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Indole inhibition of *N*-acylated homoserine lactone-mediated quorum signalling is widespread in Gram-negative bacteria

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The LuxI/R quorum-sensing system and its associated *N*-acylated homoserine lactone (AHL) signal is widespread among Gram-negative bacteria. Although inhibition by indole of AHL quorum signalling in *Pseudomonas aeruginosa* and *Acinetobacter oleivorans* has been reported previously, it has not been documented among other species. Here, we show that co-culture with wild-type *Escherichia coli*, but not with *E. coli tnaA* mutants that lack tryptophanase and as a result do not produce indole, inhibits AHL-regulated pigmentation in *Chromobacterium violaceum* (violacein), *Pseudomonas chlororaphis* (phenazine) and *Serratia marcescens* (prodigiosin). Loss of pigmentation also occurred during pure culture growth of *Chro. violaceum, P. chlororaphis* and *S. marcescens* in the presence of physiologically relevant indole concentrations (0.5–1.0 mM). Inhibition of violacein production by indole was counteracted by the addition of the *Chro. violaceum* cognate autoinducer, *N*-decanoyl homoserine lactone (C10-HSL), in a dose-dependent manner. The addition of exogenous indole or co-culture with *E. coli* also affected *Chro. violaceum* transcription of *vioA* (violacein pigment production) and *chiA* (chitinase production), but had no effect on *pykF* (pyruvate kinase), which is not quorum regulated. *Chro. violaceum* AHL-regulated elastase and chitinase activity were inhibited by indole, as was motility. Growth of *Chro. violaceum* was not affected by indole or C10-HSL supplementation. Using a nematode-feeding virulence assay, we observed that survival of *Caenorhabditis elegans* exposed to *Chro. violaceum, P. chlororaphis* and *S. marcescens* was enhanced during indole supplementation. Overall, these studies suggest that indole represents a general inhibitor of AHL-based quorum signalling in Gram-negative bacteria.

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INTRODUCTION

Quorum signalling is now recognized as a mechanism of global gene regulation in most bacteria (Ng & Bassler, 2009; Whiteley *et al.*, 1999). Quorum signalling was first associated with light production in *Vibrio fischeri* (Nealson *et al.*, 1970). The *V. fischeri* system can still serve as a model for this type of regulation, in which the *N*-acylated homoserine lactone (AHL) signal is synthesized by an AHL synthase, LuxI, and when the cells reach a threshold high density the AHL signal re-enters the cell, binding to and activating the transcriptional regulator LuxR, causing transcription of a number of target genes, sometimes referred to as the quorum regulon (Visick & Fuqua, 2005). Many Gram-negative bacteria encode LuxI/R homologues, and prominent AHL-regulated genes include those associated with virulence, biofilm-formation and microbial

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Abbreviations: AHL, *N*-acylated homoserine lactone; C10-HSL, *N*-decanoyl homoserine lactone; RT-qPCR, reverse transcription-quantitative PCR.

Two supplementary figures are available with the online version of this paper.

competition (Visick & Fuqua, 2005). As a result, many eukaryotic and prokaryotic organisms use the inhibition of quorum signalling as a mechanism for competition with bacteria (Rasmussen & Givskov, 2006). The first description of quorum signalling inhibition as a competition mechanism to prevent biofilm formation was described in the macroalga *Delisea pulchra* (Givskov *et al.*, 1996). Subsequent studies have shown quorum-signalling inhibition to be present in other plants (Gao *et al.*, 2003) and marine invertebrates (Manefield *et al.*, 2000), as well as other micro-organisms (Golberg *et al.*, 2013). Mechanisms involved in quorum-signalling inhibition [reviewed by Vega *et al.* (2014)] include the production of quorum-signalling-inhibiting compounds such as furanones (de Nys *et al.*, 2006) produced by *D. pulchra* and enzymic degradation (quorum quenching) of the signal (Wang & Leadbetter, 2005). In a recent study, we observed that *Escherichia coli* enhanced its survival by producing indole during growth as a mixed culture with *Pseudomonas aeruginosa* (Chu *et al.*, 2012).

Bacterial indole production from tryptophan has been known for some time (Hopkins & Cole, 1903). Tryptophanase, encoded by *tnaA*, degrades tryptophan yielding indole, pyruvate and ammonia (Deeley & Yanofsky, 1981). As the tryptophanase reaction is reversible, it was first considered to represent an alternative mechanism for tryptophan synthesis (Deeley & Yanofsky, 1981). Many enteric bacteria produce indole (Blazevic & Ederer, 1975) and there are a number of indole derivatives produced from this compound including isatin, hydroxyindole and indole-3-acetic acid [reviewed by Lee & Lee (2010)]. Indole is also a signalling molecule (Lee *et al.*, 2007; Lee & Lee, 2010; Wang *et al.*, 2001). Several studies have shown that indole inhibits quorum signalling in *P. aeruginosa* (Chu *et al.*, 2012; Lee *et al.*, 2009; Tashiro *et al.*, 2010). More recently, indole has been shown to inhibit quorum signalling in *Acinetobacter oleivorans* by accelerating turnover of the LuxR homologue AqsR (Kim & Park, 2013). Here, we show that mixed-culture growth with indole-producing *E. coli* or supplementation with physiologically relevant indole concentrations can inhibit AHL-mediated quorum signalling in three Gram-negative bacteria, *Chromobacterium violaceum, Pseudomonas chlororaphis* and *Serratia marcescens*. These data suggest that indole is a general AHL inhibitor.

METHODS

Cultures and growth conditions. The organisms used are listed in Table 1. *Chro. violaceum* and *E. coli* cultures were grown aerobically in Luria–Bertani (LB) medium supplemented with kanamycin (50 μg ml^{-1}) for the *E. coli tnaA* mutant strain. *S. marcescens* and *P. chlororaphis* were grown in tryptic soy broth (TSB). Individual *E. coli* strains were grown at 37 °C, whereas all other strains were cultured at 30 °C. For long-term storage, bacterial cultures were grown in broth overnight and then frozen at −80 °C using 12.5 % (v/v) glycerol as a cryoprotectant. *Caenorhabditis elegans* nematodes were maintained on nematode growth medium (NGM) agar (1 l contains: 2.5 g peptone, 3 g NaCl, 17 g agar, 1 ml 1 M cholesterol, 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 1 ml 1 M potassium phosphate buffer pH 6) at 25 °C seeded with *E. coli* strain OP50 (Vidal-Gadea *et al.*, 2011). In all experiments, a minimum of three biological replicates were performed.

Reverse transcription-quantitative PCR (RT-qPCR). The genes and associated primers used for RT-qPCR analysis are listed in Table 2. Cultures were grown in the presence and absence of 1.0 mM indole for 24 h. RNA isolation was performed according to manufacturer's instructions using the RNeasy Mini kit from Qiagen. Reverse transcription was performed according to the manufacturer's instructions using the ImProm II reverse transcriptase system from Promega. RT-qPCR was performed using the SYBR Green method (Samant *et al.*, 2012).

Competition experiments. The methods used for mixed-culture experiments were described previously (Chu *et al.*, 2012). Briefly, *E. coli* and *Chro. violaceum* strains were retrieved from frozen stocks, checked for purity following 24–48 h growth on agar and then subcultured in broth overnight. Equivalent concentrations of each culture, 100 μl inoculum containing 10^6 c.f.u. ml^{-1}, were added to a 125 ml flask containing 50 ml LB and 10 silicone rubber disks (7 mm diameter × 1 mm thick; Dapro Rubber) as biofilm colonization substrata (Weber *et al.*, 2010). The growth flask set-up enabled both biofilm and planktonic populations to be tested from the same culture flask. *E. coli* and *Chro. violaceum* cultures were incubated at 37 °C with shaking. Pure and mixed cultures containing *P. chlororaphis* or *S. marcescens* were incubated at 25 °C with shaking. Planktonic bacterial concentrations were measured by dilution plating. Biofilm colonization on the silicone rubber disks was measured using the sonication and dilution plating protocol described previously (Chu *et al.*, 2012). The non-pigmented *E. coli* and pigmented *Chro. violaceum, P. chlororaphis* and *S. marcescens* colonies could be easily distinguished. While pigmentation was inhibited in mixed culture (described below), this did not appear to be a source of error during plating as dilution plating measurements immediately after co-inoculation matched the bacterial concentrations added (Fig. 3a, c, e).

Indole supplementation and analysis. A 100 mM sterile stock solution of indole was prepared by dissolving indole in dimethyl formamide and filter-sterilized using a 0.2 μm filter (Chu *et al.*, 2012).

Table 1. Bacterial strains used in this study

Strain	Source*	Reference
Chro. violaceum ATCC 12472	ATCC	McLean *et al.* (2004)
E. coli BW25113	1	Baba *et al.* (2006)
E. coli BW25113 *tnaA*	1	Baba *et al.* (2006)
E. coli OP50	2	Tan *et al.* (1999)
P. chlororaphis 30-84	3	Maddula *et al.* (2008)
S. marcescens ATCC 13880	ATCC	Horng *et al.* (2002)
Caen. elegans	2	Tan *et al.* (1999)

*The sources are as follows: 1, NBRP *E. coli*, Microbial Genetics Laboratory, National Institute of Genetics, Japan; 2, J. T. Pierce-Shimomura, Dept Neurosciences, University of Texas, Austin TX, USA; 3, L. S. Pierson III, Dept Plant Pathology and Microbiology, Texas A&M University, College Station, TX, USA; ATCC, American Type Culture Collection, Manassas, VA, USA.

Table 2. Primers of *Chro. violaceum* genes used for RT-qPCR

Gene	Function	Primer
vioA	Violacein production	R 5′-GGT TGT CGA CGA TCA GCA C-3′ F 5′-GCT GTT CAA GGC TTT CCT CA-3′
chiA	Chitinase	R 5′-TTA CAA CAC CGA GTG CAA CG-3′ F 5′-CGG CCG TAG TAT TTC TTC CA-3′
pykF	Housekeeping – pyruvate kinase	R 5′-GCA TTG CCT TCC GTT TCA T-3′ F 5′-CTG CGC ATC GGC AAG TTT-3′

F, Forward; R, reverse.

Indole was added to autoclaved media at biologically relevant concentrations (0–1.0 mM). Indole was measured using a colorimetric assay described elsewhere (Kawamura-Sato *et al.*, 1999).

Pigmentation assays. We employed solvent extraction and spectrophotometry to measure pigment production in *Chro. violaceum* (violacein), *S. marcescens* (prodigiosin) and *P. chlororaphis* (phenazine). The violacein assay consisted of removing 500 µl *Chro. violaceum* culture, harvesting the cells by centrifugation (5 min at 10 000 ***g***), resuspending the cells in 1000 µl 90 % (v/v) methanol, removing cell debris by centrifugation and measuring violacein at 585 nm (Pantanella *et al.*, 2007). For prodigiosin, the sampling and centrifugation protocols were the same, and the pigment was extracted by suspending the *S. marcescens* pellet in acidified ethanol (4 %, v/v, 1 M HCl in ethanol) and measuring absorbance at 534 nm (Morohoshi *et al.*, 2007). For phenazine production in *P. chlororaphis*, 1 ml culture was removed and the cells harvested by centrifugation. For extraction, 1 ml cell-free supernatant was extracted with 1 ml acidified benzene (4 %, v/v, 1 M HCl in benzene); the benzene phase containing the phenazine was separated and evaporated under air. Dried phenazine was dissolved in 0.1 M NaOH and quantified at 367 nm (Maddula *et al.*, 2008).

Chitinase and elastase measurement. Qualitative and quantitative measurements of chitinase (Chernin *et al.*, 1998) and elastase (Zins *et al.*, 2001) were performed on *Chro. violaceum* and *S. marcescens* cultures to determine whether indole affected these quorum-regulated phenotypes. The qualitative assays consisted of streaking cultures onto minimal agar containing chitin (0.2 %, w/v) or LB agar containing elastin (0.33 %, w/v). These media were supplemented with indole ranging from 0–2 mM. Following incubation, clearing of the insoluble chitin or elastin in the vicinity of the culture was interpreted as evidence of chitinase or elastase activity. For quantitative measurements, we used a dye-release assay involving chitin–azure (Chernin *et al.*, 1998) or elastin–Congo red (Zins *et al.*, 2001) (both reagents purchased from Sigma-Aldrich). Briefly, 100 µl culture supernatant was mixed with 1 ml phosphate buffer (10 mM sodium phosphate buffer, pH 7.0) containing either 10 mg chitin–azure or 10 mg elastin–Congo red. Following overnight incubation at 37 °C, the insoluble substrate was pelleted by centrifugation and the concentration of released dye was measured at 585 nm (azure) or 495 nm (Congo red).

Motility assay. To test the influence of indole on motility, we modified a previously described assay (O'Toole & Kolter, 1998). The medium used for the motility assay was TSB containing 0.3 % (w/v) agar, supplemented with 1 mM indole. Control plates lacked indole. An overnight culture of bacteria was stab-inoculated in the centre of the plate, and motility was measured by the colony diameter following 18 h incubation at 25 °C.

Virulence assay. NGM was supplemented with different amounts of indole (0–1.0 mM). Bacteria were added and allowed to grow for 48 h. *Caen. elegans* eggs were harvested from large populations of gravid adults using a standard bleaching protocol (30 ml 5 % bleach, 15 ml 1 M KOH, 55 ml H_2O) (Tan *et al.*, 1999). Harvested eggs were placed on lawns of fresh *E. coli* OP50 and allowed to hatch and grow to the young adult stage. Ten non-gravid young adults were transferred onto lawns of *Chro. violaceum, S. marcescens* and *P. chlororaphis* on NGM. Nematodes were scored for survival each day and transferred to fresh lawns every 2 days until all worms had expired. A total of six biological replicates were performed with each replicate consisting of ten *Caen. elegans*.

AHL extracellular complementation assay. To investigate whether indole is in competition with the acylhomoserine lactones, 1.0 mM indole and different concentrations of *N*-decanoyl homoserine lactone (C10-HSL) were added to 25 ml LB in a 50 ml flask and inoculated with 100 µl of an overnight culture of *Chro. violaceum* at a final concentration of 10^6 cells ml^{-1}. A portion (500 µl) of the planktonic culture was removed at different time intervals and violacein was extracted as previously described.

Influence of indole on *Chro. violaceum* growth. In order to determine whether the inhibitory effects of indole on pigmentation and chitinase production were due to growth inhibition, we conducted growth curve measurements in the presence or absence of indole, dimethyl formamide (the solvent used to dissolve indole), supplemented C10-HSL and ethyl acetate (the solvent used to dissolve C10-HSL). Details are presented in the supplementary information (available in the online Supplementary Material).

RESULTS

Pigmentation inhibition

In preliminary experiments we observed that co-culture of wild-type *E. coli,* but not *tnaA* mutant cells, with normally pigmented *Chro. violaceum, P. chlororaphis* and *S. marcescens* resulted in a loss of pigmentation (data not shown). Under our experimental conditions, *E. coli* indole production in pure cultures was 0.53 ± 0.01 mM and higher concentrations (~5 mM) have been reported by other investigators (Li & Young, 2013). In mixed-culture growth, indole concentrations were 0.40 ± 0.02 mM with *Chro. violaceum* and 0.41 ± 0.02 mM with *P. chlororaphis*. Unexpectedly, indole concentrations were much lower (0.058 ± 0.001 mM) following mixed-culture growth of *E.*

coli BW25113 with *S. marcescens*. This was not due to indole degradation by either *E. coli* or *S. marcescens* as neither organism degraded indole in pure or mixed culture (data not shown). In pure culture experiments, indole inhibited pigmentation of *Chro. violaceum, P. chlororaphis* and *S. marcescens* (Fig. 1a–c) cells at physiologically relevant concentrations (0.5–1.0 mM). Pigmentation inhibition of *Chro. violaceum* by 1.0 mM indole could be counteracted by the addition of the cognate quorum signal C10-HSL (Fig. 1d) in a dose-dependent manner.

Indole interferes with quorum-regulated transcription

RT-qPCR measurements (Fig. 2) showed transcription of *Chro. violaceum* quorum-regulated genes *vioA* [involved in violacein production (Balibar & Walsh, 2006)] and *chiA* [chitinase (Chernin *et al.*, 1998)] to be inhibited by indole in a dose-dependent manner. Inhibition of *vioA* and *chiA* transcription also occurred during mixed-culture growth with wild-type *E. coli*. There was a small but statistically significant inhibition of *vioA* (P=0.001) and *chiA* (P=0.018) transcription during *Chro. violaceum* mixed-culture growth with the *E. coli tnaA* mutant, suggesting that other *E. coli* metabolites may exhibit a modest inhibition on quorum-regulated genes. Transcription levels of the housekeeping gene *pykF* (encoding pyruvate kinase) were unaffected (P=0.431) by indole or mixed-culture growth.

Indole enhances *E. coli* competition

To assess the impact of indole on microbial competition, we grew *E. coli* in co-culture with *Chro. violaceum, P. chlororaphis* and *S. marcescens*. As shown in Fig. 3, *E. coli tnaA* mutants, unable to produce indole, were outcompeted by the other organisms in both planktonic (Fig. 3a, c, e) and biofilm (Fig. 3b, d, f) populations. When WT *E. coli* was cultured with *Chro. violaceum* or *P. chlororaphis* (Fig. 3a–d) the populations of *E. coli* were not significantly different from the other organisms. During co-culture of WT *E. coli* with *S. marcescens,* there was a slight but statistically significant reduction in *S. marcescens* planktonic populations at 48 h (P=0.033) but not at 24 h (P=0.885) (Fig. 3e). There was no significant difference in biofilm populations of WT *E. coli* and *S. marcescens* (Fig. 3f). In contrast, indole supplementation caused the WT *E. coli* and the *tnaA* mutant to outcompete *Chro. violaceum* (Fig. 3a, b), *P. chlororaphis* (Fig. 3c, d) and *S. marcescens* (Fig. 3e, f). Based on these results, indole production is necessary for *E. coli* to be able to compete with *Chro. violaceum, P. chlororaphis* and *S. marcescens* in mixed culture. At ambient levels (~0.5 mM indole produced by WT *E. coli*), *E. coli* maintains an equivalent population

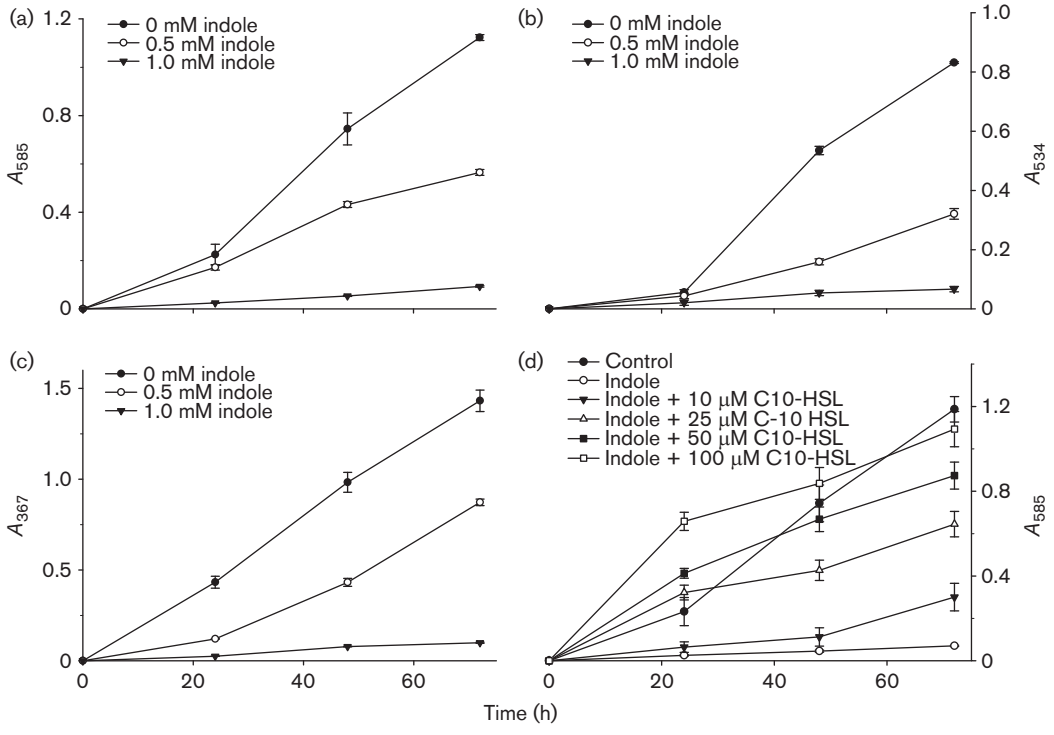

Fig. 1. Indole inhibition of AHL-regulated pigmentation during bacterial growth is evident in *Chro. violaceum* (violacein) (a), *P. chlororaphis* (phenazine) (b) and *S. marcescens* (prodigiosin) (c). Indole inhibition of violacein production in *Chro. violaceum* could be overcome by administration of the cognate autoinducer C10-HSL (Stauff & Bassler, 2011) (d). Error bars in all figures represent SE.

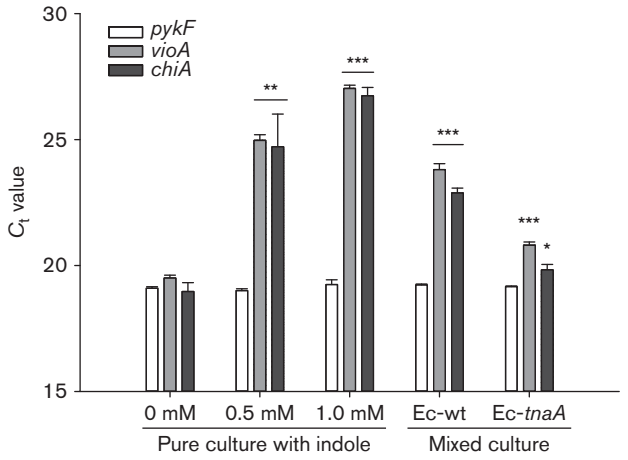

Fig. 2. Transcription of *Chro. violaceum* quorum-regulated genes *vioA* and *chiA*, reflected in increased C_t values (measured by RT-qPCR), was inhibited in the presence of indole and also by co-culture with indole-producing WT *E. coli* (Ec-wt). There was a slight inhibition seen during co-culture with the *E. coli tnaA* mutant (Ec-*tnaA*). Transcription of the housekeeping gene *pykF* was used as a reference and was not affected by the culture conditions. Statistically significant differences from *pykF* expression analysed by one-way ANOVA and Holm–Sidak analyses are denoted as *($P<0.05$), **($P<0.01$) and ***($P<0.001$).

with *Chro. violaceum, P. chlororaphis* and *S. marcescens*. However, at elevated concentrations (present in *E. coli tnaA* mutant cells supplemented with 1.0 mM indole and WT *E. coli* cells supplemented with 0.5 mM indole) the *E. coli* strains outgrew *Chro. violaceum, P. chlororaphis* and *S. marcescens* with the differences being most pronounced in both biofilm and planktonic populations after 48 h.

In order to determine whether differences in growth rate accounted for the competition results, we conducted turbidity-based assessment of *Chro. violaceum* growth in the presence and absence of indole, supplemented C10-HSL, and with the solvents dimethyl formamide and ethyl acetate, used to dissolve indole and C10-HSL, respectively. While differences were seen in culture turbidity after 24 h (Fig. S1a–c), the culture concentrations measured by dilution plating (Fig. S1d) showed no significant differences as measured by one-way ANOVA ($P=0.095$). *E. coli* growth was unaffected by ambient indole concentrations (0.5 mM), but was inhibited slightly at higher (1 mM) concentrations (Fig. S2), which is consistent with our earlier study (Chu *et al.*, 2012). These data do not support indole enhancement of *E. coli* competition with other Gram-negative bacteria as being solely due to growth rate alteration.

Indole suppression of quorum-regulated phenotypes and motility

In addition to pigmentation, several virulence factors, including chitinase and elastase, are regulated by quorum signalling in *Chro. violaceum* (Chernin *et al.*, 1998). When the activities of these enzymes were measured using dye-release assays, significant inhibition of chitinase and elastase was observed in the presence of indole (Fig. 4a). Similar inhibition of chitinase and elastase was seen in the presence of indole on qualitative agar plate assays (data not shown). We also measured the influence of indole on motility using a soft-agar plate assay (O'Toole & Kolter, 1998). Although, to our knowledge, *Chro. violaceum* motility has not been associated with quorum signalling, we did observe inhibition in the presence of indole (Fig. 4b).

Indole suppression of virulence

The bacterivorous nematode, *Caen. elegans*, has been used as a monitor for bacterial virulence (Tan *et al.*, 1999) and so we investigated whether the survival of this organism was impacted by the presence of indole. As seen in Fig. 5, indole promoted the survival of *Caen. elegans* in the presence of *Chro. violaceum* (Fig. 5a), *P. chlororaphis* (Fig. 5b) and *S. marcescens* (Fig. 5c).

DISCUSSION

Quorum-signal disruption is being increasingly recognized as a strategy whereby prokaryotes and eukaryotes can compete with bacteria (de Nys *et al.*, 2006; Schertzer *et al.*, 2009). As described earlier, indole has been shown to inhibit quorum signalling in *P. aeruginosa* (Lee *et al.*, 2009, 2011; Tashiro *et al.*, 2010) and in *A. oleivorans* (Kim & Park, 2013). Indole and several related compounds have been identified as quorum-inhibiting materials (Kim & Park, 2013; Lee *et al.*, 2009, 2011) against *P. aeruginosa*. During an earlier investigation of mixed-culture growth of *E. coli* and *P. aeruginosa* (Chu *et al.*, 2012), we observed that pigmentation due to *P. aeruginosa* pyocyanin production and several other quorum-regulated phenotypes was absent when this organism was co-cultured with indole-producing *E. coli*. To investigate how widespread this phenomenon was, we co-cultured *E. coli* with three other Gram-negative organisms, *Chro. violaceum, P. chlororaphis* and *S. marcescens*, and observed a reduction in pigmentation in the presence of wild-type *E. coli*, but not the *E. coli tnaA* mutant that lacks indole production (data not shown). When these organisms were grown in monoculture in the presence of indole, AHL-regulated pigmentation due to violacein (McClean *et al.*, 1997), phenazine (Wood & Pierson, 1996) and prodigiosin (Horng *et al.*, 2002) was inhibited (Fig. 1a–c). In *Chro. violaceum*, violacein pigmentation could be restored during aerobic culture in the presence of 1 mM indole by supplementation with 100 μM C10-HSL.

During co-culture studies, we also wanted to address the possibility that one or more of the three organisms (*Chro. violaceum, P. chlororaphis* and *S. marcescens*) could produce indole, degrade indole or else affect *E. coli* indole

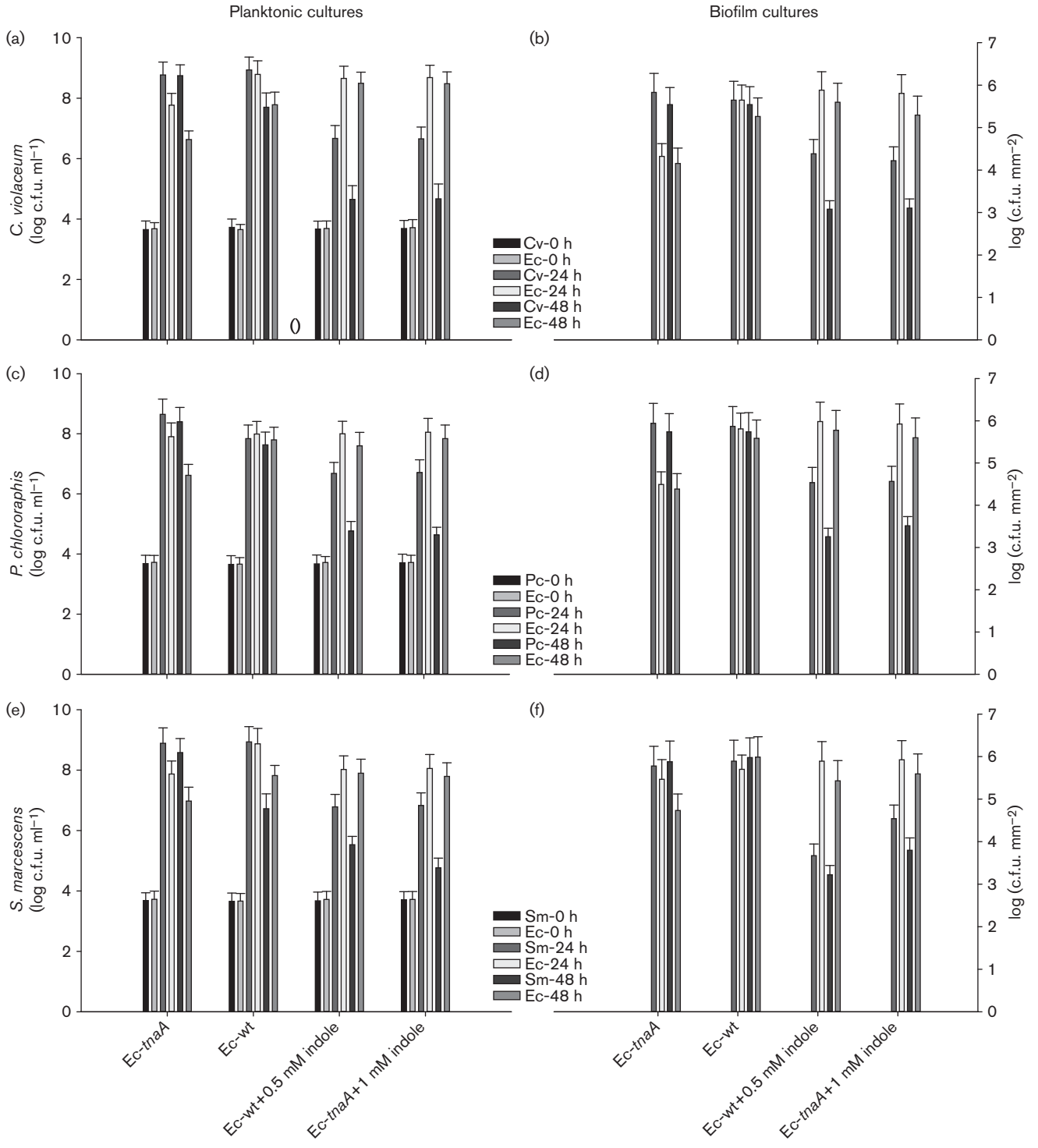

Fig. 3. Population levels after 24 and 48 h of planktonic and biofilm cultures of *Chro. violaceum* (Cv) (a, b), *P. chlororaphis* (Pc) (c, d) and *S. marcescens* (Sm) (e, f) during co-culture with *E. coli* (Ec). The initial planktonic populations of the organisms at 0 h are also shown.

production. As described in Results, indole production by WT *E. coli* was 0.53 ± 0.01 mM in pure culture, 0.40 ± 0.02 mM in mixed culture with *Chro. violaceum* and 0.41 ± 0.02 mM in mixed culture with *P. chlororaphis*. During mixed-culture growth of WT *E. coli* with *S. marcescens*, indole concentrations were considerably lower,

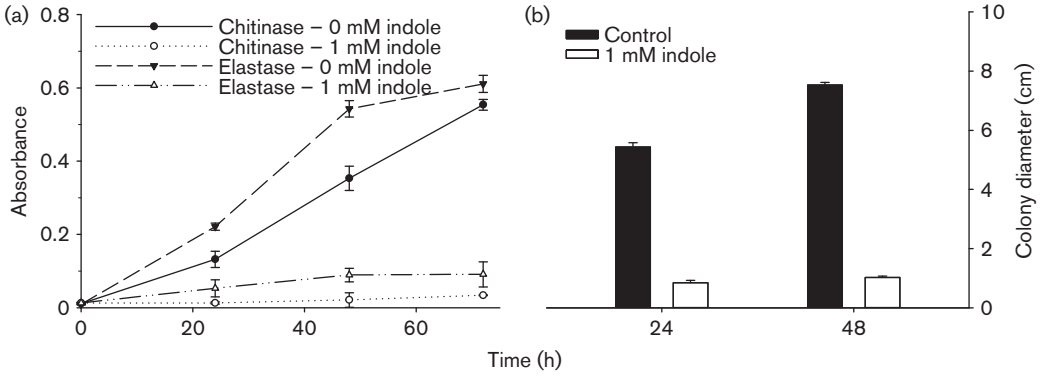

Fig. 4. Indole inhibition of AHL-regulated *Chro. violaceum* chitinase (A_{585}) and *S. marcescens* elastase (A_{495}) (a) as shown by a dye-release assay. Indole also inhibited *Chro. violaceum* motility (b), although this phenotype has not been associated with quorum regulation.

0.058 ± 0.001 mM. To address the possibility of indole degradation being responsible, we tested indole concentrations in pure cultures of *S. marcescens* that had been supplemented with 0.5 and 1.0 mM indole, and found no significant differences in indole concentrations following 24 h growth (data not shown). Based on these results, *E. coli* indole production is inhibited slightly by co-culture with *Chro. violaceum* and *P. chlororaphis*, and markedly by *S. marcescens*. Mechanisms for *S. marcescens* inhibition of *E. coli* indole production are presently unknown.

We tested the influence of mixed-culture growth and indole on *Chro. violaceum* gene transcription using RT-qPCR. As seen in Fig. 2, there was a significant inhibition of transcription (reflected in the significantly increased C_t values) of quorum-regulated *vioA* (involved in violacein production) (Morohoshi *et al.*, 2010) and *chiA* (chitinase) (Chernin *et al.*, 1998). In contrast, the housekeeping gene *pykF* (pyruvate kinase) (Brito *et al.*, 2004), which to the best of our knowledge is not quorum regulated, was not significantly affected by indole or mixed-culture growth.

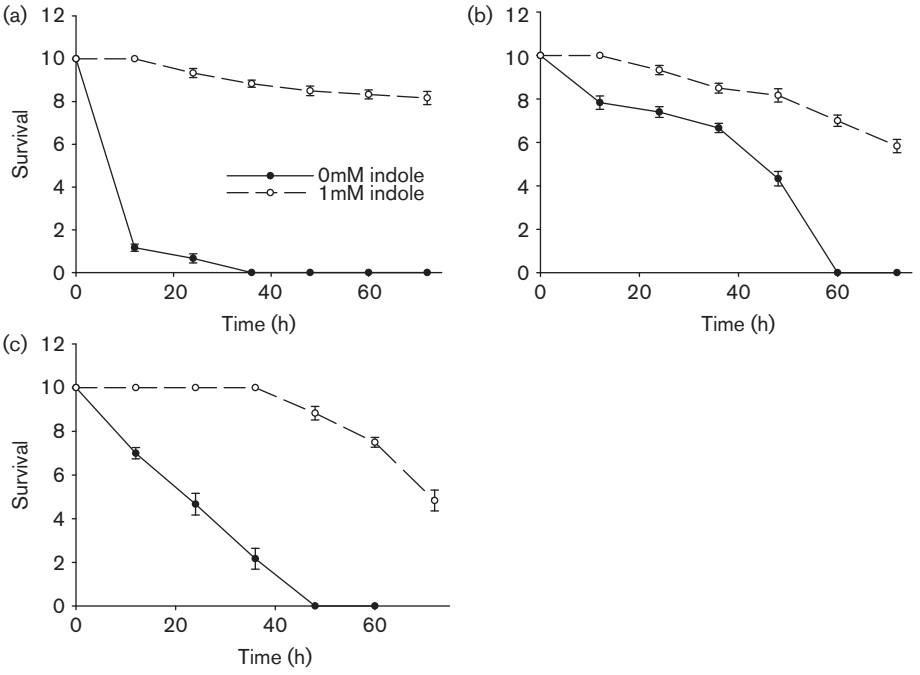

Fig. 5. Survival of *Caen. elegans* (expressed as the number of viable nematodes remaining from an initial inoculum of ten) during exposure to *Chro. violaceum* (a), *P. chlororaphis* (b) and *S. marcescens* (c) was enhanced by indole inhibition of AHL-regulated virulence.

Interestingly, there was a smaller but statistically significant inhibition of *vioA* and *chiA* transcription during mixed-culture growth with the *E. coli tnaA* mutant cells. Although there was no detectable indole present, it is conceivable that some other *E. coli* metabolite may influence *Chro. violaceum* gene expression. It is also possible that differences in transcript levels may be due to the relative stability of mRNA under different growth conditions, rather than levels of transcription, but we have no data to support this concept.

Several other quorum-regulated genes have been identified in *Chro. violaceum* and *S. marcescens*, including those encoding chitinase (Chernin *et al.*, 1998) and elastase (Zins *et al.*, 2001). Using dye-release enzyme assays, we also observed that chitinase and elastase were inhibited by indole (Fig. 4a). Of interest, we also found that indole inhibits motility (Fig. 4b). To our knowledge, there is no experimental evidence associating quorum signalling with motility in *Chro. violaceum*. However, AHL-based signalling has been associated with swarming motility in several other bacteria including *Serratia liquefaciens* (Givskov *et al.*, 1998) and *P. aeruginosa* (Köhler *et al.*, 2000). Based on our results (Fig. 4b), we speculate that there is either a direct or an indirect link between quorum signalling and motility in *Chro. violaceum*. Flagella-based motility does play a role in biofilm formation through enabling organisms to reach and colonize surfaces (Costerton *et al.*, 1987). It is also an important aspect of biofilm detachment (Sauer & Camper, 2001) in that it allows cells to leave biofilms and return to the planktonic mode of growth. In this context, indole may play an important role in certain stages of biofilm growth, notably initial adhesion and detachment (Petrova & Sauer, 2012a, b).

In mixed-culture growth with *P. aeruginosa*, *E. coli* indole production was very important for survival (Chu *et al.*, 2012). In the present study, we noted a similar pattern in which the *E. coli tnaA* cells competed less well after 24 h and especially after 48 h with *Chro. violaceum* (Fig. 3a, b), *P. chlororaphis* (Fig. 3c, d) and *S. marcescens* (Fig. 3e, f). With one exception [48 h planktonic data (Fig. 3e) where the population of *S. marcescens* was less ($P=0.033$) than *E. coli*], the populations of WT *E. coli* were statistically similar to the populations of the other three Gram-negative bacteria in both planktonic and biofilm populations. When the indole concentration was increased from 0.5 mM (wild-type *E. coli* co-culture) to 1 mM (WT *E. coli* + 0.5 mM indole and *E. coli tnaA* mutant + 1 mM co-cultures), *E. coli* outcompeted *Chro. violaceum* (Fig. 3a, b), *P. chlororaphis* (Fig. 3c, d) and *S. marcescens* (Fig. 3e, f). Similar trends were seen in both planktonic (Fig. 3a, c, e) and biofilm (Fig. 3b, d, f) populations. As stated earlier, indole has been shown to interfere with AHL-based quorum regulation (Lee *et al.*, 2009) in *P. aeruginosa* by repressing expression of the antimicrobial compound pyocyanin. In the current study, we observed inhibition of phenazine production in *P. chlororaphis* (Fig. 1b). As production of antimicrobial phenazine in *P. chlororaphis* is quorum regulated (Wood & Pierson, 1996), indole production by *E. coli* would certainly provide a mechanism for the survival of this organism in co-culture with *P. chlororaphis*. During laboratory investigations of *Chro. violaceum* competition with *Burkholderia thailandensis* (Chandler *et al.*, 2012), *Chro. violaceum* was shown to compete with *B. thailandensis* through the AHL-regulated production of antimicrobial compounds, as well as by scavenging AHLs from *B. thailandensis* via the broad spectrum LuxR homologue CviR. Mechanisms of bacterial competition in *S. marcescens* are associated with antimicrobial peptide production, type 6 secretion (Fritsch *et al.*, 2013) and bacteriocin production (Kuo *et al.*, 2013). Kuo *et al.* (2013) speculated that bacteriocin production might be regulated by quorum signalling and it was definitely regulated by phosphate limitation.

Indole has been shown to interfere with AHL regulation in *A. oleivorans* through misfolding of the LuxR homologue AqsR (Kim & Park, 2013). However, this situation may be more complex in *Chro. violaceum*. Our data (Fig. 1d) would suggest C10-HSL to have a higher affinity than indole for CviR (LuxR homologue) (McClean *et al.*, 1997), since inhibition by 1 mM indole could be reversed in the presence of a 10-fold lower (100 μM) C10-HSL concentration (Fig. 1d). The concentration of AHLs produced by *Chro. violaceum* and many other Gram-negative bacteria is unknown and it would be worth investigating the impact of indole and other inhibitors on AHL production (LuxI effect), as well as AHL response (LuxR effect). Nevertheless, our results suggest the physiological concentrations of indole normally present (0.5 mM) during mixed culture are necessary for *E. coli* growth with other Gram-negative organisms in biofilm and planktonic populations.

In a number of bacteria, virulence is regulated by quorum signalling (Anand *et al.*, 2013; Williams, 2007), and organisms in which quorum signalling is diminished through mutation or by quorum-inhibiting compounds are less able to harm eukaryotic hosts or tissues (Jensen *et al.*, 2007; Wu *et al.*, 2004). In this context, we tested the ability of indole to protect the bacterivorous nematode *Caen. elegans* from *Chro. violaceum*. As seen in Fig. 5, *Caen. elegans* survival during exposure to *Chro. violaceum* (Fig. 5a), *P. chlororaphis* (Fig. 5b) and *S. marcescens* (Fig. 5c) was enhanced by the presence of 1 mM indole, and certainly supports our conclusion that indole inhibits quorum-regulated virulence in *Chro. violaceum*, *P. chlororaphis* and *S. marcescens*. There were certainly differences in *Caen. elegans* survival noted among the three bacteria tested. The likely explanation for these differences would be the relative susceptibility of *Caen. elegans* to the three organisms or alternatively the different nutrients available from these three organisms, when compared to the *E. coli* OP50 strain on which *Caen. elegans* is normally cultured (Tan *et al.*, 1999).

Bacterial indole production has been known for some time (Hopkins & Cole, 1903) and this compound has been

shown to inhibit AHL-mediated quorum signalling in *P. aeruginosa* (Chu *et al.*, 2012; Lee *et al.*, 2009; Tashiro *et al.*, 2010) and *A. oleivorans* (Kim & Park, 2013). Indole production was also shown to promote *E. coli* survival during co-culture with *P. aeruginosa* (Chu *et al.*, 2012). Based on this current study, we conclude that indole production plays an important role in the competition between *E. coli* and AHL-producing Gram-negative bacteria, and that indole is a general inhibitor of AHL-based quorum signalling.

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