Role of Indole Production on Virulence of *Vibrio cholerae* Using *Galleria mellonella* Larvae Model

Taiyeebah Nuidate¹ · Natta Tansila² · Suwat Saengkerdsub³ · Jetnaphang Kongreung¹ ·
Dhamodharan Bakkiyaraj¹ · Varaporn Vuddhakul¹

Received: 9 February 2016 / Accepted: 29 April 2016 / Published online: 6 May 2016
© Association of Microbiologists of India 2016

Abstract Cell to cell communication facilitated by chemical signals plays crucial roles in regulating various cellular functions in bacteria. Indole, one such signaling molecule has been demonstrated to control various bacterial phenotypes such as biofilm formation and virulence in diverse bacteria including *Vibrio cholerae*. The present study explores some key factors involved in indole production and the subsequent pathogenesis of *V. cholerae*. Indole production was higher at 37 °C than at 30 °C, although the growth at 37 °C was slightly higher. A positive correlation was observed between indole production and biofilm formation in *V. cholerae*. Maximum indole production was detected at pH 7. There was no significant difference in indole production between clinical and environmental *V. cholerae* isolates, although indole production in one environmental isolate was significantly different. Both growth and indole production showed relevant changes with differences in salinity. An indole negative mutant strain was constructed using transposon mutagenesis and the direct effect of indole on the virulence of *V. cholerae* was evaluated using *Galleria mellonella* larvae model. Comparison to the wild type strain, the mutant significantly reduced the mortality of *G. mellonella* larvae which regained its virulence after complementation

with exogenous indole. A gene involved in indole production and the virulence of *V. cholerae* was identified.

Keywords *Vibrio cholerae* · Indole · *Galleria mellonella* · Biofilm

Introduction

In environmental niches that composed of variety of bacteria, bacterial signaling molecules play important roles in space and nutrition competition. These signaling molecules including extracellular and intracellular signals affect both inter- and intra-species of bacteria [1–3]. *Vibrio cholerae* is a Gram-negative bacterium that exists in aquatic environments and most strains belonging to the O1 serogroup cause cholera outbreaks. The outbreaks are usually associated with poverty and poor sanitation in southern Asia, Africa and Latin America. The incubation period is from several hours to 5 days and the symptoms include watery diarrhea, vomiting and dehydration [4]. The signaling molecules, cholerae autoinducer-1 (CAI-1) and autoinducer-2 (AI-2), demonstrated in this bacterium are activated at high cell densities and control the production of virulence factors and biofilm formation [5]. Recently, indole has been proposed as a novel signal involved in quorum sensing and demonstrated to be an extracellular signaling molecule of *V. cholerae* that activates the genes involved in polysaccharide production, increase biofilm formation and grazing resistance to *Dictyostelium discoideum*, a soil-dwelling amoeba [2, 3]. In addition, indole influences genes expression of *V. cholerae* such as flagella synthesis, amino acid transport, iron uptake and virulence-associated secretion genes. Although indole has been reported in both Gram-negative and Gram-positive

✉ Varaporn Vuddhakul
varaporn.v@psu.ac.th

¹ Food Safety and Health Research Unit, Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai 90110, Thailand

² Faculty of Medical Technology, Prince of Songkla University, Hat Yai 90110, Thailand

³ Department of Food Technology, Faculty of Agroindustry, Prince of Songkla University, Hat Yai 90110, Thailand

bacteria, biosynthesis of indole has been studied mostly in *Escherichia coli*. Indole is produced from tryptophan by tryptophanase (encoded by *tnaA*) and its production is regulated by the tryptophan (*trpABCDE*) and *tna* operons (*tnaCAB*) [6–8]. The level of tryptophan controls transcription-terminating factor (Rho) that affects *tna* operon and indole production [6, 8]. In the low tryptophan level, *trp* operon is elevated and *tna* operon is repressed, as Rho occurs in this operon resulting in low indole production. In contrast, in the high tryptophan condition, Rho is eliminated causing high production of indole. In addition, the level of glucose also influences indole production. In *E. coli*, catabolism of glucose represses *tnaA* and decreases indole production [9]. *E. coli* and *V. cholerae* start producing indole during their exponential growth phase and the maximum concentration is detected and stable in the stationary phase [3, 10]. The presence of indole affects drug resistance, plasmid stability and virulence of *E. coli* and high concentration of indole decreases biofilm formation in some *E. coli* strains such as *E. coli* ATCC25404 and JM109 [7, 11–13]. Environmental factors that affect extracellular indole production have been demonstrated in *E. coli* [14]. Low pH inhibits indole production but it increases at high pH at both 30 °C as well as 37 °C. This directly corroborates with the decreased biofilm formation in alkaline conditions. In the presence of bactericidal antibiotics (ampicillin and kanamycin) indole production is induced, whereas the bacteriostatic agent chloramphenicol has no effect. Furthermore, adding ampicillin and indole to *E. coli* increases the cell density by cell elongation but not by cell division. High temperatures up to 50 °C stimulate more indole production than at 25 or 37 °C. Sodium chloride, ferrous chloride and cadmium chloride that greatly affect bacteria in the environment, have no effect on indole production [14]. The known roles of indole produced by *V. cholerae* in response to environmental conditions and its association to pathogenesis are still limited. Therefore, in this study, factors that influence indole production by *V. cholerae* were investigated. In addition, virulence of a *V. cholerae* wild type and an indole mutant strain was compared to determine the role of indole in pathogenicity of *V. cholerae* in vivo.

Materials and Methods

Bacterial Strain

The clinical and environmental isolates of *V. cholerae* O1 and non-O1 were obtained from the bacterial stock culture collection in the Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand. One clinical isolate of *V. cholerae* O1 designated as PSU

966 was used throughout this study unless indicated otherwise.

Growth Rate Determination

An overnight culture of *V. cholerae* O1 (PSU 966) was inoculated into the flask containing 50 mL of Luria–Bertani (LB) broth to give the cell density of OD₆₀₀ 0.01, the flask was shaken at 37 °C, 180 rpm for 24 h unless other conditions were indicated. The growth rate was determined by OD measurement at 600 nm using a spectrophotometer (UV-160, Shimadzu, Japan).

Estimation of Indole in the Medium

The concentration of indole was measured as described previously with a slight modification [15]. Briefly, 0.4 mL of Kovac's reagent was mixed with 1 mL of the bacterial supernatant and the absorbance of the mixture was measured at 540 nm with a spectrophotometer using Kovac's reagent as a blank. The concentration of indole was calculated according to the indole standard curve.

Effect of Environmental Factors on Indole Production

Vibrio cholerae was incubated at 30 and 37 °C and indole production was determined to compare the effects of temperature and the growth rate. The suitable temperature was used to evaluate the effects of pH and NaCl concentration on indole production. Briefly, *V. cholerae* was cultured in LB broth with varying pH between 6 and 9. To investigate the effect of the concentration of NaCl on indole production, *V. cholerae* was grown in 1 % peptone and 0.5 % yeast extract broth without NaCl or supplemented with NaCl at concentrations of 0.5, 1, 3, 5 and 6 %. The concentration of indole was evaluated as described above.

Biofilm Assay

Formation of biofilm by *V. cholerae* was assessed by quantitative determination of bacterial adherence to a 96-well microtiter plate as described earlier with minor modifications [16]. Briefly, an overnight culture of *V. cholerae* (200 µL) was inoculated into the wells of a microtiter plate. After incubation at 37 °C for 24 h, the unattached bacterial cells were discarded and the plate was gently washed three times with water. The cells were fixed with glutaraldehyde and stained with 0.4 % (w/v) crystal violet for 15 min at room temperature. After being rinsed with water, the wells were destained with ethanol–acetone

(80:20) and biofilm production was measured using a spectrophotometer at an OD₅₇₀.

The Indole Deficient Mutant Strain

An indole mutant strain was constructed using transposon mutagenesis with a mini-Tn5-ermR delivery vectors pEVS168 because it generates a single Tn insertion in each strain examined [17]. Briefly, a triparental mating of each single colony of a wild type *V. cholerae* (PSU 966), donor *E. coli* DH5 α with pEVS168 (erm^R) and helper *E. coli* DH5 α with pRK2013 (kan^R) was performed on LB agar at 37 °C, over a 6 h period. The mixture was then resuspended in LB broth and spread on thiosulfate citrate bile salt sucrose (TCBS) agar supplemented with erythromycin at a final concentration of 10 μ g/mL. After overnight incubation at 37 °C, indole negative colonies were selected using a replica plate agar containing 1 % peptone. The plate was incubated overnight at 37 °C before flooding with Kovac's reagent.

To identify the insertion region in the genome of the *V. cholerae* indole negative mutant strains, chromosomal DNA was extracted and cut with the restriction enzyme *Hha*I. Self-ligation products of the gene fragments were transformed into *E. coli* DH5 α - λ pir and the transformants were selected using LB agar supplemented with erythromycin at a final concentration of 350 μ g/mL. Plasmid extraction was performed and the M13 forward primer was used to sequence the regions flanking the inserted transposon.

Effect of Indole on *V. cholerae* Virulence

Previously, ligated rabbit ileal loop or infant mice model were used for pathogenicity investigation of *Vibrio* spp. causing gastrointestinal tract infections. However, due to ethic complaints, *Dictyostelium discoideum* (soil-living amoeba) or *Caenorhabditis elegans* (nematode), have been selected for *V. cholerae* and *V. alginolyticus* infection models [3, 18]. In this work, *Galleria mellonella* caterpillars were used to investigate the virulence of *V. cholerae* by comparing the wild type and an indole negative mutant. The larvae are more attractive than other non-animal model because they possess well characterized phagocytic system and have been used to study various bacterial pathogens including *Acinetobacter baumannii* and *Campylobacter jejuni* [19, 20]. In this study, caterpillars with an approximate weight of 250–350 mg and 2.0–3.0 cm in length were selected for the virulence investigation. The most suitable concentration of bacterial cells used for testing was evaluated with the wild type strain. To prepare the *V. cholerae* inocula, bacteria were grown overnight on trypticase soy agar at 37 °C. A selected colony was inoculated

into LB broth and incubated at 37 °C with shaking for 4 h. Bacterial cells were harvested and adjusted to 0.3×10^6 to 2.4×10^6 CFU/mL in phosphate buffered saline (PBS) pH 7.0. The bacterial concentration was confirmed by a viable cell count assay.

Galleria mellonella larvae (n = 15/concentration) were injected with 20 μ L of *V. cholerae* suspension into the haemocoel through the last left proleg using an insulin syringe (Terumo Myjector, USA). Larvae were then incubated at room temperature and the number of dead larvae was enumerated at 24, 36, 48 and 72 h after injection. Larvae were considered dead when no response was observed after touching. A control inoculation (n = 15) was performed with 20 μ L of PBS. The most suitable concentration and time that caused 50 % or more of caterpillars to die was used for virulence investigation between the *V. cholerae* wild type and mutant strains. All experiments were carried out in duplicate.

Statistical Analysis

One way ANOVA was performed for statistical analysis using SPSS software version 14 (SPSS inc., USA). The *p* value of <0.05 was considered statistically significant result.

Results and Discussion

Factors Involved in Indole Production in *V. cholerae*

The natural habitat of *V. cholerae* is the aquatic environment in which it exists either as a free living organism or attached to copepods [21]. In this situation, competition for space and perhaps nutrition may be lower than bacteria in the human intestine where the bacteria are exposed to both extreme environments and the body's defense mechanisms. *V. cholerae* can form biofilm to aid its survival under adverse conditions [22, 23]. Indole has been reported to be a bacterial signal that induces biofilm formation in *V. cholerae* [3]. In this study, we demonstrated that *V. cholerae* significantly produces more indole at 37 °C than at 30 °C, although, its growth rate at 37 °C was slightly higher than the growth rate at 30 °C (Fig. 1). It has been reported that growth rate of 6 isolates of *V. cholerae* after growing at 37 °C was slightly increased than that of 30 °C during culture in different conditions [24]. Thus, in the tropical environment where average ambient temperature is around 30 °C, the association of indole with colonization may be less critical than at 37 °C. This was confirmed by the increase in biofilm formation in the presence of higher concentrations of indole (Fig. 2). In *E. coli*, investigations of gene expression at 30 and 43 °C revealed that the *tnaAB*

Fig. 1 Effect of temperature on cell growth and indole production in *Vibrio cholerae*. *V. cholerae* O1 was cultured in 50 mL of LB broth at 30 and 37 °C separately, growth and indole production (after mixed with Kovac's reagent) were determined by spectrophotometer at 600 and 540 nm respectively

Fig. 2 Correlation between biofilm formation and indole production. 200 μ L of *Vibrio cholerae* O1 was cultured in microtiter plate at 37 °C for 24 h, biofilm formation was determined by staining the attached cells with crystal violet

genes were induced at higher temperature [25]. The *V. cholerae* *tnaA* gene is 82–85 % homologous to the *tnaA* of *E. coli* [2] and hence expression of this gene may correspond to that of *E. coli*.

It has been known that *V. cholerae* has an ability to withstand alkaline pH especially in the presence of plankton or algae [26]. Under laboratory conditions that were designed to mimic the natural environment, the optimal pH for attachment and multiplication of *V. cholerae* O1 to copepods over 48 h cultivation was 8.5 [27]. Our finding revealed no significant difference in the growth rate of *V. cholerae* between pH 6 and 9, however, the indole production was much better at pH 7 (Fig. 3). This might be due to the test bacterium was a clinical isolate and preferred the conditions close to that of the human intestine (neutral pH). In *E. coli*, it has been found that alkalinity could induce the expression of *tnaA* gene leading to high tryptophanase activity and active indole production [13, 28, 29]. In this study, we found that at pH 7, all clinical isolates

Fig. 3 Effect of pH on indole production. *Vibrio cholerae* O1 was cultured in 50 mL of LB broth with various pH at 37 °C, 24 h

Table 1 Indole production in clinical and environmental *Vibrio cholerae*

<i>V. cholerae</i> strains	Indole (mM) Mean $\pm$ SD
C1 (O1)	3.2 $\pm$ 0.3
C2 (O1)	3.0 $\pm$ 0.1
C3 (non-O1)	3.1 $\pm$ 0.1
C4 (non-O1)	2.8 $\pm$ 0.2
E1 (non-O1)	2.8 $\pm$ 0.2
E2 (non-O1)	3.0 $\pm$ 0.2
E3 (O1)	2.9 $\pm$ 0.2
E4 (O1)	4.2 $\pm$ 0.2 ^a

V. cholerae O1 was cultured in 50 mL of LB broth at 37 °C, 24 h and indole production was determined

C clinical isolates, E environmental isolates

^a Significant difference ($p < 0.05$)

of *V. cholerae* and environmental isolates produced similar amounts of indole, although indole production in one environmental isolate was significantly different (Table 1). The normal pH of freshwater and seawater is between 6.5–7.5 and 7.5–8.5 respectively. Thus, pH 7 may be the optimal pH for the metabolic activity of this organism. Therefore, due to pH, the mechanism for regulating *tnaA* gene expression as well as indole production differing from that of *E. coli* may be postulated in *V. cholerae*.

Vibrio cholerae occurs in both marine and freshwater habitats. Different salinities of these sources including humans are addressed; humans (0.85 %), freshwater (0.5 %) and seawater (3.5 %). To investigate the effect of salinity on indole production, *V. cholerae* was cultured in different concentrations of NaCl (Fig. 4). It is of interest that indole was increased at the concentration of NaCl 5 and 6 % whereas the growth rate of *V. cholerae* started decreasing at the concentration of NaCl between 1 and 3 %. It has been reported that 3 % NaCl could stress *V. cholerae* to exist in a viable but non-culturable cells [30].

Fig. 4 Effect of NaCl on indole production. *Vibrio cholerae* O1 was cultured in 50 mL of LB broth with various concentrations of NaCl at 37 °C, 24 h

In this condition, *V. cholerae* might utilize indole to protect itself. The protective role of indole towards various stresses has been described in several microorganisms possibly through inducing efflux system or biofilm formation [31–33]. High level of extracellular indole was detected in *E. coli* in the stress condition of antibiotics treatment and supplementation or increase production of indole enhanced cell survival [10, 13, 14].

Effect of Indole on *V. cholerae* Virulence

A correlation between indole production and virulence of bacteria has been demonstrated. Most pathogenic *Haemophilus influenzae* isolates (94–100 %) were indole positive whereas 70–75 % of the normal microbiota isolated from the respiratory tract were indole positive [34]. It has been reported that *tnaA* gene is necessary for killing and paralysis of *C. elegans* by enteropathogenic *E. coli* (EPEC) presumably through direct or indirect regulation of shiga toxin production [11]. In enterohaemorrhagic *E. coli* (EHEC), a *tnaA* mutant had decreased EspA and EspB (type III secreted proteins) secretion and reduced its ability to form attaching and effacing (A/E) lesions in HeLa cells. However, those abilities were restored upon exogenous addition of indole [35]. It was therefore concluded that indole regulated the expression of the *espA* and *espB* genes and were involved in the formation of A/E lesions in EHEC. In *V. cholerae*, using *D. discoideum* as the grazing model, a *tnaA* mutant had a decreased ability to resist *D. discoideum* phagocytosis but the grazing resistance was restored after adding indole [3]. In this work, we have now confirmed the involvement of indole in the virulence of *V. cholerae* using *G. mellonella* as the infection model. The optimal concentration of 1.2×10^6 CFU/mL *V. cholerae* wild type isolate was selected to cause 60 % mortality in caterpillars after 36 h injection (Table 2). This condition

Table 2 Investigation of the infective dose of *V. cholerae* against *Galleria mellonella*

<i>V. cholerae</i> concentration (CFU/mL)	<i>G. mellonella</i> mortality (%)			
	24 h	36 h	48 h	72 h
Control (PBS)	0	0	0	0
2.4×10^6	33.3	73.3	86.7	100
1.2×10^6	26.7	60.0	73.3	100
0.6×10^6	13.3	46.7	66.7	100
0.3×10^6	6.7	46.7	66.7	100

V. cholerae O1 was cultured in 5 mL of LB broth at 37 °C for 3 h and the concentration of cells was adjusted in PBS. Fifteen of *G. mellonella* were injected in each concentration

Table 3 Mortality of *Galleria mellonella* after injected with *V. cholerae* wild type and indole deficient mutant

<i>V. cholerae</i> strains	<i>G. mellonella</i> mortality within 36 h (%) Mean $\pm$ SD
Control (indole)	0
Wild type	63.3 ± 4.7^a
Mutant	43.0 ± 4.2^b
Mutant + 0.5 mM indole	49.5 ± 4.9^{bc}
Mutant + 1.0 mM indole	56.5 ± 4.9^{ac}
Mutant + 2.0 mM indole	63.0 ± 4.2^a
Mutant + 4.0 mM indole	66.0 ± 0^a

Both wild type and mutant strains were cultured in 5 mL of LB broth at 37 °C for 3 h and the concentration of cells was adjusted in PBS. Fifteen of *G. mellonella* were injected in each category

The difference in super subscribe indicates the significant difference. In control group, the larvae were injected only with indole at the concentration of 0.5–4 mM

was used to investigate the difference in virulence between the wild type strain and an indole negative mutant. This mutant strain was constructed by transposon mutagenesis and its phenotype including colony morphology, oxidase production, sucrose fermentation, was not different from the wild type strain except indole production was negative (data not shown). The mortality of *G. mellonella* injected with the wild type was 63.3 ± 4.7 % whereas it was 43.0 ± 4.2 % for the mutant strain. However, the mutant supplemented with 0.5–4 mM indole gradually increased its mortality rate comparable to that of wild type (Table 3). The transposon insertion site of the *V. cholerae* mutant was then analyzed by gene sequencing and it was demonstrated that the disrupted gene was 99.9 % homologous to the *V. cholerae oadB* gene (GenBank accession no. AE003852.1). Thus, this gene may be involved in indole production and virulence of *V. cholerae*.

The gene *oadB* encodes for the oxaloacetate decarboxylase β subunit. In *V. cholerae*, the oxaloacetate

decarboxylase enzyme consists of α , β and γ subunits. The α subunit is a peripheral protein that possesses carboxyltransferase domain and a biotin-containing CO₂ acceptor domain, the β subunit harbors the decarboxylase domain which also acts as a Na⁺ transporter and the γ subunit ties the 2 subunits together [36]. Initially, α subunit catalyses the conversion of oxaloacetate to pyruvate while transferring the carboxy group to its attached prosthetic biotin. Upon transferring the carboxybiotin to the β subunit, decarboxylation coupled Na⁺ transport across the membrane is initiated resulting in the release of CO₂ from carboxybiotin besides generating an electrochemical ion gradient [37]. Such electrochemical ion gradient on cell membrane, mainly the Na⁺ ion gradient has been reported to control diverse physiological functions including motility and virulence in many bacteria including *V. cholerae* [38]. Thus, it is possible that in *V. cholerae* mutant, oxaloacetate and Na⁺ ions are accumulated inside the cell and electrochemical ion gradient may be enforced to shift and subsequently reduce the virulence and motility of *V. cholerae*. Accumulation of oxaloacetate itself could result in virulence suppression because this has been reported to inhibit the expression of ToxT, a global regulator of virulence in *V. cholerae* [39].

In addition, the enzyme tryptophanase (*TnaA*) which regulates the synthesis of indole from tryptophan has been shown to be sensitive to Na⁺ ions [40]. Thus, accumulation of Na⁺ ions in an oxaloacetate decarboxylase mutant of *V. cholerae* could be the plausible mechanism behind indole negative phenotype and virulence reduction in the *V. cholerae* mutant.

Quorum sensing inhibitors have been demonstrated to influence bacterial signal molecules of *V. harveyi*, *E. coli*, *P. aeruginosa* etc. [41]. Although, they operate in lower concentration than those of antibiotics, strategy development of bacteria to avoid quorum sensing inhibitors may occur [42]. Different interruption mechanisms of quorum sensing inhibitors such as signal degradation, inhibition of signal molecule synthesis, reduction of receptor proteins and interference by signal molecule analogues have been demonstrated [2, 41]. However, enzymatic degradation of signal molecules has been well documented [43]. *V. cholerae* is one of the important human pathogens, therefore, regulation of indole by quorum sensing inhibitors is critical to control this bacterium in the near future.

In conclusion, this study demonstrates that temperature, pH and salinity affect indole production in *V. cholerae*. Indole production correlates to virulence of *V. cholerae* and a gene encoded for oxaloacetate decarboxylase β subunit.

Acknowledgments This work was supported by funds from The Thailand Research Fund and Royal Golden Jubilee Ph.D. program (Grant No. PHD/0213/2556). Thanks to Dr. Brian Hodgson for assistance with the manuscript.

References

- Camilli A, Bassler BL (2006) Bacterial small-molecule signaling pathways. *Science* 311:1113–1116. doi:10.1126/science.1121357
- Lee JH, Lee J (2010) Indole as an intercellular signal in microbial communities. *FEMS Microbiol Rev* 34:426–444. doi:10.1111/j.1574-6976.2009.00204.x
- Mueller RS, Beyhan S, Saini SG, Yildiz FH, Bartlett DH (2009) Indole acts as an extracellular cue regulating gene expression in *Vibrio cholerae*. *J Bacteriol* 191:3504–3516. doi:10.1128/JB.01240-08
- Kaper JB, Morris JG, Levine MM (1995) Cholera. *Clin Microbiol Rev* 8:48–86
- Higgins DA, Pomianek ME, Kraml CM, Taylor RK, Semmelhack MF, Bassler BL (2007) The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* 450:883–886. doi:10.1038/nature06284
- Gong F, Yanofsky C (2002) Analysis of tryptophanase operon expression in vitro: accumulation of TnaC-peptidyl-tRNA in a release factor 2-depleted S-30 extract prevents Rho factor action, simulating induction. *J Biol Chem* 277:17095–17100. doi:10.1074/jbc.M201213200
- Lee J, Jayaraman A, Wood TK (2007) Indole is an inter-species biofilm signal mediated by SdiA. *BMC Microbiol* 7:42. doi:10.1186/1471-2180-7-42
- Yanofsky C, Horn V, Gollnick P (1991) Physiological studies of tryptophan transport and tryptophanase operon induction in *Escherichia coli*. *J Bacteriol* 173:6009–6017
- Botsford JL, DeMoss RD (1971) Catabolite repression of tryptophanase in *Escherichia coli*. *J Bacteriol* 105:303–312
- Kobayashi A, Hirakawa H, Hirata T, Nishino K, Yamaguchi A (2006) Growth phase-dependent expression of drug exporters in *Escherichia coli* and its contribution to drug tolerance. *J Bacteriol* 188:5693–5703. doi:10.1128/JB.00217-06
- Anyanful A, Dolan-Livengood JM, Lewis T, Sheth S, Dezalia MN, Sherman MA, Kalman LV, Benian GM, Kalman D (2005) Paralysis and killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli* requires the bacterial tryptophanase gene. *Mol Microbiol* 57:988–1007. doi:10.1111/j.1365-2958.2005.04739.x
- Chant EL, Summers DK (2007) Indole signalling contributes to the stable maintenance of *Escherichia coli* multicopy plasmids. *Mol Microbiol* 63:35–43. doi:10.1111/j.1365-2958.2006.05481.x
- Hirakawa H, Inazumi Y, Masaki T, Hirata T, Yamaguchi A (2005) Indole induces the expression of multidrug exporter genes in *Escherichia coli*. *Mol Microbiol* 55:1113–1126. doi:10.1111/j.1365-2958.2004.04449.x
- Han TH, Lee JH, Cho MH, Wood TK, Lee J (2011) Environmental factors affecting indole production in *Escherichia coli*. *Res Microbiol* 162:108–116. doi:10.1016/j.resmic.2010.11.005
- Kawamura-Sato K, Shibayama K, Horii T, Iimura Y, Arakawa Y, Ohta M (1999) Role of multiple efflux pumps in *Escherichia coli* in indole expulsion. *FEMS Microbiol Lett* 179:345–352. doi:10.1111/j.1574-6968.1999.tb08748.x
- Nesper J, Lauriano CM, Klose KE, Kapfhammer D, Kraiss A, Reidl J (2001) Characterization of *Vibrio cholerae* O1 El tor galU and galE mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. *Infect Immun* 69:435–445. doi:10.1128/IAI.69.1.435-445.2001
- Lyell NL, Dunn AK, Bose JL, Vescovi SL, Stabb EV (2008) Effective mutagenesis of *Vibrio fischeri* by using hyperactive mini-Tn5 derivatives. *Appl Environ Microbiol* 74:7059–7063. doi:10.1128/AEM.01330-08
- Durai S, Pandian SK, Balamurugan K (2011) Establishment of a *Caenorhabditis elegans* infection model for *Vibrio alginolyticus*. *J Basic Microbiol* 51:243–252. doi:10.1002/jobm.201000303

19. Peleg AY, Jara S, Monga D, Eliopoulos GM, Moellering RC Jr, Mylonakis E (2009) *Galleria mellonella* as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. *Antimicrob Agents Chemother* 53:2605–2609. doi:[10.1128/AAC.01533-08](https://doi.org/10.1128/AAC.01533-08)
20. Senior NJ, Bagnall MC, Champion OL, Reynolds SE, La Ragione RM, Woodward MJ, Salguero FJ, Titball RW (2011) *Galleria mellonella* as an infection model for *Campylobacter jejuni* virulence. *J Med Microbiol* 60:661–669. doi:[10.1099/jmm.0.026658-0](https://doi.org/10.1099/jmm.0.026658-0)
21. Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR (1983) Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Appl Environ Microbiol* 45:275–283
22. Alam M, Sultana M, Nair GB, Siddique AK, Hasan NA, Sack RB, Sack DA, Ahmed KU, Sadique A, Watanabe H, Grim CJ, Huq A, Colwell RR (2007) Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proc Natl Acad Sci USA* 104:17801–17806. doi:[10.1073/pnas.0705599104](https://doi.org/10.1073/pnas.0705599104)
23. Colwell RR, Huq A (1994) Environmental reservoir of *Vibrio cholerae*. The causative agent of cholera. *Ann N Y Acad Sci* 740:44–54. doi:[10.1111/j.1749-6632.1994.tb19852.x](https://doi.org/10.1111/j.1749-6632.1994.tb19852.x)
24. Muic V, Ljubcic M, Vodopija I, Mayer V (1999) Basing a selective method for isolating environmental *Vibrio cholerae* on differences in the growth rate of competing *Vibrio metschnikovii*. *Veterinarski Arhiv* 69:125–134
25. Li Y, Cole K, Altman S (2003) The effect of a single, temperature-sensitive mutation on global gene expression in *Escherichia coli*. *RNA* 9:518–532. doi:[10.1261/rna.2198203](https://doi.org/10.1261/rna.2198203)
26. Cockburn TA, Cassanos JG (1960) Epidemiology of endemic cholera. *Public Health Rep* 75:791–803
27. Huq A, West PA, Small EB, Huq MI, Colwell RR (1984) Influence of water temperature, salinity, and pH on survival and growth of toxigenic *Vibrio cholerae* serovar O1 associated with live copepods in laboratory microcosms. *Appl Environ Microbiol* 48:420–424
28. Blankenhorn D, Phillips J, Slonczewski JL (1999) Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J Bacteriol* 181:2209–2216
29. Yohannes E, Barnhart DM, Slonczewski JL (2004) pH-dependent catabolic protein expression during anaerobic growth of *Escherichia coli* K-12. *J Bacteriol* 186:192–199. doi:[10.1128/JB.186.1.192-199.2004](https://doi.org/10.1128/JB.186.1.192-199.2004)
30. Lyon WJ (2001) TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. *Appl Environ Microbiol* 67:4685–4693. doi:[10.1128/AEM.67.10.4685-4693.2001](https://doi.org/10.1128/AEM.67.10.4685-4693.2001)
31. Lee J, Attila C, Cirillo SL, Cirillo JD, Wood TK (2009) Indole and 7-hydroxyindole diminish *Pseudomonas aeruginosa* virulence. *Microb Biotechnol* 2:75–90. doi:[10.1111/j.1751-7915.2008.00061.x](https://doi.org/10.1111/j.1751-7915.2008.00061.x)
32. Molina-Santiago C, Daddaoua A, Fillet S, Duque E, Ramos JL (2014) Interspecies signalling: *Pseudomonas putida* efflux pump TtgGHI is activated by indole to increase antibiotic resistance. *Environ Microbiol* 16:1267–1281. doi:[10.1111/1462-2920.12368](https://doi.org/10.1111/1462-2920.12368)
33. Nikaido E, Giraud E, Baucheron S, Yamasaki S, Wiedemann A, Okamoto K, Takagi T, Yamaguchi A, Cloeckaert A, Nishino K (2012) Effects of indole on drug resistance and virulence of *Salmonella enterica* serovar Typhimurium revealed by genome-wide analyses. *Gut Pathog* 4:5. doi:[10.1186/1757-4749-4-5](https://doi.org/10.1186/1757-4749-4-5)
34. Martin K, Morlin G, Smith A, Nordyke A, Eisenstark A, Golomb M (1998) The tryptophanase gene cluster of *Haemophilus influenzae* type b: evidence for horizontal gene transfer. *J Bacteriol* 180:107–118
35. Hirakawa H, Kodama T, Takumi-Kobayashi A, Honda T, Yamaguchi A (2009) Secreted indole serves as a signal for expression of type III secretion system translocators in enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiology* 155:541–550. doi:[10.1099/mic.0.020420-0](https://doi.org/10.1099/mic.0.020420-0)
36. Dimroth P (1997) Primary sodium ion translocating enzymes. *Biochim Biophys Acta* 1318:11–51. doi:[10.1016/S0005-2728\(96\)00127-2](https://doi.org/10.1016/S0005-2728(96)00127-2)
37. Balsera M, Buey RM, Li XD (2011) Quaternary structure of the oxaloacetate decarboxylase membrane complex and mechanistic relationships to pyruvate carboxylases. *J Biol Chem* 286:9457–9467. doi:[10.1074/jbc.M110.197442](https://doi.org/10.1074/jbc.M110.197442)
38. Hase CC, Mekalanos JJ (1999) Effects of changes in membrane sodium flux on virulence gene expression in *Vibrio cholerae*. *Proc Natl Acad Sci USA* 96:3183–3187. doi:[10.1073/pnas.96.6.3183](https://doi.org/10.1073/pnas.96.6.3183)
39. Minato Y, Fassio SR, Wolfe AJ, Hase CC (2013) Central metabolism controls transcription of a virulence gene regulator in *Vibrio cholerae*. *Microbiology* 159:792–802. doi:[10.1099/mic.0.064865-0](https://doi.org/10.1099/mic.0.064865-0)
40. Snell EE (1975) Tryptophanase: structure, catalytic activities, and mechanism of action. *Adv Enzymol Relat Areas Mol Biol* 42:287–333. doi:[10.1002/9780470122877.ch6](https://doi.org/10.1002/9780470122877.ch6)
41. Kalia VC (2013) Quorum sensing inhibitors: an overview. *Biotechnol Adv* 31:224–245. doi:[10.1016/j.biotechadv.2012.10.00](https://doi.org/10.1016/j.biotechadv.2012.10.00)
42. Koul S, Prakash J, Mishra A, Kalia VC (2016) Potential emergence of multi-quorum sensing inhibitor resistant (MQSIR) bacteria. *Indian J Microbiol* 56:1–18. doi:[10.1007/s12088-015-0558-0](https://doi.org/10.1007/s12088-015-0558-0)
43. Kalia VC, Purohit HJ (2011) Quenching the quorum sensing system: potential antibacterial drug targets. *Crit Rev Microbiol* 37:121–140. doi:[10.3109/1040841X.2010.532479](https://doi.org/10.3109/1040841X.2010.532479)