGTTGCGCTTCTGA-3′
egl8 rp	5′-AGTAGTGACACAGCGGTTG-3′
sod-fp	5′-GGATGGTGGAGAACCTTCAA-3′
sod-rp	5′-CATAGTCTGGGCGGACATTT-3′
β- actin fp	5′-ATCGTCCTCGACTCTGGAGATG-3′
β- actin rp	5′-TCACGTCCAGCCAAGTCAAG-3′

Supplementary table 3. Thermal cyclic conditions used for Q-PCR analysis

Initial denaturation	**95°C for 10 min**	
Denaturation	95°C for 45 sec	40 cycles
Annealing	58°C for 45 sec	
Extension	72°C for 45 sec	

References

1. M. Sandasi, C. M. Leonard and A. M. Viljoen, *Lett. Appl. Microbiol*., 2010, **50,** 30.
2. J. N. Walker and A. R. Horswill, *Front. Cell Infect. Microbiol.*, 2012, **2**,162.
3. S. Gowrishankar, N. M. Duncun and S. K. Pandian, *Evid. Based. Complement. Alternat. Med.*, 2012, 862374.
4. F. G. Sorroche, M. B. Spesia, Á. Zorreguieta and W. Giordano, *Appl. Environ. Microbiol*., 2012, **78,** 4092.
5. A. Annapoorani, R. Parameswari, S. K. Pandian and A. V. Ravi, *J. Microbiol. Methods,* 2012, **91**, 208.
6. K. Riedel, D. Talker-Huiber, M. Givskov, H. Schwab and L. Eberl, *Appl. Environ. Microbiol*, 2003, **69**, 3901.
7. N. A. Mani, P. H. Tobin and R. K. Jayaswal, *J. Bacteriol*., **175,** 1493.
8. Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, Bastian JF, Fierer J, Nizet V, *J. Exp. Med*., 2005, **202**, 209.