SUPPLEMENTARY MATERIAL

Antimicrobial activity of prodigiosin is attributable to plasma-membrane damage

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The bacterial pigment prodigiosin has various biological activities; it is, for instance, an effective antimicrobial. Here, we investigate the primary site targeted by prodigiosin, using the cells of microbial pathogens of humans as model systems: *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*. Inhibitory concentrations of prodigiosin; leakage of intracellular K^+ ions, amino-acids, proteins, and sugars; impacts on activities of proteases, catalases, and oxidases; and changes in surface appearance of pathogen cells were determined. Prodigiosin was highly inhibitory (30% growth-rate reduction of *C. albicans*, *E. coli*, *S. aureus* at 0.3, 100, and 0.18 μg ml^{-1}, respectively); caused leakage of intracellular substances (most severe in *S. aureus*); was highly inhibitory to each enzyme; and caused changes to *S. aureus* indicative of cell-surface damage. Collectively, these findings suggest that prodigiosin, log $P_{octanol\text{-}water}$ 5.16, is not a toxin but is a hydrophobic stressor able to disrupt the plasma membrane via a chaotropicity mediated mode-of-action.

Keywords: Antimicrobial activity; competitive interactions; mode-of-action, membrane leakage, microbial pigment prodigiosin, *Staphylococcus aureus*, *Serratia marcescens*.

1. Experimental

1.1 Microbial cultures

Serratia marcescens SV1 (Gene bank accession no. KT351651) was isolated by the authors and is available from the Satish Patil laboratory the lab isolate isolated from soil sample (Jalgaon, India). The human pathogens *Candida albicans* SV was isolated by the authors and is available from the Satish Patil laboratory (Gene bank accession no. KP406581), *Escherichia coli* (NCIM 2931) and *Staphylococcus aureus* (NCIM 2492);

1.2 Source of prodigiosin

The bacterium *S. marcescens* SV1 was cultured in nutrient broth (HiMedia Laboratories, Bangalore, India) at 28 C for 24 h. Cells were harvested by centrifugation as described by Suryawanshi et al. (2014). Prodigiosin was isolated from these cells by resuspending the cell pellet in ethanol (100% v/v) followed by centrifugation. The pigment was further purified by heat treatment and making the crude extract insoluble in MilliQ water and the precipitate was further purified using column chromatography employing n hexane: butanol (1:2) as a solvent system. The characterization of pigment was performed using spectroscopy, mass spectroscopy, Furrier transform infrared spectroscopy, and nuclear magnetic resonance (see Suryawanshi et al., 2014).

1.3 Determination of prodigiosin concentrations which inhibit growth of human-pathogenic microbes

Cultures of *Candida albicans*, *Escherichia coli* (NCIM 2931) and *Staphylococcus aureus* (NCIM 2492) were incubated in 96-well plate system. A stock solution of prodigiosin (1 mg ml^{-1}) was made up and then used to make a dilution series by adding nutrient broth (HiMedia, Bangalore, India). The nutrient broth contained NaCl (5.0 g l^{-1}), peptic digest (5.0 g l^{-1}), beef extract (1.5 g l^{-1}), and yeast extract (1.5 g l^{-1}). A two-fold dilution series was prepared, and each dilution was added to the 96 wells plate. Wells were also inoculated with bacterial cell suspensions (100 µL) in duplicate, to give a 0.5 McFarland density. As a positive control, the antibiotic streptomycin (8 µM) was used in place of prodigiosin. As a negative control, bacterial cells were suspended in Nutrient Broth without any inhibitor (neither a pigment nor antibiotic). Plates were incubated for 24 h at 37°C and then OD was determined at 600 nm. Growth curves (data not shown) were plotted and percentage growth

inhibition was calculated by comparison with the negative control. For each pathogen, the prodigiosin concentration that corresponded to 30% inhibition of growth rate (relative to the control; no prodigiosin added) was determined.

1.4 Prodigiosin-induced leakage of K+ ions and cellular metabolites

Cultures of *C. albicans*, *E. coli,* and *S. aureus* were grown, in duplicate, at 37 °C for 24 h in nutrient broth in a 250 ml conical flask. Samples (3 ml) were taken from each culture and then centrifuged at 10,000 rpm (Multifuge 1 S-R, Heraeus, Germany). The supernatant was discarded and the pellet washed three times with sterile MilliQ water (1 ml). The pellet was transferred to a sterile test tube containing MilliQ water (10 ml). One set of control- and prodigiosin-treated cultures for each pathogen were taken from the inhibition assays (see above), one duplicate was treated with a concentration of prodigiosin which corresponded to 30% growth-rate inhibition (see above) and the second duplicate was used as a control in the leakage experiments. After 2 h, the tubes were again centrifuged at 10,000 rpm (see above) and then the supernatants were analyzed for K^+ ion concentrations using atomic absorption spectrophotometry.

Conical flasks (250 ml volume) containing 100 ml nutrient broth (HiMedia Laboratories) were inoculated using 10^6 cfu ml^{-1} of each pathogen, incubated 37°C, with shaking at 120 rpm, for 24 h. A sample (3 ml) was taken from each flask and then centrifuged (10,000 rpm for 10 min., Multifuge 1 S-R, Heraeus, Germany), the pellet was washed three times with 0.85% w/v NaCl. Prodigiosin (1 ml – made up using 10% v/v ethanol – at concentrations corresponding to 30% growth-rate inhibition; see above) was added to cells from these samples and then incubated at 37°C for 2 h. For each pathogen, control (no prodigiosin added) were incubated in the same way. All samples were then centrifuged at 10,000 rpm (see above), and the supernatant used to determine concentrations of reducing sugars, amino acids and proteins as described by Moore and Stein (1948), Miller (1959), and Bradford (1975).

1.5 Determination of enzyme activities

For each of the microbial pathogens, *in-vivo* enzyme activities were determined for proteases, catalases, and oxidases for both control cultures and prodigiosin-treated cells. For proteases, hydrolysis of the synthetic substrate benzoyel DL arginine-paranitroanilide was determined by spectrophotometric measurement of P-nitroanilidine at 410 nm. For catalase assays, the pathogens were incubated with sublethal concentration of prodigiosin and then macerated mechanically and were treated with substrate hydrogen peroxide and observed for formation of bubbles; the latter

indicates catalase activity (Gagnon, Hunting, & Esselen, 1959). For oxidases, assays were performed using Oxidase Discs (HiMedia Laboratores) which were impregnated with redox indicator that turns blue upon oxidation (*N, N*-dimethyl-*p*-phenylenediamine) (Gordon, & McLeod, 1928).

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