RESEARCH ARTICLE

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

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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.

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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.

Keywords: protozoan predation; biofilms; environmental fitness; Vibrio 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...
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 chlora 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; Abdul, 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, Rhyhcomonas nasuta and the amoeba A. castellanii (Lutz et al. 2013; Sun, Kjelleberg and McDougal 2013). The extracellular protease, PrtV, provides grazing resistance against the flagellate Cafeteria roenbergensis and the ciliate, T. pyriformis (Vaiteckivicius 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, violcein, which acts as a chemical defence for several bacterial genera (Chromobacterium, Janthinobacterium, Pseudalteromonas) (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 (Jouset 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-oxides to form pyomelanin (Turiick 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 μg ml⁻¹). A. castellanii was routinely passaged in 15 ml growth medium containing peptone-yeast-glucose (PYG) (20 g l⁻¹ proteose peptone, 1 g l⁻¹ yeast extract) supplemented with 1 litre 0.1 × M minimal medium (6 g l⁻¹ Na₂HPO₄, 3 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ NaCl, 1 g l⁻¹ NH₄Cl) and 0.1 M sterile-filtered glucose in 25 cm² tissue culture flasks with ventilated caps (Sarstedt Inc., Nürnberg, 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 was passed in 20 ml of 0.5× NSS medium (8.8 g l⁻¹ NaCl, 0.735 g l⁻¹ Na₂SO₄, 0.04 g l⁻¹ NaHCO₃, 0.125 g l⁻¹ KCl, 0.02 g l⁻¹ KBr, 0.935 g l⁻¹ MgCl₂·6H₂O, 0.205 g l⁻¹ CaCl₂·2H₂O, 0.004 g l⁻¹ SrCl₂·6H₂O and 0.004 g l⁻¹ H₃BO₃) (Mardén et al. 1985) supplemented with 1% (v/v) of heat-killed Pseudomonas aeruginosa PAO1 (heat-killed bacteria [HKB]) in a 25 cm² 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 °C with shaking at 200 rpm and adjusted to OD₆₀₀ = 1.0 (10⁶ cells ml⁻¹) in 0.5× NSS. The tubes were then transferred to a water bath at 65 °C for 2 h, 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, three biological replicates of V. cholerae biofilms were cultivated on the interior surfaces of Silastic® laboratory tubing (Dow Corning, MI, 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 FeSO₄·7H₂O
Wild type ATCC 205063

A. castellanii

Protozoan strains

V. cholerae

pUC18 Cloning vector, pMB1 ori, LAC pr, lacZ

Plasmids

genes.

considered to be significantly differentially expressed (SDE)


cance. Transcripts with an FDR-adjusted

was calculated to give the statistical validity level of signifi-

lated the log fold change in fragments per kilobase of exon per

A1552 pUC18 O1 El Tor, Inaba, smooth, Rif r, A pr 

This study

This study

Protozoan strains

A. castellanii

Wild type

ATCC 30234

A. castellanii

T. pyriformis

Wild type

ATCC 205063

Table 1. Strains and plasmids used in this study.

| Strain                          | Properties                              | Reference/source |
|---------------------------------|-----------------------------------------|------------------|
| Bacterial strains               |                                         |                  |
| V. cholerae A1552               | Wild type, O1 El Tor, Inaba, smooth, Rif' | Valeru et al. (2009) |
| V. cholerae A1552 hmgA          | O1 El Tor, Inaba, smooth, ΔhmgA, Rif', Km' | Valeru et al. (2009) |
| V. cholerae A1552 hmgA complement| O1 El Tor, Inaba, smooth, ΔhmgA::pUC18, Rif', Ap', Km' | Valeru et al. (2009) |
| V. cholerae A1552 pUC18         | O1 El Tor, Inaba, smooth, ΔhmgA, pUC18, Rif', Ap' | This study |
| V. cholerae A1552 hmgA pUC18    | O1 El Tor, Inaba, smooth, ΔhmgA, pUC18, Rif', Ap', Km' | This study |
| Plasmids                        |                                         |                  |
| pUC18                           | Cloning vector, pMB1 ori, LAC pr, lacZ, Ap' | Yanisch-Perron et al. (1985) |
| Protozoan strains               |                                         |                  |
| A. castellanii                  | Wild type                               |                  |
| T. pyriformis                   | Wild type                               |                  |

and 0.01 g Na2HPO4) in 1 l 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 re-suspended in 0.5× VNSS,

injected into the tubing and incubated without flow for 2 h. A

prost-fre control biofilm was treated the same to exclude oxy-

gen 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 plank-

tonic bacteria, and immediately re-suspended in 2 volumes of

RNAlater (Qiagen, Hilden, Germany) and harvested from the in-

terior surface of the tubing by mechanical manipulation (man-

ually 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

(F = 0.05) as implemented by SolexaQA version 3.1.3 (Cox, Pe-

terson 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. castel-

lanii 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 calcu-
lated 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 signifi-
cance. 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 con-
served domain search (Tatusov, Koonin and Lipman 1997). With

the assistance of the Database for Annotation, Visualization and

Integrated Discovery Bioinformatics Resources 6.7 (National In-

stitute of Allergy and Infectious Diseases), the differentially ex-

pressed 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 Inter-

Pro/UniProt (to analyse the protein domains).

Early and late biofilm grazing assay with A. castellanii

Overnight cultures of V. cholerae were adjusted so that 105 cells

ml−1 in 0.5× VNSS were added to 24-well microtitre plates (Fal-

кон, 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 × 104 cells ml−1) 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 spectrophotome-

try at OD650 nm (Wallac Victor2 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 re-

moved 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 OD490 nm was deter-

mined by spectrophotometry.

To determine if the cell-free supernatants from the hmgA

mutant would provide protection against grazing by A. castellani-
in 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 × 104 cells ml−1) and in-
cubated 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 spec-

trophotometry (OD490 nm) 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 (OD490 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 O2 was used (Owusu-Ansah, Yavari and Banerjee 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 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.

H2O2 treatment of V. cholerae biofilms

Overnight cultures were inoculated at a final concentration of 10⁶ 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 H2O2 for 30 min, after which the H2O2 was removed and fresh 0.5× VNSS medium with or without A. castellanii (2 × 10⁶ 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 10⁷ 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 × 10⁶ 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.

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, RNA later (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 (qRTPCR) 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 Supplemental Table 1, Supporting Information. The expression was determined 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 (10⁴ cells 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 OD600 nm. Planktonic fractions were collected for enumeration of CFU ml⁻¹ and biofilm biomass determined by CV staining and spectrophotometry (OD490 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 × 10⁷ cells 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 (10⁵ cells 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 three times with 0.5× NSS and treatment with gentamicin (300 μg 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 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 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 antiprototrophic 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 (Vaikevicius et al. 2006) and the T6SS (Pukatzki et al. 2006). Although a QS mutant of V. cholerae was more sensitive to
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 log2 fold change in FPKM varies from −1.964 to −0.724 for the downregulated 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 (amino transferases). 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. The protective mechanism of melanin is 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 subunit 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 neoforms, from antibody-mediated phagocytosis by macrophages (Wang, Aisen and Casadevall 1995), as well as from digestion of phagocytosed cells by the amoeba A. castellanii (Steenberg, Shuman and Casadevall 2001). Melanised C. neoforms are significantly less susceptible to hydrolytic enzymes commonly used by environmental predators than non-melanised cells (Rossas 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.
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 production 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...
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 [OD405nm/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 [OD405nm/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.
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 mutant 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$_2$- 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 (Fig. 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$_2$O$_2$ induced oxidative stress responses and virulence factor expression (Valeru *et al.* 2009). The auto-oxidation of HGA can generate superoxide radicals and H$_2$O$_2$ in eukaryotic cells at physiological pH (Martin and Batkoff 1987). Here, the effect of addition of H$_2$O$_2$ as a substitute for pyomelanin-associated ROS on resistance of *V. cholerae* biofilms to amoeba 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$_2$O$_2$ for 30 min (Fig. 6B). A. castellanii was added after H$_2$O$_2$ 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$_2$O$_2$ compared to the controls with HKB. After 3 days of grazing by *A. castellanii*, the biomass of the untreated *V. cholerae* biofilms was significantly reduced, while biofilms that had been treated with H$_2$O$_2$ 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$^{-1}$ 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 hypertoxinogenic 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 nontoxicigen 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$_2$O$_2$ were also more grazing resistant.

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Figure 6. Effect of cell-free supernatants (A), H$_2$O$_2$ (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 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.
This project provides insight into the genes involved in defense 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 colonization ability (Yaleru 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.

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