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Original Article

Altered virulence of non-indole-producing pathogenic bacteria by indole signaling

Paramaporn Rattanaphan[1, 2], Thunchanok Yaikhan[1, 2], Pawina Rachniyom[2], Pimonsri Mittraparp-Arthorn[1], Varaporn Vuddhakul[1], and Natta Tansila[2*]

[1] *Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

[2] *Faculty of Medical Technology, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

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Abstract

Bacteria naturally live in a multispecies community. Cell-to-cell communication is therefore crucial for bacteria to adapt, survive, and regulate virulence. Indole has been recently reported as an intercellular signal that regulates diverse bacterial physiologies such as biofilm formation, antibiotic tolerance, and virulence factor production. The role of indole on bacterial virulence was mainly studied in indole-producing bacteria. Therefore, this study aimed to study the effect of indole on pathogenic non-indole-producing bacteria including *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterobacter cloacae*. Bacteria were cultured in a physiological concentration of indole (1 mM), and then growth, biofilm formation, autoaggregation, and motility were evaluated. Indole could increase biofilm formation and autoaggregation of *K. pneumoniae*, whereas biofilm formation and autoaggregation decreased in *E. cloacae* and *P. mirabilis*. Growth of *K. pneumoniae* and *E. cloacae* was slightly affected by exposure to indole. This finding indicated that indole influences the virulence of pathogenic non-indole-producing bacteria in different ways. Further studies are still required for a better understanding of the biological functions of indole signaling.

Keywords: indole, biofilm formation, virulence, non-indole-producing bacteria, signaling molecule

1. Introduction

Bacteria reside in a complex community. Various genera and species of organisms naturally live together in both supportive and competitive conditions. Consequently, bacterial communication via a signaling molecule is essential for survival, space, nutrients, and virulence. Indole signaling has increasingly been of interest as a new class of signaling molecule for communication and adaptation within the microbial community (Lee & Lee, 2010). In addition to using indole as a biochemical test for bacterial identification, many studies have reported that indole regulates biofilm formation (Lee, Jayaraman, & Wood, 2007), antibiotic tolerance (Lee, Molla, Cantor, & Collins, 2010), virulence factor production (Hirakawa, Kodama, Takumi-Kobayashi, Honda, & Yama guchi, 2009), plasmid stability (Chant & Summers, 2007), growth, and cell division (Hu, Zhang, Mu, Shen, & Feng, 2010). Tryptophanase encoded by *tnaA* gene degrades tryptophan amino acid into indole, pyruvate, and ammonia. More than 80 bacterial species have been reported as indole producers such as *Escherichia coli* (Lee & Lee, 2010) and *Vibrio cholerae* in which the functional role of indole on bacterial physiology has been primarily studied (Nuidate *et al.*, 2016). In fact, indole signaling may also affect other non-indole-producing species coexisting in a microbial community, e.g., gastrointestinal (GI) tract. It has been reported that the physiological concentration of indole in the human GI tract is approximately 0.5–1.0 mM (Bansal, Alaniz, Wood, & Jayaraman, 2010). Non-indole producers are capable of

*Corresponding author
Email address: natta.t@psu.ac.th

encountering this substantial amount of indole by means of several monooxygenases or dioxygenases (Lee & Lee, 2010) and may use this signal molecule to change their physiological activities (Lee, Wood, & Lee, 2015). So far, however, a potential receptor of indole signaling in microorganisms has not been identified (Lee & Lee, 2010; Kim & Park, 2015). To better understand the functional role, this study aims to investigate the effect of indole on the virulence of pathogenic non-indole-producing bacteria including *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterobacter cloacae* which are naturally exposed to indole in the human digestive tract. Four phenotypic tests were assessed: growth, biofilm formation, autoaggregation, and motility. We aimed to clarify the role of indole as a signaling molecule on enteric pathogens which may lead to discovering or designing new antivirulence agents against pathogenic bacteria.

2. Materials and Methods

2.1 Chemicals, bacteria, and culture preparation

Clinical isolates of *K. pneumoniae*, *P. mirabilis*, and *E. cloacae* were used in this study. Strains were recovered from stock cultures (stored at −20 ºC in Luria Bertani (LB) broth with 25% glycerol) and the correct bacterial identifications were checked by biochemical tests prior to starting the experiments. All bacterial isolates were grown in LB broth and incubated at 35 ºC for 18−24 h unless stated otherwise. Indole was obtained from Sigma-Aldrich.

2.2 Indole toxicity testing and effect of indole on bacterial growth

Overnight cultures were adjusted to 0.5 McFarland in LB broth and cultured with or without indole at final concentrations of 0.01, 0.1, and 1.0 mM in 0.2% methanol (MeOH) to assess the toxicity to the tested bacteria. Incubation was at 35 ºC for 18 h. Bacterial growth was evaluated by measuring the optical density at 600 nm (PowerWave™ XS Microplate Reader, USA). A suitable indole concentration for other experiments was determined as the concentration that did not reduce the growth ability of the bacteria. The bacterial cultures were adjusted to 0.5 McFarland and cultured with or without 1.0 mM indole in 0.2% MeOH at 35 ºC to observe the effect on growth. Cell turbidity was monitored by measuring the optical density (OD) at 600 nm (OD600). The measurements were recorded at different time intervals until 36 h by PowerWave™ XS Microplate Reader. Cells treated with 0.2% MeOH were used as a vehicle control. Growth curves were created from a triplicate experiment.

2.3 Effect of indole on biofilm production

This assay was adapted from (Han, Lee, Cho, Wood & Lee, 2011). After adjusting to 0.5 McFarland, the bacteria were cultured in a 96-microtiter plate at 35 ºC with or without 1.0 mM indole in 0.2% MeOH for up to 48 h. The OD at 600 nm was then measured to evaluate the bacterial growth using the PowerWave™ XS Microplate Reader. A vehicle control was always performed by treating the cells with 0.2% MeOH. After that the planktonic cells were removed by pouring and washed at least three times by distilled water. The plate was dried at room temperature for 30 min and the biofilm was then stained by addition of 1.0% crystal violet (CV). The liquid was discarded and unbound CV was removed by washing with distilled water until transparent liquid was visually observed. The biofilm CV was solubilized in 95% ethanol for 30 min, transferred to new microtiter plate and the absorbance was measured at 595 nm (OD595 nm). The amount of biofilm formation was calculated by OD595 nm/OD600 nm to normalize the bacterial turbidity. All results were obtained from an average of at least 6 replicates.

2.4 Motility assay

The effect of indole on both swimming and swarming motility was evaluated. In brief, the bacteria cultures were adjusted to 0.5 McFarland. The cell suspension was stabbed into an LB plate with 0.4% or 1.0% agar for swimming or swarming motility assay, respectively, in the absence or presence of 1 mM indole supplementation. The bacteria were incubated at 35 ºC for 24–48 h, and the diameter of bacterial motility was measured. The mean (SD) from a triplicate experiment was then calculated. A vehicle control was always performed by adding 0.2% MeOH to an LB agar instead of indole solution.

2.5 Autoaggregation assay

Bacterial autoaggregation was performed as previously reported with a slight modification (Sorroche, Spesia, Zorreguieta, & Giordano, 2012). The bacterial cultures were adjusted to 0.5 McFarland and then cultured in the absence or presence of 1 mM indole in 0.2% MeOH at 35 ºC for 24 h. Five milliliters of bacterial solutions were transferred to a new centrifuge tube and allowed sedimentation at 4 ºC for 24 h. Two hundred microliters of the upper phase were taken to determine the absorbance at 600 nm (OD_{final}). The remaining cell suspension was vortexed vigorously for 30 s and then subjected to OD600 nm measurement ($OD_{initial}$). The autoaggregation percentage was calculated using this formula: $100 \times [1 - (OD_{final}/OD_{initial})]$. Cells treated with 0.2% MeOH were used as a vehicle control. Values were an average of a triplicate experiment.

2.6 Statistical analysis

Significant differences of each phenotypic difference between the indole- and MeOH-treated cultures were determined by paired samples *t*-test. A *p*-value <0.05 was considered significant.

3. Results and Discussion

Non-indole-producing enteric pathogens may be exposed to indole when they reach animal intestines due the presence of indole-producing gut microbiota, especially *E. coli* (Lee & Lee, 2010; Lee, Wood, & Lee, 2015). Alteration of the virulence factors and pathogenicity may be anticipated through indole signaling. However, indole toxicity has been reported which may be attributed to cell membrane damage and inhibition of cell growth and division especially at a high concentration (> 2 mM) (Chant & Summers, 2007; Garbe, Kobayashi, & Yukawa, 2000; Lee, Maeda, Hong, & Wood,

2009). In this work, three indole concentrations (0.01, 0.1, and 1.0 mM) were tested for toxicity and the results found that none were toxic to *K. pneumoniae, P. mirabilis*, or *E. cloacae* (data not shown). Therefore, all further experiments were done at an indole concentration of 1.0 mM which resembles the physiological concentration found in the human GI tract (Bansal, Alaniz, Wood, & Jayaraman, 2010; Karlin, Mastro marino, Jones, Stroehlein, & Lorentz, 1985). No significant differences in the growth of any of the bacteria were found following indole exposure compared with those of control at 30 to 36 h (Figure 1). Indole treatment slightly decreased the growth of *K. pneumoniae* and *E. cloacae* during log phase as demonstrated in Figures 1A and 1B, respectively. Nevertheless all tested bacteria reached a stationary phase at 24 or 30 h regardless of the presence of indole.

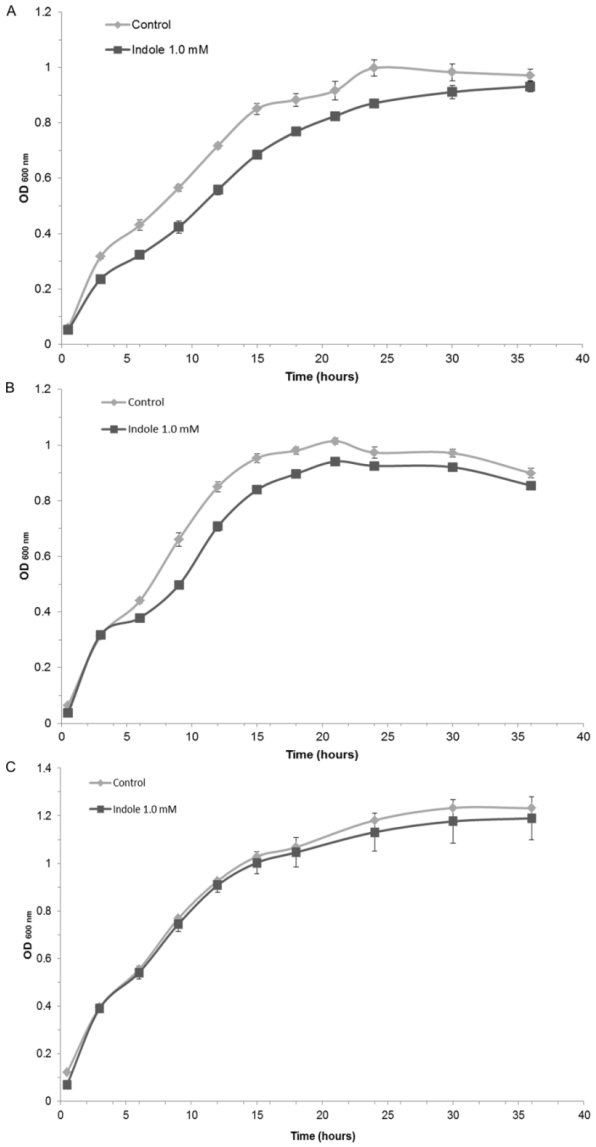

Figure 1. Effect of indole on growth of *K. pneumoniae* (A), *E. cloacae* (B), and *P. mirabilis* (C). The mean (SD) values of triplicate individual experiments are shown.

Biofilm formation is one of the most studied virulence factors regulated by indole signaling in various microorganisms (Lee & Lee, 2010; Lee, Wood, & Lee, 2015). Each enteric pathogen responded differently in biofilm formation to indole exposure (Figure 2). Compared with the control, biofilm production was significantly lower (p <0.05) in *E. cloacae* and *P. mirabilis*, while higher (p <0.05) in indole-treated *K. pneumoniae* (Figure 2). The bacteria initiated biofilm formation when an appropriate cell density was reached and subsequently executed several steps including cell aggregation, surface adherence, production of extracellular polymeric substance (EPS), cell proliferation, and biofilm maturation in order to obtain a complete biofilm community. After nutrients become limited and the excreted toxic waste products accumulate, bacterial cells detach from the biofilm, disperse, and initiate biofilm formation again elsewhere (Joo & Otto, 2012; Koo, Allan, Howlin, Stoodley, & Hall-Stoodley, 2017). Biofilm development is considerably complex and involves several regulatory and signaling systems (Jakobsen, Tolker-Nielsen, & Givskov, 2017; Solano, Echeverz, & Lasa, 2014). The role of a particular signal molecule may vary by function as well as microorganisms. Similar to *E. cloacae* and *P. mirabilis* in this work, indole was found to decrease biofilm formation in *E. coli* by lowering the motility, chemotaxis, and cell attachment (Bansal *et al.*, 2007; Domka, Lee, & Wood, 2006; Lee, Jayaraman, & Wood, 2007) in indole-producing *Vibrio anguillarum* and *V. campbellii* by reducing exopolysaccharide production and motility (Li, Yang, Dierckens, Milton, & Defoirdt, 2014; Yang *et al.*, 2017), and in non-indole-producing *Acinetobacter oleivorans* by inhibiting quorum sensing (QS) (Kim & Park, 2013). On the other hand, the induction of biofilm formation by indole was reported in *V. cholerae* (Mueller, Beyhan, Saini, Yildiz, & Bartlett, 2009; Nuidate *et al.*, 2016) and *P. aeruginosa* (Lee, Attila, Cirillo, Cirillo, & Wood, 2009). *Vibrio* polysaccharide (VPS) genes, responsible for production of *V. cholerae* biofilm, are up-regulated in indole-treated cells could antagonize the biofilm-deteriorating effect of

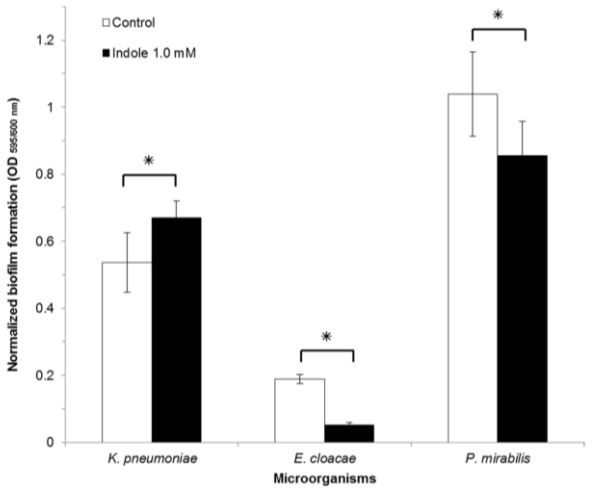

Figure 2. Effect of indole on biofilm formation. The mean and SD values of at least 6 replicates of individual experiments are shown. The statistical difference between the indole-treated cultures and control was calculated by paired samples t-test (p <0.05) is shown as asterisk (*).

anthranilate (degradation products of tryptophan metabolism), and also slightly activate genes involved in fimbria and pili in *P. aeruginosa* which may contribute to increased cell attachment, motility, or biofilm formation (Kim, Park, & Lee, 2015; Lee, Attila, Cirillo, Cirillo, & Wood, 2009). Various indole effects on the bacterial biofilm in our study possibly resulted from a different mechanism of indole action in each type of bacteria as described previously.

The influence of indole exposure to bacterial auto-aggregation, one of the early steps in biofilm formation, was also examined. Only *K. pneumoniae* had a statistically significant increase in cell aggregation compared to the control (p <0.05) (Figure 3). This finding is probably attributable to the increased biofilm formation induced by indole in this bacterium (Figure 2). Enhanced autoaggregation of bacteria seems to facilitate their biofilm initiation as a result of increased EPS synthesis (Dorken, Ferguson, French, & Poon, 2012; Sorroche, Spesia, Zorreguieta, & Giordano, 2012). This consequently elevates cell stickiness and surface conditioning leading to their self-aggregation and adhesion to surfaces (Deziel, Comeau, & Villemur, 2001; Kragh *et al.*, 2016; Sorroche, Spesia, Zorreguieta, & Giordano, 2012). Interestingly, the functional role of indole in bacterial aggregation was previously investigated in *Pantoea agglomerans*, which is an endophytic bacterium isolated from rice (Yu *et al.*, 2016). Furthermore, swimming and swarming motility after indole exposure were assessed. However, no significant differences in the motility in all tested bacteria were observed (Table 1). It can be postulated that the suppressing activity on biofilm formation in *E. cloacae* and *P. mirabilis* was unlikely due to the effect of indole on autoaggregation and motility. However, the effect of indole signaling to EPS production, adherence to an abiotic surface, or other QS regulation cannot be excluded. A detailed investigation including the molecular mechanism of action as well as potential receptors of indole is therefore needed.

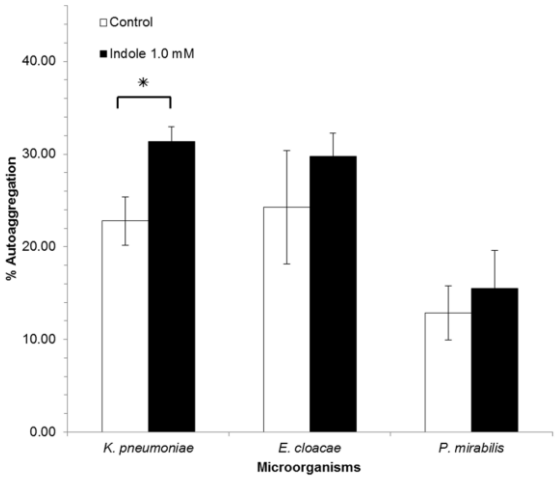

Figure 3. Effect of indole on autoaggregation. The mean and SD values of triplicate individual experiments are shown. The statistical difference between the indole-treated cultures and control was calculated by paired samples t-test (p <0.05) is shown as asterisk (*).

4. Conclusions

All findings elucidate the different responses of each pathogenic non-indole-producing bacterium towards this novel signaling molecule. Further studies are still required for a better understanding of the diverse roles of indole in the bacterial community consisting of both indole-producing and non-indole-producing bacteria. This knowledge will be helpful for the discovery or design of new anti-virulence therapeutics against bacterial pathogens.

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Table 1. Effect of indole on bacterial motility.

Microorganisms	Swarming motility[a]			Swimming motility[a]		
	Control (mm)	Indole 1 mM (mm)	p -value	Control (mm)	Indole 1 mM (mm)	p -value
Klebsiella pneumoniae	11.25±0.48	10.88±0.25	0.2282	2.83±0.58	2.25±0.65	0.2722
Enterobacter cloacae	13.00±2.16	11.63±0.48	0.2603	≥60	≥60	ND
Proteus mirabilis	≥60	≥60	ND	≥60	≥60	ND

[a] Values are expressed as mean±standard deviation (SD) of three independent replicates. ND=not determined.

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