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REVIEW ARTICLE

Indole Affects Biofilm Formation in Bacteria

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Abstract Biofilm is bacterial population adherent to each other and to surfaces or interfaces, often enclosed by a matrix. Various biomolecules contribute to the establishment of biofilms, yet the process of building a biofilm is still under active investigation. Indole is known as a metabolite of amino acid tryptophan, which, however, has recently been proved to participate in various aspects of bacterial life including virulence induction, cell cycle regulation, acid resistance, and especially, signaling biofilm formation. Moreover, indole is also proposed to be a novel signal involved in quorum sensing, a bacterial cooperation behavior sometimes concerning the biofilm formation. Here the signaling role and molecular mechanism of indole on bacterial biofilm formation are reviewed, as well discussed is its relation to bacterial living adaptivity.

Keywords Indole · Biofilm · Bacterial signal · Quorum sensing

Introduction

Bacteria do not always adopt planktonic lifestyle, instead they usually aggregate in clusters or attach to surfaces, forming what is called "biofilm". Then, it is natural to ask how and when bacteria decide to build a biofilm. As it is required in collective society, bacteria should have their own "languages". Although studies on quorum sensing system have provided a lot instructive information [1, 2], it is still unable to draw a clear picture of bacterial biofilm constitution at present. Nevertheless, it has been recently revealed that indole, which had been useful only for bacterial taxonomy and clinical diagnoses during the past over 100 years [3], plays real biological roles as a signal molecule participating in biofilm formation and multiple physiological pathways in many bacteria. In this paper, we reviewed those researching advancements, summarized the factors involved in this signal process, and finally discussed the possible regulatory mechanism of indole on biofilm formation.

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Indole is a Bacterial Signal

Indole is widespread in the natural environment. By far, at least 85 bacterial species have been shown to produce large quantities of this molecule, including both Gram-positive and Gram-negative bacteria of which many are pathogens [3]. So it is intriguing to understand why so many bacteria produce indole and what the functions are.

Indole Biosynthesis and Influencing Factors

For indole-producing bacteria like *Escherichia coli*, the primary resource for indole production is an amino acid tryptophan: an enzyme tryptophanase encoded by gene *tnaA* reduces tryptophan to pyruvate, ammonia, and indole in a reversible reaction. The tryptophan pathway is participated and controlled by many other genes including *aroP*, *tnaB* (encoding permeases for transporting tryptophan into or outside a cell), *trpE* (encoding anthranilate synthase component I), *tnaC* (encoding the tryptophanase leader peptide), *trpL* (encoding *Trp* operon leader peptide),

acrEF (encoding multidrug transporter for indole efflux) and *mtr* (encoding permease to transport indole into the cell). Furthermore, carbon sources like glucose can repress *tnaA* expression [4], which is reasonable considering the first choice of bacterial catabolism should be carbohydrates rather than amino acids.

Besides, some environmental factors like temperature and pH also can affect indole biosynthesis in *E. coli*. For example, gene expression of *tnaAB* can be induced when the temperature shifting from 30 to 43°C, but cells lost indole biosynthesis ability at 44.5°C [3]; the effect of indole signaling is more significant at a lower temperature (30°C) rather than 37°C, which is different to the quorum sensing signal AI-2 [5]; and a low pH can inhibit indole production, while TnaA is among the most induced proteins at pH 9.0 [3].

Physiological Functions of Indole

It is recently observed that indole means more than a metabolite of amino acid, and influences bacteria in many ways [3].

Firstly, it is related to bacterial toxicity, as well as drug defense. On the one hand, indole promotes bacterial excretion of toxins. Anyanful et al. noticed that the toxicity of Enteropathogenic *E. coli* (EPEC) toward *Caenorhabditis elegans* does not require direct contact, but surprisingly, this virulent effect relies on the existence of amino acid tryptophan, as lack of tryptophan in growth media or deletion of tryptophanase gene deprives its toxicity [6]. Either complementing the enzyme tryptophanase or co-cultivating EPEC with another tryptophanase expression strain restores its virulence. In this case, certain metabolite of tryptophan or tryptophan itself should regulate toxin production. Moreover, Hirakawa et al. demonstrated that in enterohaemorrhagic *E. coli* (EHEC) O157:H7, indole serves to signal EspAB expression/secretion and stimulates the ability of EHEC to form A/E lesions in HeLa cells [7]. On the other hand, indole improves bacterial drug resistance. It was found that indole induces the expression of multidrug exporter genes like *acrD*, *acrE*, *cusB*, *emrK*, *mdtA*, *mdtE* and *yceL* [8], which protect the bacteria from extracellular toxic substances.

Secondly, indole has a role in maintaining genetic stability. For instance, it contributes to the maintenance of plasmids copies [9]. In rapidly replicating cells, plasmid multimer may disrupt steady heredity. In order to counter this challenge, bacteria like *E. coli* adopt a multimer resolution system; in which cell division is delayed until all the plasmid multimers are resolved. In another study, tryptophanase is surprisingly found as a binding protein in the Xer-*cer* multimer resolution system. In the system, dimers of plasmid ColE1 encode a sRNA that interacts directly with tryptophanase and enhances its affinity to tryptophan. The breakdown product indole then arrests cell division until the plasmid dimers are resolved to monomers [10]. This mechanism is further confirmed by the observation that adding 4 mM indole to *E. coli* culture stopped cell division. It is tempting to suggest that indole is a far-ranging signal busily participating in various activities from virulence expression, metabolic feedback control, to cell cycle regulation.

Thirdly, indole is an active signal in metabolic control. Indole can activate genes involved in amino acids metabolism, including *cysK* (encoding cysteine synthase), *astD* (encoding succinylglutamic semialdehyde dehydrogenase), *gabT* (encoding 4-aminobutyrate aminotransferase), as well as aforementioned *tnaB* [11]. However, the mechanism by which indole regulates these genes is unknown. As there has been no direct evidence that *E. coli* produces *N*-acyl homoserine lactones (AHLs), Wang et al. proposed that *E. coli* may have evolved to utilize metabolites like indole instead [11]. One advantage of signaling via the accumulation of amino acid metabolites is that bacteria can adjust its activity in response to changing nutritional conditions. Wang et al. also suggest that for as much as the ability to catabolize amino acids is an important index of bacteria to persist in stationary phase, signaling by indole prepares the cells for a nutrition-poor environment where the catabolism of amino acid becomes an important energy source.

Additionally, indole is a chemo-repellent and decreases motility [3], while AI-2 is chemo-attractant in *E. coli* [12]. Englert et al. developed a microfluidic flow-based device to investigate interactions between different bacterial chemo-effectors. They found that attraction to AI-2 can overcome repulsion by indole in equal, competing gradients. So the interactions between these two concentration-dependent signals may be important determinants of the extent of bacterial colonization [12].

Indole Affects Biofilm Formation

The mechanism of bacterial biofilm formation has been investigated comprehensively since its first description as early as in 1936 [13]. Scientists are dedicated in discovering modulate signals and molecules [14–16], dissecting the proteins involved in the process [17], and searching for the mechanism used by biofilm on resistance against unfriendly environments [18], among which the roles of indole are seemed to be more and more outstanding.

Regulators Involved in the Signaling Pathway

During constant efforts searching for alternative quorum sensing signals apart from AHLs in the model organism

E. coli, a protein SdiA was discovered to detect the AHLs signal produced by other bacterial species [19, 20]. SdiA which belongs to the LuxR family is a transcriptional regulator of *ftsQ2p* gene involved in cell division. Surprisingly, it has been found that indole also interacts with SdiA either directly or through some intermediates, and regulates SdiA-mediated transcription in the absence of AHLs [21]. For example, it is measured that addition of indole to medium decreased the acid resistance and reduced the biofilm mass in wild-type *E. coli* but had no effect on the *sdiA* mutant. Addition of 1 mM indole to *E. coli* UT481/pCX39 (*ftsQ2p::lacZ*) led to about 33% decrease in *ftsQ2p* expression, suggesting indole affects SdiA-mediated transcription.

Besides, two proteins named YliH and YceP were found to regulate indole transport, and then affect biofilm formation. It has been reported that the deletion of *yliH* and *yceP* in *E. coli* greatly enhanced biofilm formation [22]. The increased biofilm formation in *yliH* and *yceP* mutants paralleled the reduction in extracellular indole concentration, while addition of indole into the culture restores the wild-type biofilm phenotype. Interestingly, indole transporters (Mtr and AcrEF, for indole importation and exportation, respectively) are differently expressed in *yliH* and *yceP* mutants. This information implies that YliH and YceP repress biofilm formation by controlling the extracellular indole concentration via regulation on the indole transporters.

Stress-Response Genes Regulated by Indole

Biofilm formation is significant for bacterial stress-resistance, where indole exhibits multiple regulating effects on the stress-related genes. YmgB (renamed as AriR), a dimeric protein structurally homologous to the gene regulatory protein Hha in *E. coli*, plays an important role in biofilm formation and acid resistance responding to AI-2 or indole [23]. It is evaluated that 2 mM supplementary indole decreased acid survival (by 350- to 650-fold) of wild-type *E. coli* K12, while addition of the same concentration of indole to the *ymgB* mutant did not appreciably change acid survival (1.7-fold decrease) [24], suggesting that indole affects acid resistance ability of *E. coli* through YmgB. Likewise, YmgB represses biofilm formation in rich medium containing glucose, decreases motility, and protects cell from acid.

The relationship between indole-repressed biofilm formation and stress conditions has also been observed to be associated with another protein YcfR. *E. coli* YcfR has ability to render cells more resistant to antimicrobial agents [25]. Deletion of *ycfR* made the cells more sensitive to environmental stresses like heat, H_2O_2, low pH, and cadmium. Noticeably, *ycfR* mutant exhibits a decreased indole concentration and enhanced biofilm formation ability. Zhang et al. suggest that it is *ycfR* mediates the stress response in *E. coli* through a pathway inducing indole synthesis and repressing biofilm formation, while the stress itself increases bacterial biofilm formation.

Moreover, Kuczyńska-Wisnik et al. demonstrated that the mutant strain of *ibpAB* operon (among the stress-response genes most induced during growth of the *E. coli* biofilm) experiences endogenous oxidative stress, leading to increased expression of tryptophanase (i.e., increased indole production) and then delayed biofilm formation [26]. And the study from their lab further demonstrated that antibiotics which promote ROS formation inhibit development of *E. coli* biofilm in an indole-dependent way [27], which means that bacteria also use indole signal to modulate pathways dealing with the oxidative stress and result in biofilm repression. So it is interesting to ask: why does *E. coli* use indole to render cells more resistant to stress yet to repress biofilm formation? We consider that maybe proteins induced by indole are able to ensure the bacterium to survival the stresses to a certain degree, and therefore biofilm (a relatively more expensive structure against more tough stresses) need not to be further developed.

Meanwhile, indole serves to activate drug efflux pumps and oxidative-stress protective mechanisms like we mentioned above as a bacterial altruistic byproduct. Lee et al. [28] followed a continuous culture of *E. coli* facing increasing levels of antibiotic and found that the vast majority of isolates are less resistant than the population as a whole. The few highly resistant mutants improve the survival of the population, in part by producing indole. And the indole signal further provides protection to other, more vulnerable, cells, thus enhance the survival capacity of the overall population in stressful environments. It is interesting to discuss the roles of indole and other signals through a community aspect, so maybe it's tempting to investigate more about the population dynamics during the formation of biofilms and co-biofilms.

Indole and Stationary-Phase Regulation

Bacterial survival in the stationary-phase is deeply related with energy metabolism and stress resistance. Stress response is largely regulated by stationary-phase sigma S factor (σ^s) encoded by *rpoS*, which is associated with indole [29]. RpoS has been reported to repress biofilm formation and to act as a positive regulator of more than 100 stationary-phase genes [30, 31]. Deletion of *rpoS* prevents the mature of biofilm by way of regulating bacterial energy metabolism, motility, and stress responses [32]. Interestingly, these phenotypes all have connection with indole signal. Moreover, RpoS promotes tryptophan degradation by inducing *tnaA*, resulting in the production of indole and stimulation of

WrbA (tryptophan (W) repressor-binding protein) expression to strengthen negative regulation of the *trp* biosynthetic operon [33]. Besides, indole alone can also induce WrbA expression inside biofilms [33]. In addition, a regulatory protein Crl can interact with the RpoS holoenzyme, by which the transcription initiation of RpoS-regulated genes is activated [34]. Cecile et al. found that several diffusible molecules in the bacterial transition stage between the exponential and stationary phases induce the premature expression of Crl, and that indole is one of these molecules.

In *E. coli* strain E44, which carries a loss-of-function mutation in the stationary-phase regulatory gene *rpoS*, IbeR is discovered as a novel regulator contributing to the stationary-phase regulation [35]. Study on a non-invasive *ibeR* deletion mutant BR2 surprisingly revealed that *tnaA*, which is regulated by RpoS in other *E. coli* strains, is down-regulated. Chi et al. demonstrated that TnaA is necessary for E44 invasion, while indole restores the non-invasive phenotype of the *tnaA* mutant. Furthermore, the production of indole is significantly reduced in BR2, indicating that *ibeR* is required for the indole production via *tnaA*.

Besides these two regulators, Hfq protein, which is initially identified as a bacterial host factor required for replication of bacteriophage Qβ RNA [36] and involved in the stabilization of sRNAs [37], is discovered to have a novel association with *tnaA* and indole. Hfq is critical for uropathogenic *E. coli* to form biofilms, colonize effectively, and persist in the urinary tract [38]. In both *E. coli* and *Pseudomonas aeruginosa* (*rpoS* independent), Hfq mutant strains exhibit growth defects, such as decreased growth rates or early transition into stationary phase [39]. This phenomenon reminds us the impact of indole in cell division. In fact, it is tested that the extracellular indole concentration increases in Hfq mutant cells and decreases in Hfq overexpression cells in a cell-density-dependent manner. The decreased extracellular indole level in Hfq overexpression cells results in postponement of entering into stationary-phase. The production of extracellular indole is through tryptophanase, while only at cell density reaching a relatively larger value, whose activity is affected by Hfq [40], demonstrating that indole plays an important role in the stationary-phase transition process.

The Dispute About Roles of Indole in Biofilm Formation

Those documents mentioned above suggest indole as an inhibitor for biofilm formation in many different kinds of bacteria. However, Martino et al. demonstrated that indole can enhance the biofilm formation in many different microorganisms carrying the tryptophanase gene [41]. It is corresponded by recent studies on *Vibrio cholerae*, which show indole activating genes involved in vibrio polysaccharide (VPS) production (essential for *V. cholerae* biofilm formation), influencing the expression of genes involved in motility, protozoan grazing resistance, iron utilization, and ion transport [42]. Exogenous tryptophan and indole are also suggested to increase *Fusobacterium nucleatum* biofilms [43]. Moreover, even *P. aeruginosa* that can not synthesize indole, is able to respond to this molecule (e.g., in diminishing virulence, enhancing biofilm formation and antibiotic resistance) and degrades it rapidly [44].

Things are more complex in *E. coli*. Indole-decreased biofilm formation was initially found in signal pathway, stress response, stationary-phase regulatory. However, Di Martino et al. [45] found that *tnaA* mutants show a decreased ability in biofilm formation, then, they applied the competitive inhibitor (oxindolyl-L-alanine) of tryptophanase to the wild-type bacterial cultural medium, and discovered that both indole production and biofilm formation are inhibited in a dose-dependent manner. However, supplementing the culture with indole to physiological concentrations restores their biofilm formation capacity [41]. In order to understanding this conflict, whole-transcriptome profiling of the *tnaA* mutant was performed and showed repression of seven motility genes, which may be the reason of biofilm down-regulation in *tnaA* mutant. The motility assay also suggested that different from wild-type and other indole-related genes mutant, adding 0.5 mM indole enhances cell motility for onefold in *tnaA* mutant, which may explain why supplementary indole restores biofilm formation [21]. Based on these results, *tnaA* seems to be important for biofilm formation and either too little or too much indole can abolish the ability to increase biofilm formation. Maybe, further researches are needed to investigate TnaA's own function to biofilm formation involved in other pathways besides indole production, and an X gene potentially exists which enhances motility responding to indole.

Besides the controversy in *E. coli*, indole does exhibit different impact in different kinds of microorganism. Mueller et al. suggested that the effects of indole may be strain specific [42, 46]. They discovered a variant of *V. cholerae* strain 92A1552, whose biofilm formation is not affected by deletion of the *tnaA* gene. Although the underlying reason is still unclear, a possible explanation provided by Mueller et al. is that despite motility-enhancement biofilm formation in *E. coli*, it is not required for biofilm development on glass or plastic in *V. cholerae* strain SIO. As indole mainly changes biofilm formation via motility, this character may account for the observed different regulated roles in biofilm formation in response to indole by these species.

Is Indole a New Quorum Sensing Signal?

There have been numerous breakthroughs in the area of quorum sensing, but AI-1 (AHLs or oligopeptide) or AI-2 don't account for all the members engaged in bacterial signaling. What's more, the model organism *E. coli* has not been found to produce any AHL, although it can recognize and respond to it via SdiA protein. *E. coli* has been found to produce AI-2, yet its function still remains unclear [47]. As a result, more molecules have been suggested as alternative quorum sensing signals, including epinephrine/norepinephrine, or indole, etc. [48].

There exists near regulatory relation between indole and other autoinducers. As discussed above, proteins YceP and YliH are associated with indole transport, at the same time *yceP* and *yliH* mutants have also been detected to differently express proteins that are engaged in AI-2 signaling [22]. Another example, *E. coli* SdiA is the sensor of AHLs produced by other bacteria, yet indole regulates SdiA-mediated transcription in the absence of AHLs, implying a competitive relationship between the two molecules. Other investigations have also showed an interconnection between indole and autoinducers, for instance indole derivant isatin represses AI-2 transporter [49].

In this case, how do we categorize the signaling roles of indole? Some hold the view that indole is an alternative quorum sensing signal in *E. coli*, whom included are Wang et al. [50], as well as Ren et al. [51]. In contrast, Martino et al. consider that indole is not a quorum-sensing signal molecule but rather a signaling molecule that may share some modes of action with quorum sensing systems [41]. We agree with the opinion proposed by Ahmer et al. that the response of certain genes to an extracellular factor like indole may be purely metabolic, but a quorum-sensing role still cannot be excluded. The labeling of indole as either a metabolic signal or a quorum sensing signal will probably depend on whether or not genes that play a role in coordinated behavior are found to be regulated by indole [47].

Indole Derivants Also Involve in Biofilm Formation

The molecule indole has been demonstrated to trigger the signaling cascade during biofilm formation, besides, it could still be further processed by bacteria to generate various derivants that might be involved in biofilm formations. For example, many bacterial oxygenases readily convert indole to oxidized compounds, like 2-hydroxyindole, 3-hydroxyindole, 4-hydroxyindole, isatin, indigo, isoindigo, and indirubin [52–54].

Lee et al. found that biofilm formation is decreased by hydroxyindoles (including 7-hydroxyindole and 5-hydroxyindole) while increased by isatin [49]. These derivants were supposed to either act as bacterial signals, or compete with indole, or promote indole in binding the signal receptor. In vivo signaling by indole derivants has been reported by Guan et al. too: an indole-based, indoxyl derivant QSM-1, from gypsy moth gut microbiota is able to induce quorum sensing [55]. Interestingly, the gypsy moth midgut is highly alkaline (pH ranging from 9 to 12), which reminds us the fact that alkalinization activates tryptophanase expression and dramatically induce indole production [56]. Perhaps in this gypsy moth microbiota, QSM-1, as a derivant of indole, signals biofilm formation.

Another promising indole derivant is indole-acetic acid (IAA), which has been well known as a phytohormone. Diverse bacterial strains produce IAA, especially those plant associated endophytes. The interaction between IAA-producing endophytes and plants may lead to certain effects on their association, such as pathogenesis and phyto-stimulation [57]. For example, we found that IAA-producing endophyte *Pantoea agglomeran* YS19 isolated from rice can aggregate to form biofilm-like structure symplasmata and influence the physiology of its host [58–60]. As IAA and indole are metabolically interconnected, it is possible that there is a crossover in their functions, too. This point has been confirmed by Bianco et al. that IAA-treated cells increased the biofilm formation by promoting the production of biofilm-forming matrices trehalose, lipopolysaccharide (LPS), and exopolysaccharide (EPS). Moreover, IAA triggers an increased tolerance to stress conditions (heat and cold shock, UV-irradiation, osmotic and acid shock and oxidative stress) and toxic compounds (antibiotics, detergents and dyes) [61]. A recent research on *Rhizobium etli* showed that IAA addition regulates genes involved in plant signal processing, motility and attachment to plant roots, which clearly demonstrating a distinct role for IAA in legume–*Rhizobium* interactions [62].

In fact, as indicated by Lee et al., signals in prokaryotes and eukaryotes, such as indole, indole-3-acetic acid, serotonin, melatonin, and epinephrine, all have indole-like chemical structures [49]. Hence, although highly speculative, it is intriguing to suggest whether indole is the archetypal hormone and has been integrated into the metabolism of eukaryotes [49]. If indole derivants like IAA and epinephrine etc. are confirmed to signal biofilm formation, it then could be a signal linkage between kingdoms: eukaryotes and prokaryotes utilize the same molecule to modulate the growth of each other in micro-ecological system.

Summary

In the inquiry about bacterial communication, multiple chemical substances have been identified and each has a

biological story to tell. Indole is a direct product of amino acid catabolism, signals in multidrug exportation, cell division inhibition, stresses resistance, and biofilm formation (this review). Underlying the building of biofilms, there are essentially a network of signal molecules and proteins. The effects of indole are probably highly dependent on the status of the members in this network, and a slight change in one factor (either biotic or abiotic) can set off a chain of events that eventually result in the fluctuation of indole concentrations, which in turn nicely fits the bacterial response to the changed factor. In the perspective of evolution, this kind of network can help bacteria readily and precisely respond to various environmental/biological changes and properly modulate the cells to adapt to the changes, thus achieving the ultimate goal of all organisms: survival.

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