

## RESEARCH ARTICLE

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

Parisa Noorian<sup>1,2,†</sup>, Jie Hu<sup>3,†</sup>, Zhiliang Chen<sup>1,4</sup>, Staffan Kjelleberg<sup>5</sup>, Marc R Wilkins<sup>1,4</sup>, Shuyang Sun<sup>2</sup> and Diane McDougald<sup>1,2,5,\*</sup>

<sup>1</sup>School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia, <sup>2</sup>The ithree Institute, University of Technology Sydney, Sydney, NSW 2007, Australia, <sup>3</sup>School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore, <sup>4</sup>Systems Biology Initiative, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia and <sup>5</sup>Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Singapore 637551, Singapore

\*Corresponding author: The ithree Institute, University of Technology Sydney, PO Box 123 Broadway, Sydney, NSW 2007, Australia. Tel: +61-2-9514-4869; Fax: +61-2-9514-4143; E-mail: [diane.mcdougald@uts.edu.au](mailto:diane.mcdougald@uts.edu.au)

†These authors contributed equally to this work.

**One sentence summary:** Pigment production by the pathogenic bacterium that causes cholera protects the bacterium from being consumed by the natural predatory amoeba *A. castellanii*.

**Editor:** Rolf Kümmerli

## 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*.

**Keywords:** protozoan predation; biofilms; environmental fitness; *V. cholerae*; grazing resistance; pigment production

## INTRODUCTION

*Vibrio cholerae*, the causative agent of cholera, persists in brackish and estuarine water systems (Colwell, Kaper and Joseph 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

Received: 26 April 2017; Accepted: 30 October 2017

© FEMS 2017. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact [journals.permissions@oup.com](mailto:journals.permissions@oup.com)

indicates its ability to respond to such stresses (Colwell and 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 and 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, Warhurst and Drasar 1992; Abd, Weintraub and Sandström 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 (Martínez Pérez, Macek and Castro Galván 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 (QS). QS has been shown to regulate antiprotozoal activities in *V. cholerae* biofilms including, the production of Vibrio polysaccharide 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, Kjelleberg and McDougald 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 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 are not completely eliminated by predation (Erken et al. 2011; Lutz et al. 2013), suggesting that 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 and 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 lipopeptide surfactants, massetolide and viscosin to protect itself against *Naegleria americana* (Mazzola et al. 2009), in addition to 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin, hydrogen

cyanide and pyoluteorin (Jousset et al. 2010). Cell surface properties have also been shown to affect grazing resistance. For example, cell surface hydrophobicity affects grazing of picoplankton cells by nanoflagellates (Monger, Landry and Brown 1999). Moreover, Wildschutte et al. (2004) showed that differences in O-antigen are sufficient to allow for prey discrimination by protozoa grazing on different serotypes of *Salmonella*.

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

## MATERIAL AND METHODS

### Strains and growth conditions

Organisms used in this study are listed in Table 1. Bacterial strains were routinely grown in Luria-Bertani (LB) broth and on agar plates (Sambrook, Fritsch and Maniatis 1989) as appropriate, with carbenicillin ( $100 \mu\text{g ml}^{-1}$ ). *A. castellanii* was routinely passaged in 15 ml growth medium containing peptone-yeast-glucose (PYG) ( $20 \text{ g l}^{-1}$  proteose peptone,  $1 \text{ g l}^{-1}$  yeast extract) supplemented with 1 litre  $0.1 \times \text{M9}$  minimal medium ( $6 \text{ g l}^{-1} \text{ Na}_2\text{HPO}_4$ ,  $3 \text{ g l}^{-1} \text{ KH}_2\text{PO}_4$ ,  $0.5 \text{ g l}^{-1} \text{ NaCl}$ ,  $1 \text{ g l}^{-1} \text{ NH}_4\text{Cl}$ ) and  $0.1 \text{ M}$  sterile-filtered glucose in  $25 \text{ cm}^2$  tissue culture flasks with ventilated caps (Sarstedt Inc., Nümbrecht, Germany) and incubated statically at  $30^\circ\text{C}$ . *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,  $500 \mu\text{l}$  of *T. pyriformis* was passaged in  $20 \text{ ml}$  of  $0.5 \times \text{NSS}$  medium ( $8.8 \text{ g l}^{-1} \text{ NaCl}$ ,  $0.735 \text{ g l}^{-1} \text{ Na}_2\text{SO}_4$ ,  $0.04 \text{ g l}^{-1} \text{ NaHCO}_3$ ,  $0.125 \text{ g l}^{-1} \text{ KCl}$ ,  $0.02 \text{ g l}^{-1} \text{ KBr}$ ,  $0.935 \text{ g l}^{-1} \text{ MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $0.205 \text{ g l}^{-1} \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $0.004 \text{ g l}^{-1} \text{ SrCl}_2 \cdot 6\text{H}_2\text{O}$  and  $0.004 \text{ g l}^{-1} \text{ H}_3\text{BO}_3$ ) (Mårdén et al. 1985) supplemented with 1% (v/v) of heat-killed *Pseudomonas aeruginosa* PAO1 (heat-killed bacteria [HKB]) in a  $25 \text{ cm}^2$  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 HKB, *P. aeruginosa* was grown overnight in LB at  $37^\circ\text{C}$  with shaking at 200 rpm and adjusted to  $\text{OD}_{600} = 1.0$  ( $10^9$  cells  $\text{ml}^{-1}$ ) in  $0.5 \times \text{NSS}$ . The tubes were then transferred to a water bath at  $65^\circ\text{C}$  for 2 h, and then tested for viability by plating on LB agar plates at  $37^\circ\text{C}$  for 2 days. HKB stocks were stored at  $-20^\circ\text{C}$ .

### 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, three biological replicates of *V. cholerae* biofilms were cultivated on the interior surfaces of Silastic® laboratory tubing (Dow Corning, MI, USA) ( $3.2 \text{ mm}$  diameter; length,  $14 \text{ cm}$ ) in  $0.5 \times \text{Vääätänen}$  nine salts solution (VNSS) ( $1 \text{ g}$  bacteriological peptone,  $0.5 \text{ g}$  yeast extract,  $0.5 \text{ g}$  D-glucose,  $0.01 \text{ g}$   $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

**Table 1.** Strains and plasmids used in this study.

Strain	Properties	Reference/source
<b>Bacterial strains</b>		
<i>V. cholerae</i> A1552	Wild type, O1 El Tor, Inaba, smooth, Rif <sup>r</sup>	Valeru et al. (2009)
<i>V. cholerae</i> A1552 <i>hmgA</i>	O1 El Tor, Inaba, smooth, $\Delta hmgA$ , Rif <sup>r</sup> , Km <sup>r</sup>	Valeru et al. (2009)
<i>V. cholerae</i> A1552 <i>hmgA</i> complement	O1 El Tor, Inaba, smooth, $\Delta hmgA$ , <i>hmgA</i> ::pUC18, Rif <sup>r</sup> , Ap <sup>r</sup> , Km <sup>r</sup>	Valeru et al. (2009)
<i>V. cholerae</i> A1552 pUC18	O1 El Tor, Inaba, smooth, pUC18, Rif <sup>r</sup> , Ap <sup>r</sup>	This study
<i>V. cholerae</i> A1552 <i>hmgA</i> pUC18	O1 El Tor, Inaba, smooth, $\Delta hmgA$ , pUC18, Rif <sup>r</sup> , Ap <sup>r</sup> , Km <sup>r</sup>	This study
<b>Plasmids</b>		
pUC18	Cloning vector, pMB1 ori, LAC pr, <i>lacZ</i> , Ap <sup>r</sup>	Yanisch-Perron et al. (1985)
<b>Protozoan strains</b>		
<i>A. castellanii</i>	Wild type	ATCC 30234
<i>T. pyriformis</i>	Wild type	ATCC 205063

and 0.01 g Na<sub>2</sub>HPO<sub>4</sub>) in 1 l of 0.5× NSS and fed at a flow rate of 9 ml h<sup>-1</sup> using a continuous flow system at RT. After 3 days, washed cells of *A. castellanii* were re-suspended in 0.5× VNSS, injected into the tubing and incubated without flow for 2 h. 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 re-suspended 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, San Diego, CA, 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, Peterson and Biggs 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, Noé and Touzet 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 and 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 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 fragments per kilobase of exon per million fragments mapped (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 downregulated SDE genes were placed into cluster of orthologous group (COG) categories by NCBI conserved domain search (Tatusov, Koonin and Lipman 1997). With the assistance of the Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (National Institute of Allergy and Infectious Diseases), 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<sup>5</sup> cells ml<sup>-1</sup> in 0.5× VNSS were added to 24-well microtitre plates (Falcon, Becton Dickinson, NJ, USA) and incubated for 24 and 72 h with shaking at 60 rpm at RT. After incubation, fresh VNSS with or without *A. castellanii* ( $2 \times 10^4$  cells ml<sup>-1</sup>) was added, and the plates were incubated at RT with shaking at 60 rpm for 3 days. The cell density in each well was measured by spectrophotometry at OD<sub>600 nm</sub> (Wallac Victor<sup>2</sup> 1420 Multilabel Counter, Perkin Elmer Life Sciences, Billerica, MA, USA). In order to quantify the biofilm biomass, crystal violet (CV) assays were performed (O'Toole and Kolter 1998). Briefly, all the planktonic cells were removed by washing three times with 0.5× NSS before adding CV (0.3%) for 15 min. The wells were washed a further three times using 0.5× NSS to remove the unattached CV, and then the stain was solubilised using 96% ethanol and the OD<sub>490 nm</sub> 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 was acquired by centrifugation at 6000×  $g$  for 10 min and filtration (0.22-mm filters, Millex-GP, Millipore, Billerica, 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* ( $2 \times 10^4$  cells ml<sup>-1</sup>) and incubated at RT with shaking at 60 rpm for 3 days. The biofilm biomass was then quantified by CV assays.

### Reactive oxygen species and pyomelanin quantification

The pyomelanin in the aqueous phase was determined by spectrophotometry (OD<sub>405 nm</sub>) of the cell-free supernatant acquired by centrifugation at 6000×  $g$  for 10 min and filtration (0.22-mm filters, Millex-GP, Millipore, Billerica, 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 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 min. 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 ( $OD_{490\text{ nm}}$ ).

To assess the level of reactive oxygen species (ROS), 25  $\mu\text{M}$  dihydroethidium (DHE) (Sigma-Aldrich, MO, USA), a fluorescent dye for detection of intracellular  $O_2^-$  was used (Owusu-Ansah, Yavari and Banerjee 2008). The biofilms were washed with 0.5× NSS after which 25  $\mu\text{M}$  DHE in fresh 0.5× VNSS medium was added and incubated in the dark for 2 h. 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  $\text{ml}^{-1}$  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 min, after which the  $H_2O_2$  was removed and fresh 0.5× VNSS medium with or without *A. castellanii* ( $2 \times 10^4$  cells  $\text{ml}^{-1}$ ) 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  $\text{ml}^{-1}$  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* ( $2 \times 10^4$  cells  $\text{ml}^{-1}$ ) was refreshed and 0.1 mg  $\text{ml}^{-1}$  catalase (Sigma-Aldrich, MO, USA) was added. After co-incubation for 3 days, the *V. cholerae* biofilm biomass was quantified using the CV assay.

### Quantitative reverse-transcriptase 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, RNeasy (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 spectrophotometry (NanoDrop ND-1000; NanoDrop Technologies) after treatment with TURBO DNase (Ambion, ThermoFisher, Waltham, MA, USA). DNA was prepared from 500 ng RNA from each sample by iScript Reverse Transcription (Bio-Rad, Hercules, CA, USA). Quantitative reverse-transcriptase PCR (qRT-PCR) experiments were done using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) by QuantStudio 6 Flex Real-Time PCR System using the primers specific for VC1344, VC1345, VC1346 and VC1347 listed in Supplementary Table 1, Supporting Information. The expression was de-

termined relative to the expression of the endogenous control gene *gyrA* using the comparative Ct (DDCt) method of RT-PCR.

### *T. pyriformis* grazing assays

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

### *Vibrio cholerae*–*A. castellanii* intracellular survival assay

To determine the role of *hmgA* in intracellular survival of *V. cholerae* internalised by *A. castellanii*, the number of internal *V. cholerae* was measured after 24 h. Briefly, *A. castellanii* ( $2 \times 10^5$  cells  $\text{ml}^{-1}$ ) in 0.5× NSS and 1% HKB were seeded in 24-well microtitre plates 1 day prior to the start of the experiment. After 24 h, the wells were washed gently with 0.5× NSS and *V. cholerae* ( $10^7$  cells  $\text{ml}^{-1}$ ) in 0.5× NSS were added. Plates were incubated for 1 h 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 ( $300 \mu\text{g ml}^{-1}$ ) for 1 h 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 h, after which the amoeba cells were lysed by addition of 1% Triton X-100 in 0.5× NSS for 20 min. 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](http://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 two-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 and 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 three 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 FPKM varies from  $-1.964$  to  $-0.724$  for the down-regulated transcripts, and from  $0.797$  to  $3.535$  for the upregulated transcripts.

SDE genes 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 upregulated and 60 were significantly downregulated in the grazed biofilm compared with the ungrazed control (see Supplementary Table S2, Supporting Information, for the complete list of differentially expressed genes).

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

The genes in the tyrosine catabolic pathway (VC1344 to VC1347) were downregulated in the grazed samples compared to ungrazed samples. These genes lead to the catabolism of tyrosine to fumarate and acetoacetate (Valeru et al. 2009; Wang et al. 2011), and are confirmed to be associated with a pigmented phenotype due to pyomelanin production (Ivins and Holmes 1980, 1981; Ruzafa, Sanchez-Amat and Solano 1995). The enzyme HmgA (VC1345) is involved in L-tyrosine catabolism in both prokaryotic and eukaryotic organisms (Fernández-Cañón and Peñalva 1995; Kotob et al. 1995) and a null mutation in *hmgA* in *V. cholerae* leads to accumulation and auto-oxidation of HGA, which in turn will lead to production of pyomelanin (Fig. 1). The downregulation of VC1344–VC1347 during predation was confirmed by qPCR ( $\log_2$  fold change of  $-1.51$ ,  $-1.21$ ,  $-1.67$  and  $-1.45$ , respectively).

Melanin production by auto-oxidation of HGA has been shown to occur in 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

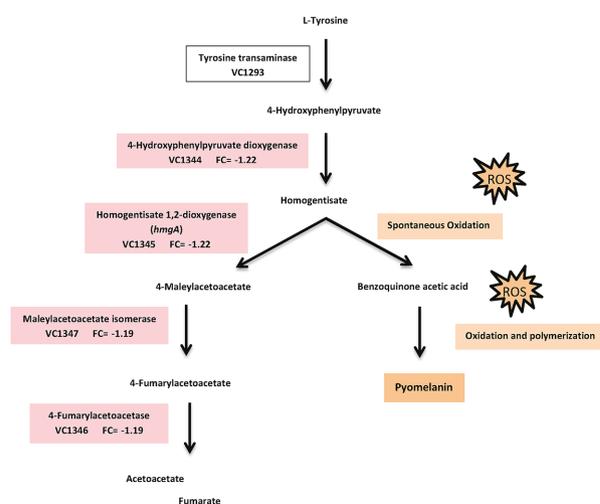


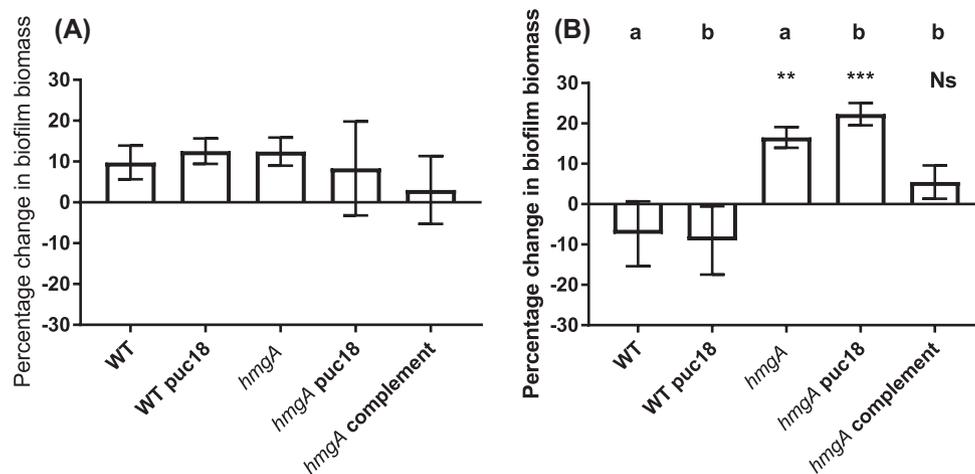
Figure 1. Differentially expressed transcripts in grazed compared to ungrazed biofilms involved in tyrosine degradation in *V. cholerae*. FC represents  $\log_2$  fold changes. The pathway in *V. cholerae* is proposed by Valeru et al. (2009).

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 and 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 sub-unit of the toxin coregulated pilus and cholera toxin, 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).

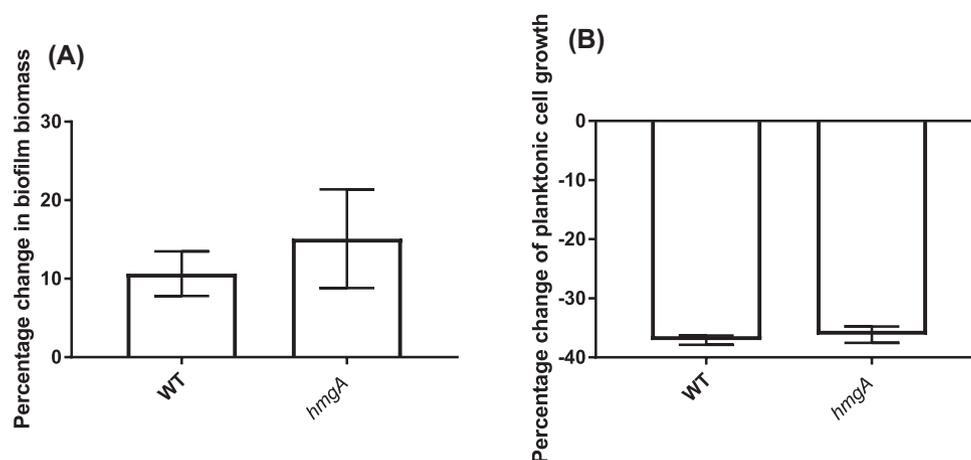
Pigment production has also been demonstrated to provide a range of functions in many different microorganisms. For example, melanin can protect the pathogenic fungus, *Cryptococcus neoformans*, from antibody-mediated phagocytosis by macrophages (Wang, Aisen and Casadevall 1995), as well as from digestion of phagocytosed cells by the amoeba *A. castellanii* (Steenbergen, Shuman and Casadevall 2001). Melanised *C. neoformans* are significantly less susceptible to hydrolytic enzymes commonly used by environmental predators than non-melanised cells (Rosas and 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, and either *T. pyriformis* or *A. castellanii* were added. After 3 days of grazing, CFU and CV measurements determined planktonic cell and biofilm biomass, respectively.



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



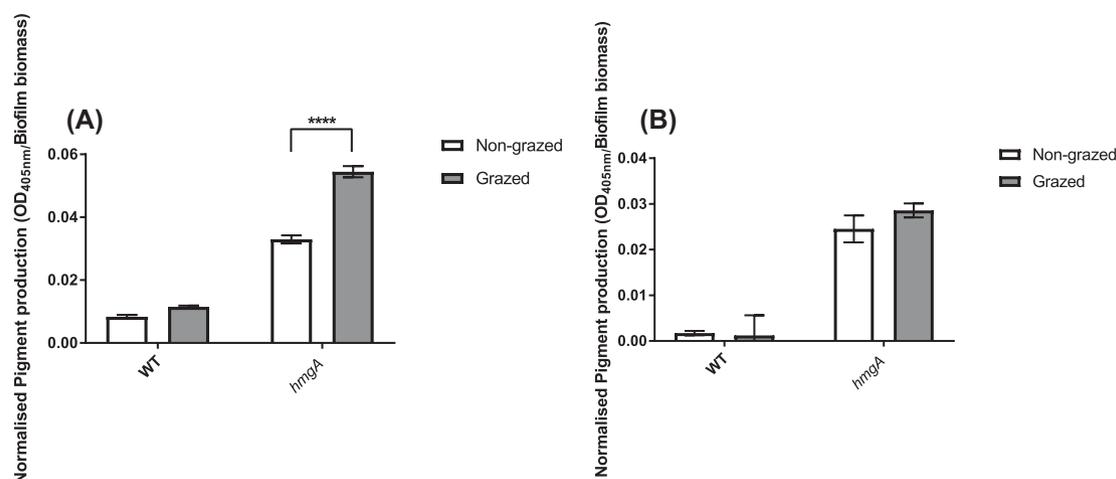
**Figure 3.** Early biofilms of *V. cholerae* A1552 exposed to *T. pyriformis* for 72 h. Biofilm biomass was determined by CV staining (A) and the planktonic cells in the supernatant enumerated by the drop plate method (B). Data were natural log transformed and the percentage change in biofilm biomass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

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 (Fig. 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 (Fig. 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 Fig. 1, Supporting Information). Furthermore, the cell-free supernatant of the *hmgA* mutant does not show toxicity towards *A. castellanii* trophozoites compared to the WT (Supplementary Fig. 2, Supporting Information).

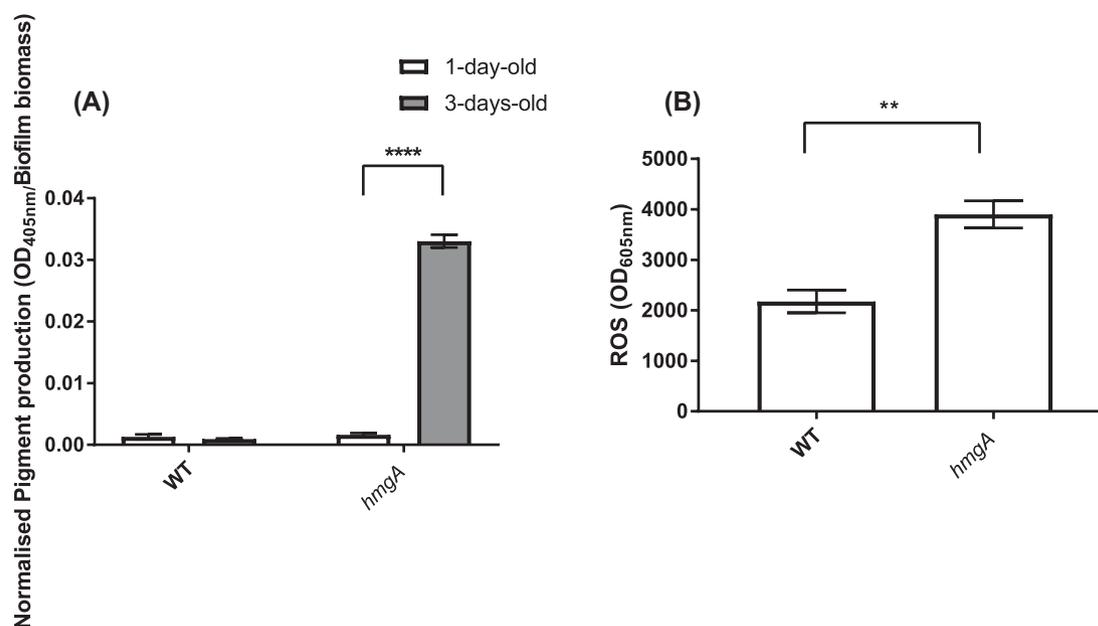
The resistance of planktonic cells of the *hmgA* mutant to predation by *T. pyriformis* was also investigated, as *A. castellanii* cannot feed efficiently on planktonic cells (Huws, McBain and

Gilbert 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 (Fig. 3A) and numbers of planktonic cells (Fig. 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 the *hmgA* mutant was observed after 3 days of grazing by *A. castellanii* but not when exposed to grazing by *T. pyriformis* (Fig. 4), which is consistent with the increased grazing resistance of the mature biofilm against *A. castellanii*.

Addition of more nutrients to WT and *hmgA* mutant strains did not result in the same increase in pyomelanin levels as active grazing by *A. castellanii*. This indicates that the extra nutrients in the *A. castellanii* culture are not responsible for induction of pyomelanin production in the *hmgA* mutant when exposed



**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<sub>405 nm</sub>) 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<sub>490 nm</sub>). Experiments were run in triplicates and repeated three times on different days. Error bars represent the standard deviation of three replicates. Bars indicate different statistical groups derived from two-way 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<sub>405 nm</sub>) 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<sub>490 nm</sub>). Error bars represent the standard deviation of three replicates. Statistical analysis was performed using two-way ANOVA and Sidak's multiple comparisons test (A) and Student's t-test (B). Statistical significance is indicated by \*\*,  $P < 0.001$ , \*\*\*\*,  $P < 0.0001$ .

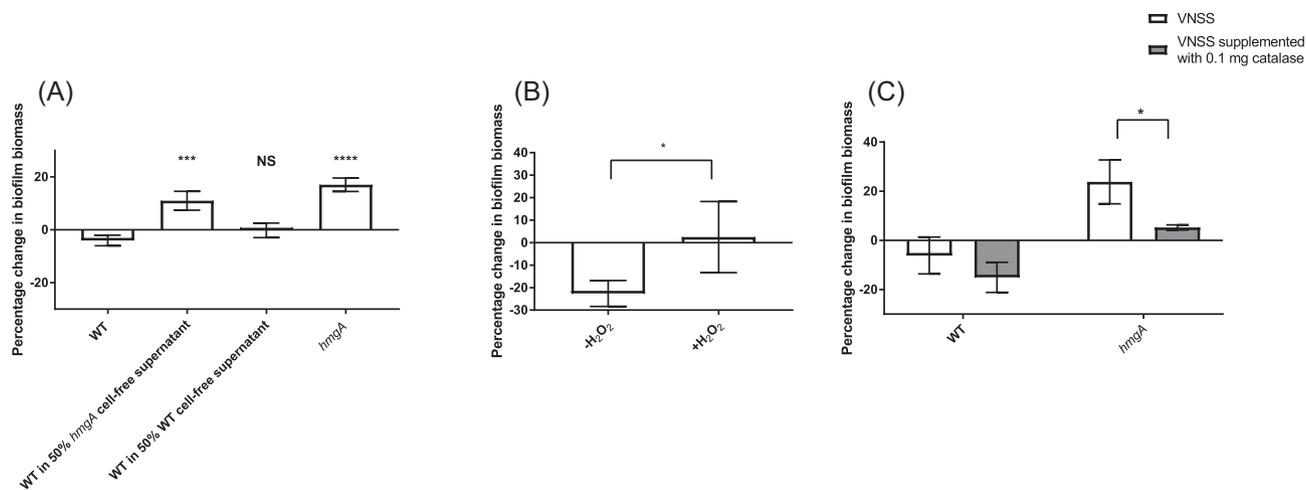
to grazing by *A. castellanii*. Furthermore, the addition of cell-free supernatants from *A. castellanii* with or without HKB and heat-killed *A. castellanii* cells did not induce overproduction of pyomelanin, supporting our hypothesis that active phagocytosis by *A. castellanii* is required (Supplementary Fig. 3, Supporting Information).

### Pyomelanin and production of ROS

The increases in grazing resistance of the *hmgA* mutant biofilms (Fig. 2B) correlated with an increase in pigment production. On

day 1, the pyomelanin concentration in both the supernatant of wild type and *hmgA* mutant strains was low (normalised pigment production [OD<sub>405nm</sub>/Biofilm biomass] = 0.0013 and 0.0009, respectively) (Fig. 5A). However, after 3 days the pyomelanin concentration in supernatants of the *hmgA* mutant was 20-fold higher than those of the wild type (normalised pigment production [OD<sub>405nm</sub>/Biofilm biomass] = 0.0016 and 0.033, respectively).

To determine if the *hmgA* cell-free supernatant would provide protection against predation by *A. castellanii* to the WT strain, the cell-free supernatant of 3-days-old established *hmgA* mutant biofilms were added to the *A. castellanii* grazing assays.



**Figure 6.** Effect of cell-free supernatants (A), H<sub>2</sub>O<sub>2</sub> (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 three times on different days. Error bars represent the standard deviation of three replicates. Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparisons test comparing all to the WT (A), Student's t-test (B) and two-way ANOVA and Sidak's multiple comparisons test (C). Statistical significance is indicated by \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$  and \*\*\*\*,  $P < 0.0001$  and Ns, not significant.

The addition of undiluted cell-free supernatants of *V. cholerae* to pre-established 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* (Fig. 6A).

In order to further investigate the relationship between grazing and pigment production, the amount of O<sub>2</sub><sup>-</sup> 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 (Fig. 5B). Biofilms of the *hmgA* mutant strain generated 79% more O<sub>2</sub><sup>-</sup> 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<sub>2</sub>O<sub>2</sub> induced oxidative stress responses and virulence factor expression (Valeru et al. 2009). The auto-oxidation of HGA can generate superoxide radicals and H<sub>2</sub>O<sub>2</sub> in eukaryotic cells at physiological pH (Martin and Batkoff 1987). Here, the effect of addition of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> for 30 min (Fig. 6B). *A. castellanii* was added after H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> compared to the controls with HKB. After 3 days of grazing by *A. castellanii*, the biomass of the untreated *V. cholerae* biofilms were significantly reduced, while biofilms that had been treated with H<sub>2</sub>O<sub>2</sub> for 30 min were not reduced ( $P = 0.0246$ ) (Fig. 6B). In addition, pre-grown 3-days-old biofilms were treated with 0.1 mg ml<sup>-1</sup> catalase to reduce ROS of the

*hmgA* mutant biofilm. After 3 days of grazing by *A. castellanii*, the biomass of the treated *V. cholerae hmgA* biofilm was significantly reduced compared to the untreated biofilm ( $P = 0.0176$ ) (Fig. 6C). Taken together, our results suggest that the production of pyomelanin results in production of ROS, which in turn, results in an increase in grazing resistance.

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

Pigmented hypertoxigenic strains of *V. cholerae* have been previously reported both in random mutagenesis experiments (Mekalanos, Sublett and Romig 1979; Parker et al. 1979; Ivins and Holmes 1980) as well as in environmental isolates. For example, *V. cholerae*, ATCC 14035, Serotype Ogawa serovar O1 strain isolated originally from a stool sample produced a reddish brown pigment when grown in low-nutrient condition media supplemented with L-glutamic acid and L-tyrosine (Ruzafa, Sanchez-Amat and Solano 1995). In addition, six non-toxicogenic serogroup O139 (water isolates) and one toxigenic O1 (clinical isolate) strains isolated from different years and from different provinces of China were pigmented. All the O139 strains had the same 15-bp deletion in *hmgA*, and a 10-bp deletion was found in the VC1345 gene of the O1 strain, indicating that the mutation of this gene may provide a fitness advantage in the environment (Wang 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 downregulated 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<sub>2</sub>O<sub>2</sub> were also more grazing resistant.

This project provides insight into the genes involved in defence against protozoan grazing of *V. cholerae*. Data presented here show 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 colonisation 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.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

## ACKNOWLEDGEMENTS

The authors would like to acknowledge two anonymous reviewers for helpful comments.

## FUNDING

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.

**Conflict of interest.** None declared.

## REFERENCES

- Abd H, Saeed A, Weintraub A et al. *Vibrio cholerae* O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii*. *FEMS Microbiol Ecol* 2007;**60**:33–9.
- Abd H, Weintraub A, Sandström G. Intracellular survival and replication of *Vibrio cholerae* O139 in aquatic free-living amoebae. *Environ Microbiol* 2005;**7**:1003–8.
- Colwell RR, Huq A. Environmental reservoir of *Vibrio cholerae* the causative agent of cholera. *Ann NY Acad Sci* 1994;**740**:44–54.
- Colwell RR, Kaper J, Joseph SW. *Vibrio cholerae*, *Vibrio parahaemolyticus*, and other vibrios: occurrence and distribution in Chesapeake Bay. *Science* 1977;**198**:394–6.
- Cox MP, Peterson DA, Biggs PJ. SolexaQA: at-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics* 2010;**11**:485.
- da Silva MB, Marques AF, Nosanchuk JD et al. Melanin in the dimorphic fungal pathogen *Paracoccidioides brasiliensis*: effects on phagocytosis, intracellular resistance and drug susceptibility. *Microbes Infect* 2006;**8**:197–205.
- Eisenman HC, Nosanchuk JD, Webber JBW et al. Microstructure of cell wall-associated melanin in the human pathogenic fungus *Cryptococcus neoformans*. *Biochemistry* 2005;**44**:3683–93.
- Elliott AM. The distribution of *Tetrahymena pyriformis*. *J Eukaryot Microbiol* 1970;**17**:162–8.
- Erken M, Weitere M, Kjelleberg S et al. In situ grazing resistance of *Vibrio cholerae* in the marine environment. *FEMS Microbiol Ecol* 2011;**76**:504–12.
- Fernández-Cañón JM, Peñalva MA. Molecular characterization of a gene encoding a Homogentisate Dioxygenase from *Aspergillus nidulans* and identification of its human and plant homologues. *J Biol Chem* 1995;**270**:21199–205.
- Huq A, Colwell RR, Rahman R et al. Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. *Appl Environ Microb* 1990;**56**:2370–3.
- Huws SA, McBain AJ, Gilbert P. Protozoan grazing and its impact upon population dynamics in biofilm communities. *J Appl Microbiol* 2005;**98**:238–44.
- Ivins BE, Holmes RK. Isolation and characterization of melanin-producing (*mel*) mutants of *Vibrio cholerae*. *Infect Immun* 1980;**27**:721–9.
- Ivins BE, Holmes RK. Factors affecting phaeomelanin production by a melanin-producing (*mel*) mutant of *Vibrio cholerae*. *Infect Immun* 1981;**34**:895–9.
- Jacobson ES. Pathogenic roles for fungal melanins. *Clin Microbiol Rev* 2000;**13**:708–17.
- Jousset A, Rochat L, Scheu S et al. Predator-prey chemical warfare determines the expression of biocontrol genes by rhizosphere-associated *Pseudomonas fluorescens*. *Appl Environ Microb* 2010;**76**:5263–8.
- Jürgens K, Matz C. Predation as a shaping force for the phenotypic and genotypic composition of planktonic bacteria. *Anton Leeuw* 2002;**81**:413–34.
- Khan NA. Acanthamoeba: biology and increasing importance in human health. *FEMS Microbiol Rev* 2006;**30**:564–95.
- Kopylova E, Noé L, Touzet H. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 2012;**28**:3211–7.
- Kotob SI, Coon SL, Quintero EJ et al. Homogentisic acid is the primary precursor of melanin synthesis in *Vibrio cholerae*, a *Hyphomonas* strain, and *Shewanella colwelliana*. *Appl Environ Microb* 1995;**61**:1620–2.
- Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Meth* 2012;**9**:357–9.
- Lei Y-L, Stumm K, Wickham SA et al. Distributions and biomass of benthic ciliates, foraminifera and amoeboid eukaryotes in marine, brackish, and freshwater sediments. *J Eukaryot Microbiol* 2014;**61**:493–508.
- Lutz C, Erken M, Noorian P et al. Environmental reservoirs and mechanisms of persistence of *Vibrio cholerae*. *Front Microbiol* 2013;**4**:375.
- Mårdén P, Tunlid A, Malmcróna-Friberg K et al. Physiological and morphological changes during short term starvation of marine bacterial isolates. *Arch Microbiol* 1985;**142**:326–32.
- Martin JP, Batkoff B. Homogentisic acid autoxidation and oxygen radical generation: implications for the etiology of alkaptonuric arthritis. *Free Radic Biol Med* 1987;**3**:241–50.
- Martínez Pérez ME, Macek M, Castro Galván MT. Do protozoa control the elimination of *Vibrio cholerae* in brackish water? *Int Rev Hydrobiol* 2004;**89**:215–27.
- Matz C, Kjelleberg S. Off the hook—how bacteria survive protozoan grazing. *Trends Microbiol* 2005;**13**:302–7.
- Matz C, Deines P, Boenigk J et al. Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates. *Appl Environ Microb* 2004;**70**:1593–9.
- Matz C, McDougald D, Moreno AM et al. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *P Natl Acad Sci USA* 2005;**102**:16819–24.
- Mazzola M, de Bruijn I, Cohen MF et al. Protozoan-induced regulation of cyclic lipopeptide biosynthesis is an effective predation defense mechanism for *Pseudomonas fluorescens*. *Appl Environ Microb* 2009;**75**:6804–11.
- Mekalanos J, Sublett R, Romig W. Genetic mapping of toxin regulatory mutations in *Vibrio cholerae*. *J Bacteriol* 1979;**139**:859–65.

- Miyata ST, Kitaoka M, Brooks TM et al. *Vibrio cholerae* requires the Type VI Secretion system virulence factor VasX to kill *Dictyostelium discoideum*. *Infect Immun* 2011;79:2941–9.
- Monger BC, Landry MR, Brown SL. Feeding selection of heterotrophic marine nanoflagellates based on the surface hydrophobicity of their picoplankton prey. *Limnol Oceanogr* 1999;44:1917–27.
- Nair GB, Sarkar B, De S et al. Ecology of *Vibrio cholerae* in the freshwater environs of Calcutta, India. *Microb Ecol* 1988;15:203–15.
- Nosanchuk JD, Casadevall A. Cellular charge of *Cryptococcus neoformans*: contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. *Infect Immun* 1997;65:1836–41.
- O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol* 1998;28:449–61.
- Owusu-Ansah E, Yavari A, Banerjee U. A protocol for in vivo detection of reactive oxygen species. *Protoc Exch* 2008, DOI: 10.1038/nprot.2008.23.
- Parker C, Gauthier D, Tate A et al. Expanded linkage map of *Vibrio cholerae*. *Genetics* 1979;91:191–214.
- Parry JD. Protozoan grazing of freshwater biofilms. *Adv Appl Microbiol* 2004;54:167–96.
- Pukatzki S, Ma AT, Sturtevant D et al. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *P Natl Acad Sci USA* 2006;103:1528–33.
- Rosas Á, Casadevall A. Melanization decreases the susceptibility of *Cryptococcus neoformans* to enzymatic degradation. *Mycopathologia* 2001;151:53–6.
- Ruzafa C, Sanchez-Amat A, Solano F. Characterization of the melanogenic system in *Vibrio cholerae*, ATCC 14035. *Pigment Cell Melanoma Res* 1995;8:147–52.
- Sambrook J, Fritsch E, Maniatis T. *Molecular Cloning: A Laboratory Manual*. NY: Cold Spring Harbor Laboratory Cold Spring Harbor, 1989.
- Schnitzler N, Peltroche-Llacsahuanga H, Bestier N et al. Effect of melanin and carotenoids of *Exophiala (Wangiella) dermatitidis* on phagocytosis, oxidative burst, and killing by human neutrophils. *Infect Immun* 1999;67:94–101.
- Shanan S, Abd H, Hedenström I et al. Detection of *Vibrio cholerae* and *Acanthamoeba* species from same natural water samples collected from different cholera endemic areas in Sudan. *BMC Res Notes* 2011;4:109.
- Steenbergen J, Shuman H, Casadevall A. *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proc Natl Acad Sci USA* 2001;98:15245–50.
- Sun S, Kjelleberg S, McDougald D. Relative contributions of *Vibrio* polysaccharide and quorum sensing to the resistance of *Vibrio cholerae* to predation by heterotrophic protists. *PLoS One* 2013;8:e56338.
- Sun S, Tay QX, Kjelleberg S et al. Quorum sensing-regulated chitin metabolism provides grazing resistance to *Vibrio cholerae* biofilms. *ISME J* 2015;9:1812–20.
- Tatusov RL, Koonin EV, Lipman DJ. A genomic perspective on protein families. *Science* 1997;278:631–7.
- Thom S, Warhurst D, Drasar BS. Association of *Vibrio cholerae* with fresh water amoebae. *J Med Microbiol* 1992;36:303–6.
- Turick CE, Knox AS, Becnel JM et al. Properties and Function of Pyomelanin. In: Elnashar M (ed). *Biopolymers*. In Tech, 2010.
- Vaitkevicius K, Lindmark B, Ou G et al. A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. *P Natl Acad Sci USA* 2006;103:9280–5.
- Valeru SP, Rompikuntal PK, Ishikawa T et al. Role of melanin pigment in expression of *Vibrio cholerae* virulence factors. *Infect Immun* 2009;77:935–42.
- Van der Henst C, Scrignari T, Maclachlan C et al. An intracellular replication niche for *Vibrio cholerae* in the amoeba *Acanthamoeba castellanii*. *ISME J* 2016;10:897–910.
- Wang Y, Aisen P, Casadevall A. *Cryptococcus neoformans* melanin and virulence: mechanism of action. *Infect Immun* 1995;63:3131–6.
- Wang H, Jiang L, Weitz JS. Bacterivorous grazers facilitate organic matter decomposition: a stoichiometric modeling approach. *FEMS Microbiol Ecol* 2009;69:170–9.
- Wang R, Wang H, Zhou H et al. Characters of homogenisate oxygenase gene mutation and high clonality of the natural pigment-producing *Vibrio cholerae* strains. *BMC Microbiol* 2011;11:109.
- Wang Z, Lin B, Mostaghim A et al. *Vibrio campbellii* hmgA-mediated pyomelanization impairs quorum sensing, virulence, and cellular fitness. *Front Microbiol* 2013;4:379.
- Wildschutte H, Wolfe DM, Tamewitz A et al. Protozoan predation, diversifying selection, and the evolution of antigenic diversity in *Salmonella*. *P Natl Acad Sci USA* 2004;101:10644–9.
- Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. *Gene* 1985;33:103–119.