Pyomelanin produced by *Vibrio cholerae* confers resistance to predation by *Acanthamoeba castellanii*

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Running title: *hmgA*-mediated pyomelanization confers grazing resistance

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Abstract

Protozoan predation is one of the main environmental factors constraining bacterial growth in aquatic environments, and thus has led to the evolution of a number of defence mechanisms that protect bacteria from predation. These mechanisms may also function as virulence factors in infection of animal and human hosts. Whole transcriptome shotgun sequencing of *Vibrio cholerae* biofilms during predation by the amoebae, *Acanthamoeba castellanii,* revealed that 131 transcripts were significantly differentially regulated when compared to the non-grazed control. Differentially regulated transcripts included those involved in biosynthetic and metabolic pathways. The transcripts of genes involved in tyrosine metabolism were down-regulated in the grazed population, which indicates that the tyrosine metabolic regulon may have a role in the response of *V. cholerae* biofilms to *A. castellanii* predation. Homogentisate 1, 2-dioxygenase (HGA) is the main intermediate of the normal L-tyrosine catabolic pathway which is known to auto-oxidize, leading to the formation of the pigment, pyomelanin. Indeed, a pigmented mutant, disrupted in *hmgA*, was more resistant to amoebae predation than the wild type. Increased grazing resistance was correlated with increased production of pyomelanin and thus reactive oxygen species (ROS), suggesting that ROS production is a defensive mechanism used by bacterial biofilms against predation by amoebae *A. castellanii*.

Introduction

Vibrio cholerae, the causative agent of cholera, persists in brackish and estuarine water systems (Colwell *et al.*, 1977, Huq *et al.*, 1990) where it is exposed to starvation conditions, fluctuations in temperature and salinity, and predators (Lutz *et al.*, 2013). The persistence of *V. cholerae* in the environment indicates its ability to respond to such stresses (Colwell & Huq, 1994, Lutz *et al.*, 2013, Sun *et al.*, 2015). Heterotrophic protists are the biggest consumers of bacteria in the environment and are thus, a major mortality factor for bacteria (Jürgens & Matz, 2002).

In benthic marine, brackish, and freshwater sediments where *V. cholerae* naturally occurs, ciliates are the most abundant protists, while amoebae contribute most of the biomass (Lei *et al.*, 2014). *V. cholerae* shares an ecological niche with the model protozoa, *Acanthamoeba castellanii* and *Tetrahymena pyriformis.* The free-living amoeba, *Acanthamoeba* spp. have been isolated from various fresh and salt water sources (Khan, 2006) where they feed on bacterial biofilms. *V. cholerae* and *Acanthamoeba* spp. were detected in water samples collected from different cholera endemic areas in Sudan (Shanan *et al.*, 2011). *V. cholerae* is often isolated from freshwater systems (Nair *et al.*, 1988) where *T. pyriformis* typically occurs, feeding on bacterioplankton (Elliott, 1970). These predators are among the few axenic protozoan cultures available, making them ideal ecologically relevant model organisms.

Both clinical and environmental strains of *V. cholerae* have been shown to survive intracellularly within a range of amoeba (Thom *et al.*, 1992, Abd *et al.*, 2005, Abd *et al.*, 2007), and Van der Henst *et al.* (2016) showed that *V. cholerae* can grow inside *A. castellanii.* A study using laboratory microcosms of natural bacterioplankton communities from the Gulf of Mexico showed elimination of *V. cholerae* by ciliates and heterotrophic nanoflagellates (HNFs) (Martínez Pérez *et al.*,

2004). In contrast, when *V. cholerae* biofilms were exposed to predation by flagellates, there was little effect on biofilm biomass, indicating that biofilms are protected from predation (Matz *et al.*, 2005).

Biofilms provide physical protection as well as a high cell density population that enables cell-to-cell communication, or quorum sensing. Quorum sensing (QS) has been shown to regulate antiprotozoal activities in *V. cholerae* biofilms including, the production of Vibrio polysaccharide (VPS) that protects both early and late stage biofilms from predation by the surface-feeding nanoflagellate, *Rhynchomonas nasuta* and the amoeba *A. castellanii* (Lutz *et al.*, 2013, Sun *et al.*, 2013). The extracellular protease, PrtV provides grazing resistance against the flagellate *Cafeteria roenbergensis* and the ciliate, *T. pyriformis* (Vaitkevicius *et al.*, 2006). The type VI secretion system (T6SS) uses virulence-associated secretion (VAS) proteins to deliver effector proteins that are cytotoxic to the amoebae, *Dictyostelium discoideum* and mammalian macrophages (Pukatzki *et al.*, 2006, Miyata *et al.*, 2011). Despite the fact that the early and late biofilms of a *V. cholerae* QS mutant were more susceptible to grazing by *A. castellanii*, *C. roenbergensis* and *R. nasuta* than the wild type, the biofilms not completely eliminated by predation (Erken *et al.*, 2011, Lutz *et al.*, 2013), suggesting other anti-predation strategies could be present.

Studies on bacterial prey and protozoan predators have shown several potential defences against grazing, including production of toxins, microcolony formation and changes in cell surface properties (Matz & Kjelleberg, 2005). Examples of secondary metabolites that are active against protists include an alkaloid purple-pigmented metabolite, violacein, which acts as a chemical defence for several bacterial genera (*Chromobacterium, Janthinobacterium, Pseudoalteromonas*) (Matz *et al.*, 2004). Similarly, *Pseudomonas fluorescens* is known to employ the cyclic

93 lipopeptide surfactants, massetolide and viscosin to protect itself against *Naegleria*
94 *americana* (Mazzola *et al.*, 2009), in addition to 2,4-diacetylphloroglucinol (DAPG),
95 pyrrolnitrin, hydrogen cyanide, and pyoluteorin (Jousset *et al.*, 2010). Cell surface
96 properties have also been shown to affect grazing resistance. For example cell surface
97 hydrophobicity affects grazing of picoplankton cells by nanoflagellates (Monger *et*
98 *al.*, 1999). Moreover, Wildschutte *et al.* (2004) showed that differences in O-antigen
99 are sufficient to allow for prey discrimination by protozoa grazing on different
100 serotypes of *Salmonella*.

101 In order to study the factors contributing to grazing resistance of *V. cholerae*,
102 the transcriptome of biofilms exposed to *A. castellanii* was analysed to identify
103 genetic features that likely contribute to survival during predation. Here, we examine
104 the effect of down-regulation of genes involved in tyrosine degradation on grazing
105 resistance of *V. cholerae.* A decrease in the activity of homogentisate 1, 2 –
106 dioxygenase (HmgA) leads to accumulation of homogentisic acid (HGA) which auto-
107 oxidizes to form pyomelanin (Turick *et al.*, 2010). Results show that the production
108 of pyomelanin has a protective effect against predation by *A. castellanii*.

109 **Material and methods**

110 **Strains and growth conditions**

111 Organisms used in this study are listed in (Table 1). Bacterial strains were routinely
112 grown in Luria-Bertani (LB) broth and on agar plates (Sambrook *et al.*, 1989) as
113 appropriate, with carbenicillin (100 μg ml^{-1}). *A. castellanii* was routinely passaged in
114 15 ml growth medium containing peptone-yeast-glucose (PYG) (20 g l^{-1} proteose
115 peptone, 1 g l^{-1} yeast extract) supplemented with 1 litre 0.1 × M9 minimal medium (6
116 g l^{-1} NaH_2PO_4, 3 g l^{-1} K_2PO_4, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH_4Cl) and 0.1 M sterile-filtered

glucose in 25 cm^2 tissue culture flasks with ventilated caps (Sarstedt Inc., Nümbrecht, Germany) and incubated statically at 30°C. *A. castellanii* was passaged 3 days prior to harvesting for experiments and enumerated microscopically using a haemocytometer.

The browsing ciliate, *T. pyriformis* was maintained as above but incubated statically at room temperature (RT). Prior to experiments, 500 µl of *T. pyriformis* were passaged in 20 ml of 0.5 × NSS medium (8.8 g l^{-1} NaCl, 0.735 g l^{-1} Na_2SO_4, 0.04 g l^{-1} $NaHCO_3$, 0.125 g l^{-1} KCl, 0.02 g l^{-1} KBr, 0.935 g l^{-1} $MgCl_2.6H_2O$, 0.205 g l^{-1} $CaCl_2.2H_2O$, 0.004 g l^{-1} $SrCl_2.6H_2O$ and 0.004 g l^{-1} H_3BO_3) (Mårdén *et al.*, 1985) supplemented with 1% (v/v) of heat- killed *Pseudomonas aeruginosa* PAO1 (HKB) in a 25 cm^2 tissue culture flask, and further incubated at RT statically for 2 days before enumeration and use. This process is necessary to remove the nutrient media and to acclimatise the ciliate to phagotrophic feeding.

To prepare heat-killed bacteria (HKB), *P. aeruginosa* was grown overnight in LB at 37°C with shaking at 200 rpm and adjusted to (OD_{600}=1.0; 10^9 cells ml^{-1}) in 0.5 × NSS. The tubes were then transferred to a water bath at 65°C for 2 hours, and then tested for viability by plating on LB agar plates at 37°C for 2 days. HKB stocks were stored at -20°C.

Transcriptomic profiling of continuous-culture biofilms

For the transcriptomic analysis, 3 day-old *V. cholerae* biofilms were exposed to grazing by *A. castellanii* in a continuous flow system. Briefly, 3 biological replicates of *V. cholerae* biofilms were cultivated on the interior surfaces of Silastic® laboratory tubing (Dow Corning, Michigan, USA) (3.2 mm diameter; length, 14 cm) in 0.5 × Väätänen nine salts solution (VNSS) (1 g bacteriological peptone, 0.5 g yeast extract, 0.5 g D-glucose, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.01 g Na_2HPO_4) in 1 litre of 0.5 × NSS and

fed at a flow rate of 9 ml h^{-1} using a continuous flow system at RT. After 3 days, washed cells of *A. castellanii* were resuspended in 0.5 × VNSS, injected into the tubing and incubated without flow for 2 hours. A protist free control biofilm was treated the same to exclude oxygen or starvation effects.

The *V. cholerae* biofilms on the walls of the tubing were washed by a flow of 2 volumes of 0.5 × VNSS to remove planktonic bacteria, and immediately resuspended in 2 volumes of RNAlater (Qiagen, Hilden, Germany) and harvested from the interior surface of the tubing by mechanical manipulation (manually squeezing out of the tubing). Total RNA was extracted by lysozyme digestion and use of the RNeasy plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the mRNA-Seq sample preparation, the Illumina standard kit was used, according to the manufacturer's protocol (Illumina, USA).

Transcriptome data analysis

Prior to RNA-Seq analysis, filters were applied to remove low quality reads from all pair-end samples. Pair-end raw reads were trimmed with the BWA trimming mode at a threshold of Q13 (P = 0.05) as implemented by SolexaQA version 3.1.3 (Cox *et al.*, 2010). Low-quality 3' ends of each read were filtered and reads that were less than 25 bp in length were discarded.

The trimmed reads were subsequently depleted of ribosomal RNA with SortMeRNA version 1.8 (Kopylova *et al.*, 2012). Trimmed reads (102 bp) were first mapped to the *A. castellanii* contigs (GenBank accession ID GCA_000826485.1*)* using Bowtie (version 2.2.3) (Langmead & Salzberg, 2012) with default parameters. Reads that were not mapped to amoeba contigs were then mapped to the reference genome, *V. cholerae* O1 biovar El Tor str. N16961 and the *V. cholerae* A1552 indel correction table (http://microbes.ucsc.edu/lists/vibrChol1/StrainA1552-list.html) using

Bowtie2 with parameters set to -N 1. Cuffdiff (Cufflinks version 2.2.1) with default parameters was finally used to identify differentially expressed transcripts of *V. cholerae* biofilms grazed by *A. castellanii* compared to ungrazed controls. Cuffdiff calculated the log fold change in FPKM, and then the significance of the fold change. A false discovery rate (FDR) adjusted p value was calculated to give the statistical validity level of significance. Transcripts with an FDR-adjusted p-value of <0.05 were considered to be significantly differentially expressed (SDE) genes.

The lists of up and down regulated SDE genes were placed into cluster of orthologous group (COG) categories by NCBI conserved domain search (Tatusov *et al.*, 1997). With the assistance of the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 (National Institute of Allergy and Infectious Diseases, NIH), the differentially expressed transcripts were further analysed using databases such as Gene Ontology Annotation Database (to analyse the biological processes, molecular functions and cellular components), KEGG Pathway (to analyse the metabolic pathways), and InterPro/UniProt (to analyse the protein domains).

Early and late biofilm grazing assay with *A. castellanii*

Overnight cultures of *V. cholerae* were adjusted so that 10^5 cells ml^{-1} in 0.5 × VNSS were added to 24-well microtitre plates (Falcon™, Becton Dickinson, New Jersey, USA) and incubated for 24 and 72 hours with shaking at 60 rpm at RT. After incubation, fresh with or without *A. castellanii* (2×10^4 cells ml^{-1}) was added and the plates incubated at RT with shaking at 60 rpm for 3 days. The cell density in each well was measured by spectrophotometry at $OD_{600\ nm}$ (Wallac Victor^2 1420 Multilabel Counter, Perkin Elmer Life Sciences, Massachusetts, USA). In order to quantify the biofilm biomass, crystal violet (CV) assays were performed (O'Toole & Kolter,

1998). Briefly, all the planktonic cells were removed by washing three times with 0.5 × NSS before adding CV (0.3%) for 15 minutes. The wells were washed a further three times using 0.5 × NSS to remove the unattached CV and then the stain was solubilized using 96% ethanol and the $OD_{490\ nm}$ was determined by spectrophotometry.

To determine if the cell-free supernatants from the *hmgA* mutant would provide protection against grazing by *A. castellanii* to the WT, the cell-free supernatant of 3-days old established biofilms were acquired by centrifugation at 6 000 × *g* for 10 minutes and filtration (0.22 mm filters, Millex-GP, Millipore, MA, USA). The cell free supernatants were then added to 3-days old established biofilms of the WT strain at a ratio of 50% with fresh VNSS with or without *A. castellanii* (2×10^4 cells ml^{-1}) and incubated at RT with shaking at 60 rpm for 3 days. The biofilm biomass was then quantified by CV assays.

ROS and pyomelanin quantification

The pyomelanin in the aqueous phase was determined by spectrophotometry ($OD_{405\ nm}$) of the cell-free supernatant acquired by centrifugation at 6 000 × *g* for 10 minutes and filtration (0.22 mm filters, Millex-GP, Millipore, MA, USA). In order to study the effect of nutrients released from *A. castellanii* on pyomelanin production by *V. cholerae*; *A. castellanii* was incubated in 0.5 × VNSS with or without 1% (v/v) of heat killed bacteria (HKB) for 3 days at RT. Furthermore, to assess if phagocytosis by the amoeba predator is required for induction of pyomelanin production, *A. castellanii* was heat inactivated in 65°C for 15 minutes. The trophozoites were confirmed to be intact by microscopy, and the viability checked by addition to PYG and incubation at RT for 3 days. The cell-free supernatant or heat-killed *A. castellanii* was added to the 3 day old established biofilm. The amount of pyomelanin in the cell free supernatant

after incubation for 3 days at RT was determined. Amount of pyomelanin was then normalised by using the corresponding biofilm biomass measured by CV assays ($OD_{490\ nm}$).

To assess the level of reactive oxygen species (ROS), 25 μM dihydroethidium (DHE) (Sigma-Aldrich, MO, USA), a fluorescent dye for detection of intracellular O^{2-} was used (Owusu-Ansah *et al.*, 2008). The biofilms were washed with 0.5 × NSS after which 25 μM DHE in fresh 0.5 × VNSS medium was added and incubated in the dark for 2 hours. After incubation, the cells were washed with 0.5 × NSS, and the ROS production was determined by spectrophotometry (518 and 605 nm for excitation and emission, respectively). The plates were incubated for 3 days before measurement of pyomelanin as described.

H_2O_2 treatment of *V. cholerae* biofilms

Overnight cultures were inoculated at a final concentration of 10^6 cells ml^{-1} in 0.5 VNSS in 24-well plates incubated at RT with shaking at 60 rpm. After 3 days, the biofilms were treated with 30 mM H_2O_2 for 30 minutes, after which the H_2O_2 was removed and fresh 0.5 × VNSS medium with or without *A. castellanii* (2×10^4 cells ml^{-1}) was added. After co-incubation for 3 days, the *V. cholerae* biofilm biomass was quantified using the CV assay.

Catalase treatment of *V. cholerae* biofilms

Overnight cultures of *V. cholerae* were inoculated at a final concentration of 10^5 cells ml^{-1} in 0.5 VNSS in 24-well plates incubated at RT with shaking at 60 rpm. After 3 days, the media with or without *A. castellanii* (2×10^4 cells ml^{-1}) was refreshed and 0.1 mg ml^{-1} catalase (Sigma-Aldrich, MO, USA) was added. After co-incubation for 3 days, the *V. cholerae* biofilm biomass was quantified using the CV assay.

qRT-PCR validation of transcriptomic data

RNA was prepared from a late biofilm grazing assay with *A. castellanii* in 24 well plates. After removal of supernatant, RNAlater (Qiagen, Hilden, Germany) was added and cells were harvested from the wells by mechanical manipulation. RNA extraction was then performed as described previously. The concentration was measured using a spectrophotometry (NanoDrop ND-1000; NanoDrop Technologies) after treatment with TURBO™ DNase (Ambion- Life Technologies). DNA was prepared from 500 ng RNA from each sample by iScript™ Reverse Transcription (Bio-Rad). Quantitative RT-PCR (qRT-PCR) experiments were done using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) by QuantStudio™ 6 Flex Real-Time PCR System using the primers specific for VC1344, VC1345, VC1346 and VC1347 listed in Supplementary Table 1. The expression was determined relative to the expression of the endogenous control gene *gyrA* using the comparative Ct (DDCt) method of reverse-transcriptase PCR (RT-PCR).

T. pyriformis grazing assays

Microtitre plates containing 1-day-old biofilms were prepared as described above. After 24 hours, the supernatants were removed and fresh 0.5 × VNSS media with or without *T. pyriformis* was added (10^3 cells ml^{-1}; determined by inverted microscopy) and the plates incubated at RT with shaking at 60 rpm for 3 days. The cell density was measured by spectrophotometry at $OD_{600\ nm}$. Planktonic fractions were collected for enumeration of CFU ml^{-1} and biofilm biomass determined by CV staining and spectrophotometry ($OD_{490\ nm}$). Numbers of *T. pyriformis* were determined by microscopy at each sampling time and pyomelanin was measured in the cell-free supernatants as described previously.

***V. cholerae* - *A. castellanii* intracellular survival assay**

To determine the role of *hmgA* in intracellular survival of *V. cholerae* internalised by *A. castellanii,* number of internal *V. cholerae* was measured after 24 hours. Briefly, *A. castellanii* (2×10^5 cells ml^{-1}) in 0.5 × NSS and 1% HKB were seeded in 24 well microtitre plates one day prior to the start of the experiment. After 24 hours, the wells were washed gently with 0.5 × NSS and *V. cholerae* (10^7 cells ml^{-1}) in 0.5 × NSS were added. Plates were incubated for 1 hour statically at RT to allow ingestion of *V. cholerae* by *A. castellanii.* Extracellular bacteria were removed by washing wells three times with 0.5 × NSS and treatment with gentamicin (300 μg ml^{-1}) for 1 hour at RT. The gentamicin was then removed by washing three times with 0.5 × NSS. The cells were then incubated in 0.5 × NSS at RT statically for 24 hours, after which the amoeba cells were lysed by addition of 1% Triton X-100 in 0.5 × NSS for 20 minutes. Bacteria were enumerated by drop plate colony counting.

Data analysis

Statistical analysis was performed using GraphPad Prism version 7.01 for Windows, GraphPad Software, La Jolla California USA, (www.graphpad.com). Data that did not follow Gaussian distribution as determined by analysing the frequency distribution graphs, was natural log transformed. Two-tailed student's t-tests were used to compare means between experimental samples and controls. For experiments including multiple samples, one-way or 2-way ANOVAs were used for the analysis and Sidak's or Dunnett's Multiple Comparison Test provided the post-hoc comparisons of means when appropriate.

Results and discussion

The current study was designed to further elucidate antiprotozoal activities generated by *V. cholerae* biofilms. Heterotrophic protists are major predators of bacteria, and consequently, bacteria have evolved both pre- and post-ingestional defence strategies

to resist predation (Matz & Kjelleberg, 2005). Such defence strategies employed by *V. cholerae* include biofilm formation (Matz *et al.*, 2005), expression of the PrtV protease (Vaitkevicius *et al.*, 2006) and the T6SS (Pukatzki *et al.*, 2006). Although a QS mutant of *V. cholerae* was more sensitive to predation than the corresponding isogenic wild type, it was still partially resistant to grazing (Erken *et al.*, 2011), implying the existence of other QS-independent anti-protozoal mechanisms.

RNA-seq revealed differences between grazed and ungrazed biofilms

In order to identify other anti-predation strategies employed by *V. cholerae* biofilms, RNA-Seq was performed. Total RNA isolated from 3 biological replicates of biofilms exposed to grazing by *A. castellanii,* was subjected to Illumina HiSeq 2000 sequencing. Between 108 and 127 million pairs of reads were generated with approximately 5 million reads per sample removed after quality filtering and trimming. Between 98.31 and 99.42% of reads were mapped to the *V. cholerae* N16961 genome and approximately 0.13% were mapped to *A. castellanii* contigs. The $\log_2$ fold change in fragments per kilobase of exon per million fragments mapped (FPKM) varies from -1.964 to -0.724 for the down-regulated transcripts, and from 0.797 to 3.535 for the up-regulated transcripts.

Significantly differentially expressed genes (SDE) were considered at fold-change of 2.0 and adjusted p-value of $p<0.05$. Cuffdiff analysis of the transcriptome revealed that 71 transcripts were significantly up-regulated and 60 were significantly down-regulated in the grazed biofilm compared with the ungrazed control (see Supplementary Table 2 for the complete list of differentially expressed genes).

A relatively large fraction of the up-regulated transcripts correspond to genes involved in metabolism, in particular nucleic acid, amino acid, lipid and carbohydrate transfer and metabolism. These transcripts encode proteins associated with amino

316 acid biosynthesis and metabolism, such as VC0027 (threonine metabolism), VC1061
317 (cysteine biosynthesis), *hisD*, *hisG*, *hisH*, VC1134, VC1135, VC1137, VC1138 and
318 VC1139 (histidine metabolism), *trpA* (tryptophan biosynthesis), *gltD* and VC2373
319 (glutamate biosynthesis), *glnA* (glutamine biosynthesis) *argC*, VC2617, VC2641,
320 VC2642, VC2643, and VC2508 (arginine metabolism and biosynthesis), VC1704
321 (cysteine and methionine metabolism), VC0162, VC0031 and VC0028 (isoleucine
322 biosynthesis) and VC0392, VCA0604 and VCA0605 (aminotransferases). The
323 increase in metabolism and energy production might be related to an increase in
324 available nutrient resources since feeding will result in the release of nutrients by
325 protozoa, either due to 'sloppy feeding' or excretion of waste products (Wang *et al.*,
326 2009).

327 The genes in the tyrosine catabolic pathway (VC1344 to VC1347) were down-
328 regulated in the grazed samples compared to ungrazed samples. These genes lead to
329 the catabolism of tyrosine to fumarate and acetoacetate (Valeru *et al.*, 2009, Wang *et*
330 *al.*, 2011), and are confirmed to be associated with a pigmented phenotype due to
331 pyomelanin production (Ivins & Holmes, 1980, Ivins & Holmes, 1981, Ruzafa *et al.*,
332 1995). The enzyme homogentisate 1, 2 – dioxygenase (HmgA) (VC1345) is involved
333 in L-tyrosine catabolism in both prokaryotic and eukaryotic organisms (Fernández-
334 Cañón & Peñalva, 1995, Kotob *et al.*, 1995) and a null mutation in *hmgA* in *V.*
335 *cholerae* leads to accumulation and auto-oxidation of homogentisic acid (HGA),
336 which in turns will lead to production of pyomelanin (Figure 1). The down regulation
337 of VC1344 - VC1347 during predation was confirmed by qPCR ($\log_2$ fold change of -
338 1.51, -1.21, -1.67, and -1.45 respectively).

339 Melanin production by auto-oxidation of HGA has been shown to occur in
340 many organisms, ranging from bacteria to humans. The gene encoding HmgA as well

as the rest of the pathway is well conserved, and a BLAST search of the NCBI database revealed that many *Vibrio* spp., including non-pathogenic environmental *Vibrio* spp. have this pathway. The protective mechanisms of melanin are unclear, but melanin (charged polymers) present in the cell wall may serve as a physical or chemical barrier (Nosanchuk & Casadevall, 1997, Jacobson, 2000, Eisenman *et al.*, 2005). An *hmgA* mutant of *V. cholerae* exhibited greater UV and oxidative stress resistance, increased expression of a subunit of the toxin co-regulated pilus and cholera toxin (CT), and was enhanced in its ability to colonise the infant mouse (Valeru *et al.*, 2009). In contrast, a *V. campbellii hmgA* mutant did not show increased UV resistance and was less virulent than the wild type strain, although the wild type strain exhibited higher resistance to oxidative stress when incubated with supernatants from the *hmgA* mutant (Wang *et al.*, 2013).

Pigment production has also been demonstrated to provide a range of functions in many different microorganisms. For example, melanin can protect the pathogenic fungus, *Cryptococcus neoformans,* from antibody-mediated phagocytosis by macrophages (Wang *et al.*, 1995), as well as from digestion of phagocytosed cells by the amoeba *A. castellanii* (Steenbergen *et al.*, 2001). Melanized *C. neoformans* are significantly less susceptible to hydrolytic enzymes commonly used by environmental predators than non-melanized cells (Rosas & Casadevall, 2001). Melanin production in the fungus, *Paracoccidioides brasiliensis,* increases protection from phagocytosis by macrophages and intracellular resistance, and decreased drug susceptibility (da Silva *et al.*, 2006), while in the yeast *Exophiala* (*Wangiella*) *dermatitidis,* melanin production prevented killing by the phagolysosomal oxidative burst of human neutrophils (Schnitzler *et al.*, 1999).

Pyomelanin production increases the grazing resistance of *V. cholerae* biofilms

In order to determine the role of pyomelanin in grazing resistance of biofilms, *V. cholerae* wild type and *hmgA* mutant strains were allowed to form biofilms and after 1 or 3 days, either *T. pyriformis* or *A. castellanii* were added. After 3 days of grazing, CFU and crystal violet (CV) measurements determined planktonic cell and biofilm biomass, respectively.

The grazing resistance of early biofilms (1-day old) of the *V. cholerae hmgA* mutant and wild type strains in the presence of *A. castellanii* were not significantly different (Figure 2A). In contrast, when late biofilms (3-days old) were exposed to grazing by *A. castellanii,* the *hmgA* mutant was significantly more grazing resistant than the wild type. The biofilm biomass of the grazed wild type was reduced by 7.3% compared to the non-grazed control, whereas the *hmgA* mutant biofilm biomass increased by 16.5% after grazing (Figure 2B). Control ungrazed biofilms of the WT and *hmgA* mutant strains were not significantly different, indicating that the biofilm growth for both strains was similar (Supplementary Figure 1). Furthermore, the cell-free supernatant of the *hmgA* mutant does not show toxicity towards *A. castellanii* trophozoites compared to the WT (Supplementary Figure 2).

The resistance of planktonic cells of the *hmgA* mutant to predation by *T. pyriformis* was also investigated, as *A. castellanii* cannot feed efficiently on planktonic cells (Huws *et al.*, 2005). *T. pyriformis* is a filter-feeding ciliate that can feed effectively on early biofilms as well as planktonic cells (Parry, 2004). The use of a second type of grazer with different feeding mechanisms and niche is important for establishment of the generality of a grazing resistance mechanism. The early biofilm (1-day old) biomass (Figure 3A) and numbers of planktonic cells (Figure 3B) of the *hmgA* mutant and wild type strains in the presence of *T. pyriformis* were not significantly different. Interestingly, a further increase in pyomelanin production by

391 the *hmgA* mutant was observed after 3 days of grazing by *A. castellanii* but not when
392 exposed to grazing by *T. pyriformis* (Figure 4), which is consistent with the increased
393 grazing resistance of the mature biofilm against *A. castellanii*.

394 Addition of more nutrients to WT and *hmgA* mutant strains did not result in
395 the same increase in pyomelanin levels as active grazing by *A. castellanii*. This
396 indicates that the extra nutrients in the *A. castellanii* culture are not responsible for
397 induction of pyomelanin production in the *hmgA* mutant when exposed to grazing by
398 *A. castellanii.* Furthermore, the addition of cell-free supernatants from *A. castellanii*
399 with or without HKB and heat-killed *A. castellanii* cells did not induce
400 overproduction of pyomelanin, supporting our hypothesis that active phagocytosis by
401 *A. castellanii* is required (Supplementary Figure 3).

402 **Pyomelanin and production of reactive oxygen species (ROS)**

403 The increases in grazing resistance of the *hmgA* mutant biofilms (Figure 2B)
404 correlated with an increase in pigment production. On day one, the pyomelanin
405 concentration in both the supernatant of wild type and *hmgA* mutant strains was low
406 (normalised pigment production (OD_{405nm}/Biofilm biomass) = 0.0013 and 0.0009
407 respectively) (Figure 5A). However, after 3 days the pyomelanin concentration in
408 supernatants of the *hmgA* mutant was twenty-fold higher than those of the wild type
409 (normalised pigment production (OD_{405nm}/Biofilm biomass) = 0.0016 and 0.033
410 respectively).

411 To determine if the *hmgA* cell-free supernatant would provide protection
412 against predation by *A. castellanii* to the WT strain, the cell-free supernatant of 3-days
413 old established *hmgA* mutant biofilms were added to the *A. castellanii* grazing assays.
414 The addition of undiluted cell-free supernatants of *V. cholerae* to pre-established
415 biofilms led to their dispersal due to lack of nutrients and accumulation of waste.

Therefore, cell-free supernatants diluted with fresh VNSS was used, and results showed that at a concentration of 50%, the cell-free supernatant of the *hmgA* strain significantly increased the resistance of the wild type biofilm to grazing by *A. castellanii* (Figure 6A).

In order to further investigate the relationship between grazing and pigment production, the amount of O^{2-} generated in biofilms of the *V. cholerae* A1552 wild type, *hmgA* mutant and complemented strain was monitored, as it has been suggested that ROS are generated during pigment production (Valeru *et al.*, 2009). Notably, when pigment production increased on day 3 in the *hmgA* mutant, the ROS level also increased (Figure 5B). Biofilms of the *hmgA* mutant strain generated 79% more O^{2-} than the A1552 biofilms ($p = 0.001$).

Hydrogen peroxide increased the grazing resistance of *V. cholerae*

Previous studies have shown that exposure of *V. cholerae* to H_2O_2 induced oxidative stress responses and virulence factor expression (Valeru *et al.*, 2009). The auto-oxidation of HGA can generate superoxide radicals and H_2O_2 in eukaryotic cells at physiological pH (Martin Jr & Batkoff, 1987). Here, the effect of addition of H_2O_2 as a substitute for pyomelanin-associated ROS on resistance of *V. cholerae* biofilms to amoebae grazing was tested in order to further confirm the pyomelanin/ROS-mediated grazing resistance.

V. cholerae biofilms were pre-grown for 3 days, followed by exposure to H_2O_2 for 30 min (Figure 6B). *A. castellanii* was added after H_2O_2 exposure and the culture incubated for 3 days. The health and number of *A. castellanii* was monitored and there was no difference between the total numbers of amoebae when co-incubated with the *V. cholerae* biofilms that had been exposed to H_2O_2 compared to the controls with HKB. After 3 days of grazing by *A. castellanii*, the biomass of the untreated *V.*

441 *cholerae* biofilms were significantly reduced while biofilms that had been treated with
442 H_2O_2 for 30 min, were not reduced ($p = 0.0246$) (Figure 6B). In addition, pre-grown
443 3-days old biofilms were treated with 0.1 mg ml^{-1} catalase to reduce ROS of the *hmgA*
444 mutant biofilm. After 3 days of grazing by *A. castellanii*, the biomass of the treated
445 *V. cholerae hmgA* biofilm was significantly reduced compared to the untreated
446 biofilm ($p = 0.0176$) (Figure 6C). Taken together, our results suggest that the
447 production of pyomelanin results in production of ROS, which in turn, results in an
448 increase in grazing resistance.

449 We investigated the effect of pyomelanin on survival of *V. cholerae*
450 intracellularly in *A. castellanii*. The total number of *V. cholerae* cells associated with
451 *A. castellanii* (extracellular and intracellular), as well as the number of intracellular *V.*
452 *cholerae* was determined, and there was no difference between wild type and *hmgA*
453 mutant strains (Supplementary Figure 4).

454 Pigmented hypertoxinogenic strains of *V. cholerae* have been previously
455 reported both in random mutagenesis experiments (Mekalanos *et al.*, 1979, Parker *et*
456 *al.*, 1979, Ivins & Holmes, 1980) as well as in environmental isolates. For example, *V.*
457 *cholerae*, ATCC 14035, Serotype Ogawa serovar O1 strain isolated originally from a
458 stool sample produced a reddish brown pigment when grown in low nutrient condition
459 media supplemented with L-glutamic acid and L-tyrosine (Ruzafa *et al.*, 1995). In
460 addition, six nontoxigenic serogroup O139 (water isolates) and one toxigenic O1
461 (clinical isolate) strains isolated from different years and from different provinces of
462 China were pigmented. All the O139 strains had the same 15-bp deletion in *hmgA*
463 and a 10-bp deletion was found in the VC1345 gene of the O1 strain, indicating that
464 the mutation of this gene may provide a fitness advantage in the environment (Wang
465 *et al.*, 2011).

Overall this study demonstrates that *V. cholerae* O1 El Tor alters its transcriptome in the presence of the predator, *A. castellanii*. One metabolic pathway that was down-regulated under grazing pressure was the tyrosine catabolic pathway, resulting in accumulation of pyomelanin. Experiments with a pyomelanin-overproducing mutant demonstrate that it is more resistant to predation by *A. castellanii* than the isogenic wild type. Furthermore, the *hmgA* mutant produces more ROS, which may account for the increased grazing resistance of the *hmgA* mutant, as *V. cholerae* biofilms pre-treated with H_2O_2 were also more grazing resistant.

This project provides insight into the genes involved in defence against protozoan grazing of *V. cholerae*. Data presented here shows that the expression of pyomelanin aids in protection of *V. cholerae* from grazing in the environment and previous reports have shown that it also plays a role in virulence factor expression and colonization ability (Valeru et al., 2009). This further supports our hypothesis that predation is a major selective factor for maintenance of virulence genes in the environment and thus melanin production may be one such dual use virulence factor.

Acknowledgements

This study was supported by the Australian Research Council [DP140102247 and DP170100453], The ithree Institute at the University of Technology Sydney and the Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Singapore. The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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637

638 **Figure Legends**

639 **Figure 1.** Differentially expressed transcripts in grazed compared to ungrazed
640 biofilms involved in tyrosine degradation in *V. cholerae*. FC represents $\log_2$ fold
641 changes. The pathway in *V. cholerae* is proposed by Valeru *et al.* (2009).

642 **Figure 2.** Biofilm biomass of early (A) and late (B) biofilms of *V. cholerae* A1552
643 exposed to grazing by *A. castellanii* for 72 hours. Biofilm biomass was determined
644 by CV staining. Data were natural log transformed and the percentage change of
645 biofilm biomass was calculated by removing the biomass of ungrazed samples from
646 the grazed samples divided by the ungrazed. The experiment was run in three
647 replicates and repeated three times separately. Error bars represent standard
648 deviation. Small letters indicate different statistical groups derived from 1way
649 ANOVA and Dunnett's multiple comparisons tests. Statistical significance is
650 indicated by (** $p < 0.001$; ***, $p < 0.0001$ and Ns, not significant).

651 **Figure 3.** Early biofilms of *V. cholerae* A1552 exposed to *T. pyriformis* for 72 hours.
652 Biofilm biomass was determined by CV staining (A) and the planktonic cells in the
653 supernatant enumerated by the drop plate method (B). Data was natural log
654 transformed and the percentage change in biofilm biomass was calculated by
655 removing the ungrazed biomass from the grazed biofilm biomass divided by the
656 ungrazed. The experiment was run in three replicates and repeated three times
657 separately. Error bars represent standard deviation. Statistical analysis was performed
658 using student's t-test which revealed no significant difference, $p=0.3$ (A) and $p= 0.1$
659 (B).

Figure 4. Amount of pyomelanin produced by non-grazed or grazed established biofilms after 3 days exposure to *A. castellanii* (A) or *T. pyriformis* (B). Pyomelanin secreted by the biofilm into the supernatant was measured by optical density ($OD_{405\ nm}$) of the cell-free supernatant obtained from the biofilms. Amount of pyomelanin was then normalised by using the corresponding biofilm biomass measured by CV assays ($OD_{490\ nm}$). Experiments were run in triplicates and repeated 3 times on different days. Error bars represent the standard deviation of three replicates. Bars indicate different statistical groups derived from 2way ANOVA and Sidak's multiple comparisons test. Statistical significance is indicated by (****, $p < 0.0001$).

Figure 5. Amount of pyomelanin produced by biofilms of *V. cholerae* A1552 wild type and *hmgA* mutant strains after 1 and 3 days (A). Amount of ROS in the cell-free supernatant of 3 days-old biofilms of *V. cholerae* A1552 wild type and *hmgA* mutant (B). Pyomelanin secreted by the biofilm into the supernatant was measured by optical density ($OD_{405\ nm}$) of the cell-free supernatant obtained from the biofilms. Amount of pyomelanin was then normalised by using the corresponding biofilm biomass measured by CV assays ($OD_{490\ nm}$). Error bars represent the standard deviation of three replicates. Statistical analysis was performed using 2way ANOVA and Sidak's multiple comparisons test (A) and Student's t-test (B). Statistical significance is indicated by (**, $p < 0.001$, ****, $p < 0.0001$).

Figure 6. Effect of cell-free supernatants (A), H_2O_2 (B) and catalase (C) on grazing resistance to *A. castellanii*. The cell-free supernatant of 3-days old *V. cholerae* biofilms were added to 3-days old biofilms at a concentration of 50% in fresh VNSS and incubated with *A. castellanii* for 3 days. Biofilm biomass was determined by CV staining. Experiments were run in triplicate and repeated 3 times on different days. Error bars represent the standard deviation of three replicates. Statistical analysis was

685 performed using 1way ANOVA and Dunnett's multiple comparisons test comparing
686 all to the WT (A), Student's t-test (B) and 2way ANOVA and Sidak's multiple
687 comparisons test (C). Statistical significance is indicated by (*, $p < 0.05$; ***, $p <$
688 0.001 and ****, $p < 0.0001$ and Ns, not significant).

689 **Supplementary Figure 1.** Biofilm biomass of *V. cholerae* wild type and *hmgA*
690 mutant grown for 1 or 3 days as determined by CV staining. The experiment was run
691 in triplicate and repeated three times. Error bars represent standard deviation.
692 Statistical analysis was performed using 1way ANOVA and Sidak's multiple
693 comparisons test which revealed no significant differences, $p>0.2$.

694 **Supplementary Figure 2.** The number of *A. castellanii* trophozoites (A) and cysts
695 (B) after 0 and 24-hour incubation in cell-free supernatants of wild type and *hmgA*
696 mutant strains of *V. cholerae*. Error bars represent standard deviation. Statistical
697 analysis was performed using 2way ANOVA and Sidak's multiple comparisons test
698 which revealed no significant differences, $p=0.8$ (A) and $p>0.9$ (B).

699 **Supplementary Figure 3.** Pyomelanin production by *V. cholerae* wild type (A) and
700 *hmgA* mutant (B) biofilms. Supernatants of *A. castellanii* were obtained after
701 incubation in 0.5 × VNSS with or without 1% HKB for 72 hours at RT. Cell-free
702 supernatants were added after 72 hours incubation of biofilms. Pyomelanin secreted
703 by the biofilm into the supernatant was measured by optical density ($OD_{405\ nm}$) of the
704 cell-free supernatant obtained from the biofilms. Amount of pyomelanin was then
705 normalised by using the corresponding biofilm biomass measured by CV assays ($OD_{490\ nm}$).
706 Experiments were run in triplicate and repeated three times. Error bars
707 represent standard deviation. Statistical analysis was performed using 1way ANOVA
708 and Dunnett's multiple comparisons tests comparing to 0.5 × VNSS controls which
709 revealed no significant differences, $p>0.1$ (A) and $p>0.5$ (B).

710 **Supplementary Figure 4.** Number of intracellular *V. cholerae* after 24 hour co-
711 incubation with *A. castellanii*. Error bars represent standard deviation. Statistical
712 analysis was performed using student's t-test which revealed no significant
713 differences, $p > 0.9$.
714