

Indole Can Act as an Extracellular Signal in *Escherichia coli*

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Previous work has shown that *lacZ* fusions to the *cysK*, *astD*, *tnaB*, and *gabT* genes in *Escherichia coli* are activated by self-produced extracellular signals. Using a combination of ethyl acetate extraction, reversed-phase C₁₈ chromatography, and thin-layer chromatography, we have purified an extracellular activating signal from *E. coli* supernatants. Mass spectrometry revealed a molecule with an *m/z* peak of 117, consistent with indole. Nuclear magnetic resonance analysis of the purified *E. coli* factor and synthetic indole revealed identical profiles. Using synthetic indole, a dose-dependent activation was observed with *lacZ* fusions to the *gabT*, *astD*, and *tnaB* genes. However, *cysK::lacZ* and several control fusions were not significantly activated by indole. Conditioned medium prepared from a *tnaA* (tryptophanase) mutant, deficient in indole production, supported 26 to 41% lower activation of the *gabT* and *astD* fusions. The residual level of activation may be due to a second activating signal. Activation of the *tnaB::lacZ* fusion was reduced by greater than 70% in conditioned medium from a *tnaA* mutant.

The use of chemical signals for bacterial communication is a widespread phenomenon (10, 11, 20, 23, 33). In gram-negative bacteria, these signals can be *N*-acyl derivatives of homoserine lactone, cyclic dipeptides, and quinolones (3, 8, 17, 28–30, 43). In gram-positive bacteria, small peptides appear to be the predominant signal (7, 15, 16, 18, 25, 36). In some cases, small proteins can mediate signaling (22, 40). These signals regulate a variety of functions, including bioluminescence, differentiation, virulence, DNA transfer, and biofilm maturation (1, 2, 4, 5, 9, 19, 24, 27, 31, 32).

Indole production is a common diagnostic marker for the identification of *Escherichia coli* (37). Among the *Enterobacteriaceae*, indole is produced by *E. coli* and certain members of the *Proteaceae*, such as *Proteus vulgaris*, *Providencia* spp., and *Morganella* spp. (37). Indole is formed from tryptophan by the tryptophanase enzyme, encoded by the *tnaA* gene (35). At very high concentrations (5 mM), indole is toxic to *E. coli*, possibly by causing membrane changes that result in the generation of superoxide (12). However, the concentration at which indole is toxic is approximately 15-fold higher than the physiological concentration seen in stationary-phase supernatants of *E. coli* (see below). The efflux of indole from *E. coli* is mediated by the AcrEF pump, and *acrEF* mutants exhibit enhanced indole sensitivity (21). The primary pathway for indole transport into the cell is via the Mtr permease (42).

For *E. coli*, the role of cell-to-cell signaling in a variety of functions, including regulation of *ftsQAZ*, expression of type III secretion systems, inhibition of DNA replication, and activation of degradative pathways, has been described (1, 13, 34, 38, 39, 41). However, the extracellular signals involved in these processes are poorly understood. Previous studies from our lab have identified the *E. coli* genes *cysK*, *astD*, *tnaB*, and *gabT*,

which are activated by extracellular signals (1). We have utilized a *lacZ* fusion to one of these genes (*gabT*) as a biosensor to purify an activating signal from *E. coli* supernatants. Our data indicate that this signal is indole. In addition to the activation of *gabT*, indole is also capable of activating *lacZ* fusions to the *astD* and *tnaB* genes, indicating that it may affect a specific signaling pathway.

MATERIALS AND METHODS

Strains and growth conditions. A *luxS* mutant of *E. coli* strain DH5 α , obtained from B. Bassler, Princeton University, was used for the preparation of conditioned medium for signal purification. Strains MT9 (*cysK::lacZ*), MT48 (*astD::lacZ*), MT113 (*tnaB::lacZ*), and MT114 (*gabT::lacZ*) have been described previously (1). Strain TM1061 is an MC1061 derivative that contains a *tnaA::mini-Tn5* Cm null allele and is unable to produce indole. All strains were grown in 0.5 \times Luria broth (LB) at pH 7.5 for β -galactosidase assays by the method of Miller (26).

Bioassay conditions. Strain MT114 *gabT::lacZ* was used to monitor purification of the activating signal. Assays were conducted in 3 ml of 0.5 \times LB at pH 7.5, and mixtures were shaken at 280 rpm in 13- by 100-mm test tubes. Cultures were inoculated at a 1:1,000 dilution with a dilute overnight culture of MT114, and cells were harvested at an optical density at 600 nm (OD₆₀₀) of 0.35. This represented approximately 5 to 6 h of growth. The effects of indole were examined on MT9, MT48, MT113, and MT114 using the above conditions. Crude preparations of conditioned medium were prepared as described previously (1).

Signal purification. For factor purification, 900 ml of LB (three preparations of 300 ml each) was inoculated with a dilute suspension of log-phase DH5 α and allowed to shake overnight at 300 rpm. Cells were harvested at an *A*₆₀₀ of 1.5 and were pelleted by centrifugation at 4,300 \times g. The resulting supernatant was filter sterilized, and the pH was adjusted to 7.5. The supernatant was then sequentially extracted three times with 200 ml of ethyl acetate. The ethyl acetate phase was dried under a rotary evaporator at 40°C. The material was resuspended in 1 ml of ethyl acetate and loaded on a 5-g C₁₈ column (Waters Corp.). The column was washed sequentially with 20-ml portions of H₂O, 20% methanol, 50% methanol, 60% methanol, 80% methanol, and 100% methanol. The material in the 60% wash activated the *gabT::lacZ* fusion. The 60% methanol fraction was dried on a rotary evaporator and redissolved in 300 μ l of ethyl acetate. The resulting material was applied to a silica gel thin-layer chromatography plate and eluted with hexane-ethyl acetate (3:2). Six individual bands were typically observed, and each band was cut out. The resulting material from each band was eluted in ethyl acetate and tested for activity in the bioassay described above.

Structural analysis. The high-resolution electron impact mass spectrum was recorded on a KRATOS MS25FA spectrometer. For the activating material, an

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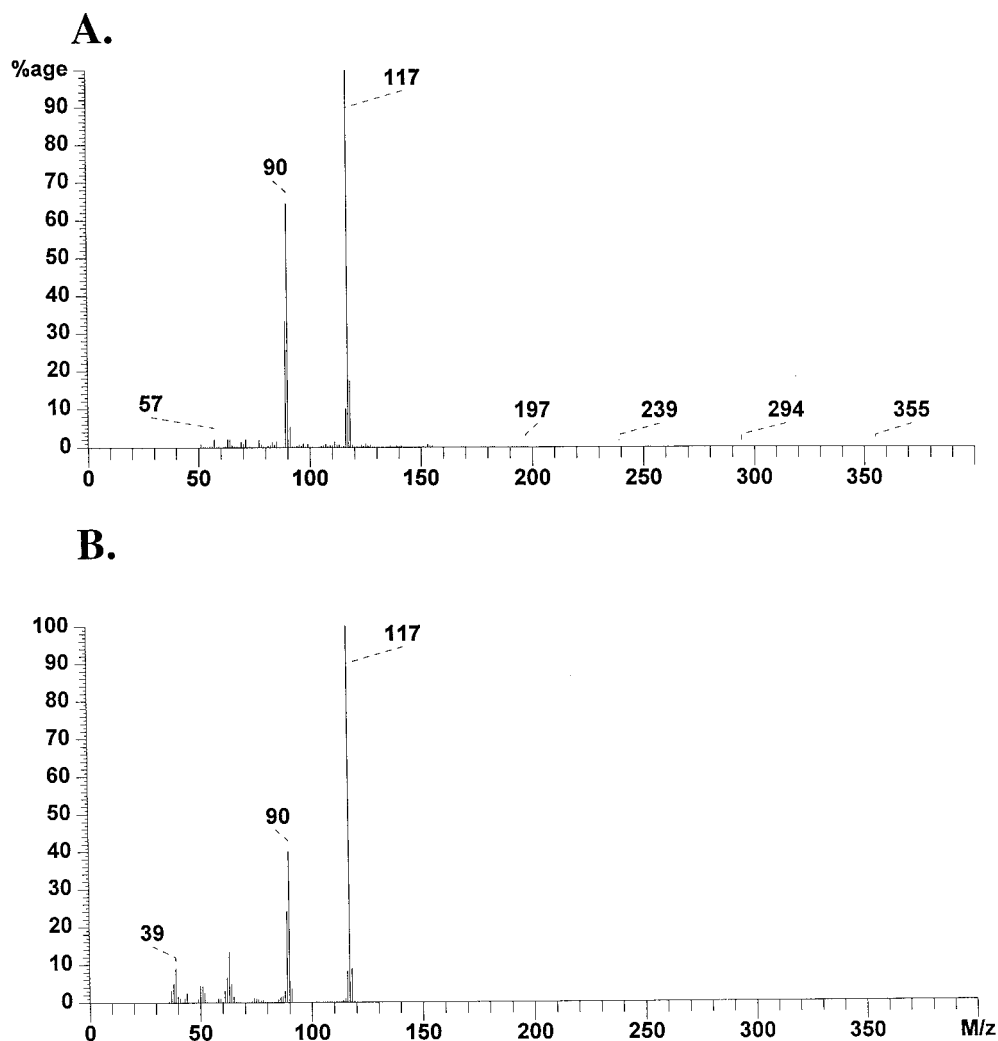


FIG. 1. Electron impact mass spectra of the activating fraction (A) and of synthetic indole (B). The m/z peaks were 117.0584 (C_8H_7N ; calculated, 117.0578) and 90.04767 ($C_7H_6-CHN^+$).

m/z of 117.0584 was observed, corresponding to C_8H_7N with a calculated value of 117.0578. A database search of the spectrum for the activating factor identified a match with indole. 1H nuclear magnetic resonance (NMR) spectra in $CDCl_3$ were recorded on a Varian 300-MHz spectrometer. The chemical shifts are reported in δ (parts per million). The 1H NMR spectra for the activating factor and synthetic indole (Aldrich Chemical Co. Inc.) were identical: 6.55 (m, 1H), 7.09 to 7.21 (m, 3H), 7.39 (d, J 8.1, 1H), 7.65 (d, J 7.8, 1H), 7.96 (br, s, 1H).

RESULTS

Purification of an extracellular signal that activates the *gabT::lacZ* fusion. Strain MT114 *gabT::lacZ* was used as a biosensor to purify an activating signal. Previous work indicated that the LuxS-dependent signal of *E. coli* was not involved in the activation of *gabT::lacZ* (1). Preliminary extractions of conditioned medium indicated that both chloroform and ethyl acetate were capable of extracting an activating signal. However, ethyl acetate extracts gave higher activity and were used for further experiments. The stage in growth for optimal factor production was also examined. Although activation of *gabT::lacZ* was observed using conditioned medium from cells

at mid-log phase (A_{600} of 0.5), conditioned medium prepared at an A_{600} of 1.5 gave the highest activity (data not shown).

To purify an activating signal, conditioned medium was prepared and extracted with ethyl acetate as described in Materials and Methods. The extract was applied to a reversed-phase C_{18} column and eluted with increasing concentrations of methanol. The material contained within the 60% elution displayed activity when tested with MT114 (*gabT::lacZ*) (data not shown). Thin-layer chromatography of this material resulted in six prominent bands under UV illumination. Individual bands were cut out, eluted with ethyl acetate and tested for activity. The material from one band was capable of activating the *gabT::lacZ* fusion approximately five-fold (data not shown).

Structural analysis of the activating signal. The high-resolution electron impact mass spectra of the activating material indicated a primary ion with an m/z of 117.0584 and a second peak at an m/z of 90.04767 (Fig. 1A). A database search indicated a match with indole (C_8H_7N), with an m/z of 117.0578. A comparison of the high-resolution spectrum for indole indi-

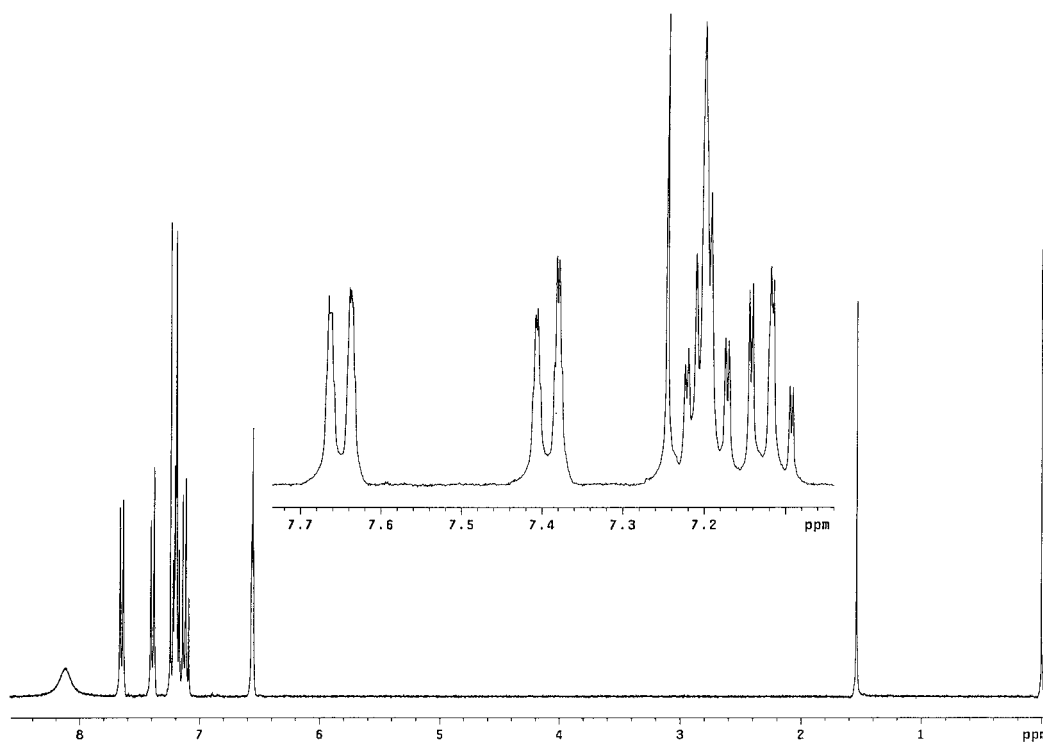
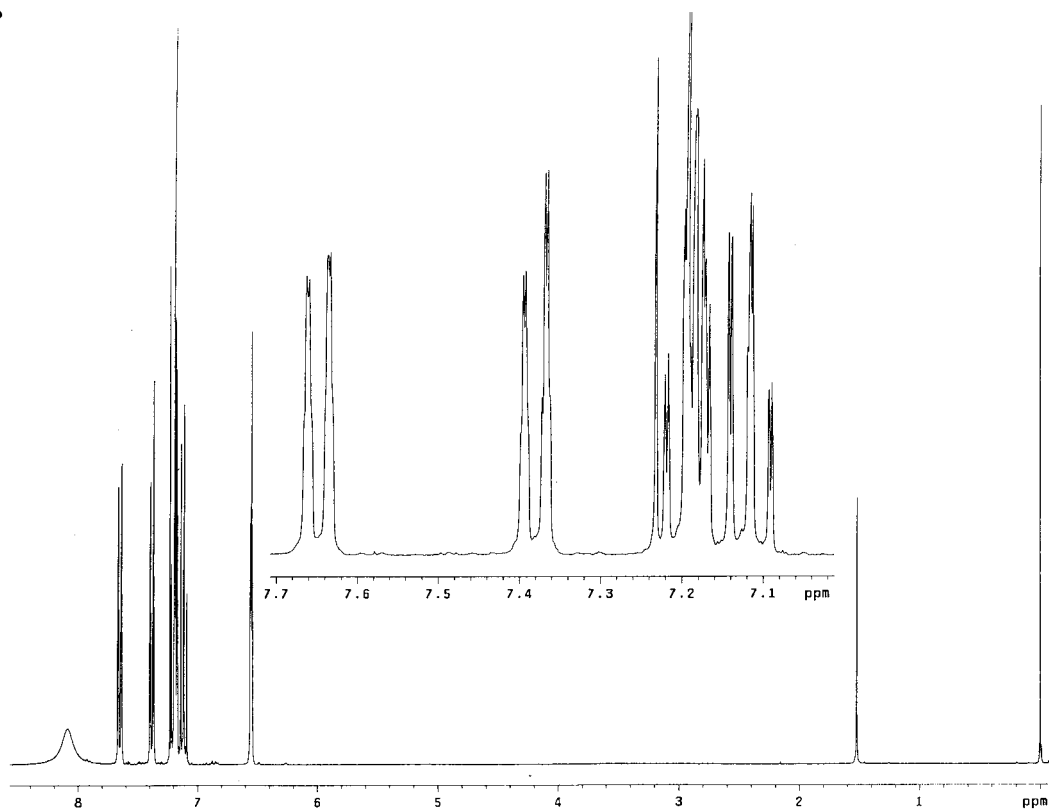
A.**B.**

FIG. 2. ¹H NMR spectra of the activating factor and synthetic indole. The chemical shifts are reported in δ (ppm) relative to residual trimethyl silane. The ¹H NMR spectra for the activating factor (A) and synthetic indole (B) were identical (see Materials and Methods).

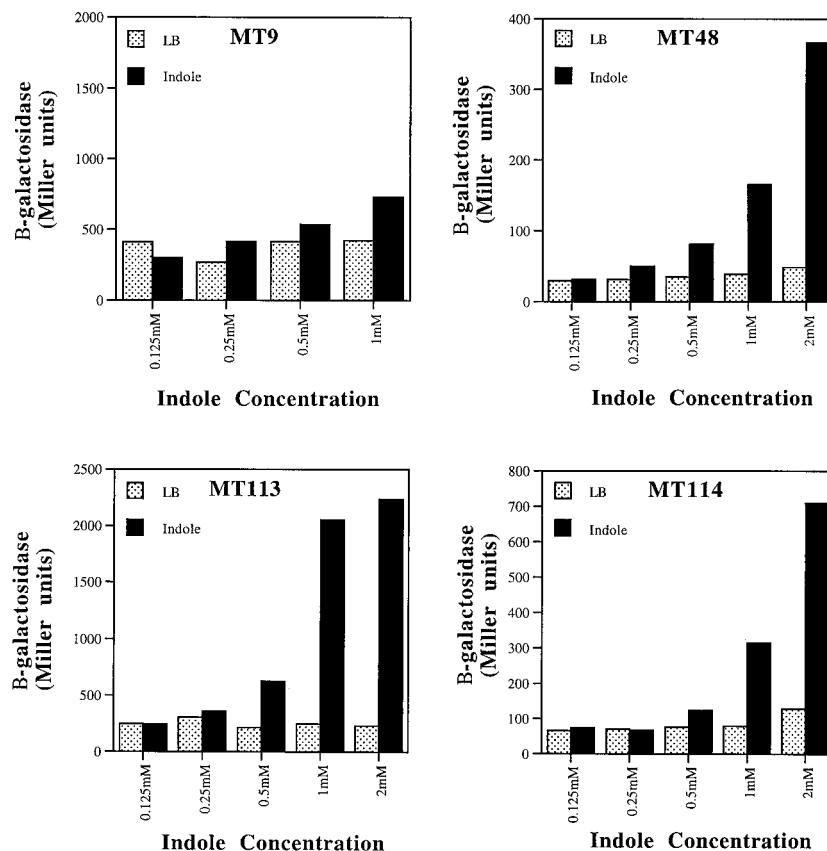


FIG. 3. Effects of indole on expression. The effects of synthetic indole on expression of *lacZ* fusions to *cysK* (MT9), *atsD* (MT48), *tnaB* (MT113), and *gabT* (MT114) were monitored by β -galactosidase expression (Miller units). Average results from duplicate experiments are shown. Standard deviations were less than 10% for each value. Duplicate experiments gave results similar to those shown.

cated that the profile was essentially identical to that for the activating signal (Fig. 1B). NMR analysis was then used to further confirm the chemical nature of the activating signal. The ^1H NMR spectra of the activating signal (Fig. 2A) and synthetic indole (Fig. 2B) revealed identical profiles. In addition, the purified activating material gave an intense purple reaction with Kovács reagent. These data taken together indicate that the activating factor is indole.

The concentration of extracellular indole produced by *E. coli* has previously been reported at 150 μM in minimal medium supplemented with tryptophan (12). However, we have observed that stationary-phase LB cultures of MG1655 have an indole concentration of 340 μM .

Synthetic indole activates the *astD*, *tnaB*, and *gabT* fusions in a dose-dependent manner. To confirm the identification of indole as an activating molecule, synthetic indole was tested for the ability to prematurely activate various quorum-sensing regulated *lacZ* fusions at early log phase. In Fig. 3, the effects of indole on β -galactosidase expression from each fusion are shown. The *cysK::lacZ* fusion (MT9) was not significantly activated by indole, with a 1.7-fold activation seen at 1 mM. In contrast, *lacZ* fusions to *astD* (MT48), *tnaB* (MT113), and *gabT* (MT114) were activated 4.3-, 8.3-, and 4.0-fold, respectively, at a concentration of 1 mM indole (Fig. 4). At 2 mM indole, the *astD* (MT48), *tnaB* (MT113), and *gabT* (MT114) fusions were activated 7.6-, 9.6-, and 6.6-fold, respectively. In-

dole did not stimulate growth, and at a concentration of 2 mM, it resulted in slower growth of MT48, MT113, and MT114. At 2 mM indole, MT9 was unable to reach an OD of 0.35. As a control, we examined the effects of indole at 1 mM on the expression of a random, uncharacterized *lacZ* fusion to a non-quorum-sensing activated gene. This fusion was not significantly activated and exhibited an induction value of 1.2-fold (data not shown). In addition, the expression of *lacZ* from its native chromosomal location in MG1655 was not altered by indole at 1 or 2 mM (data not shown).

Altered activation by conditioned medium from a *tnaA* (tryptophanase) mutant. The production of indole from tryptophan depends on the tryptophanase enzyme (TnaA) (35). We investigated the role of secreted indole in conditioned medium on the activation of the fusions. Conditioned medium was prepared from MC1061 (wild type) and the isogenic derivative TM1061 (*tnaA::mini-Tn5Cm*) at an OD₆₀₀ of 1.6 and was tested for the ability to activate *lacZ* fusions to the *cysK*, *astD*, *tnaB*, and *gabT* genes (Table 1). The TM1061 strain produced levels of indole that were below detection by standard methods (12). For the *cysK* (MT9), *astD* (MT48), and *gabT* (MT114) fusions, the activation values with conditioned medium from TM1061 were lower by 27, 41, and 26%, respectively, than the activation values with conditioned medium from MC1061 (wild type). For the *tnaB* fusion (MT113), the

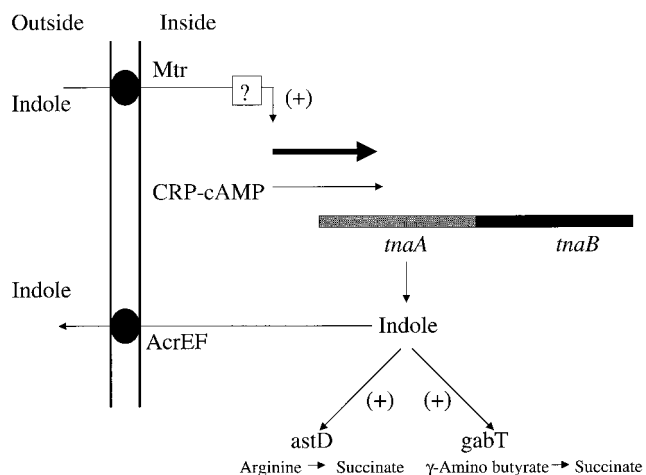


FIG. 4. Possible model for indole signaling in *E. coli*. The *tnaAB* operon is depicted and is activated by CRP-cAMP as nutrients are depleted. This results in indole production via the TnaA (tryptophanase) enzyme. The indole is secreted and acts as an extracellular signal. The components of the signal response pathway are unknown and are indicated by a question mark.

activation was lower by 70% with conditioned medium from TM1061, relative to MC1061 (Table 1).

The ability of indole at physiologically relevant concentrations to restore full activation of indole-deficient conditioned medium from the *tnaA* mutant was examined with the *tnaB* and *gabT* fusions (Table 2). Using *tnaA* mutant-conditioned medium supplemented with indole at 300 μM , the expression of β -galactosidase from the *tnaB::lacZ* and *gabT::lacZ* fusions was restored to a level that was 92 and 115%, respectively, of the levels with wild-type-conditioned medium (Table 2). With indole supplementation at 600 μM , the restored expression of β -galactosidase for the *tnaB* and *gabT* fusions corresponded to 116 and 127%, respectively, of the levels with wild-type-conditioned medium.

TABLE 1. Activation of gene expression by conditioned medium

Strain	Growth condition ^a	Mean β -galactosidase activity \pm SD (Miller units) ^b
MT9	LB	262 \pm 3
	CM (wild type)	3,466 \pm 66 (13.2)
	CM (<i>tnaA::Cm^r</i>)	2,526 \pm 61 (9.6)
MT48	LB	39 \pm 3
	CM (wild type)	341 \pm 16 (9.0)
	CM (<i>tnaA::Cm^r</i>)	200 \pm 6 (5.3)
MT113	LB	177 \pm 15
	CM (wild type)	1,500 \pm 46 (8.5)
	CM (<i>tnaA::Cm^r</i>)	464 \pm 5 (2.6)
MT114	LB	39 \pm 3
	CM (wild type)	405 \pm 21 (10.4)
	CM (<i>tnaA::Cm^r</i>)	302 \pm 8 (7.7)

^a CM, conditioned medium.

^b Values in parentheses are fold induction relative to growth in LB only. Cells were harvested at an A_{600} of = 0.35.

TABLE 2. Effects of indole addition to conditioned medium from a *tnaA* mutant

Strain	Growth condition ^a	Mean β -galactosidase activity \pm SD (Miller units) ^b
MT113	LB	299 \pm 6
	CM (wild type)	1,116 \pm 57
	CM (<i>tnaA</i> mutant)	344 \pm 17
	CM (<i>tnaA</i> mutant) + 600 μM indole	1,289 \pm 39
	CM (<i>tnaA</i> mutant) + 300 μM indole	1,030 \pm 79
MT114	LB	63 \pm 2
	CM (wild type)	252 \pm 8
	CM (<i>tnaA</i> mutant)	210 \pm 10
	CM (<i>tnaA</i> mutant) + 600 μM indole	320 \pm 12
	CM (<i>tnaA</i> mutant) + 300 μM indole	289 \pm 16
CM (<i>tnaA</i> mutant) + 150 μM indole	244 \pm 12	

^a Conditioned medium (CM) was prepared at an A_{600} of = 1.4.

^b Cells were harvested at an A_{600} of 0.35.

DISCUSSION

Previous studies of gram-negative bacteria have shown that the primary signaling molecules involved in cell-cell communication are *N*-acyl derivatives of homoserine lactone, cyclic peptides, and quinolones (10, 11, 17, 29, 33). In this study, we have demonstrated that indole can act as an extracellular signaling molecule and activate the *astD*, *tnaB*, and *gabT* genes in a concentration-dependent manner. To date, there is no direct evidence that *E. coli* produces any of the *N*-acyl homoserine lactone signals commonly used in other gram-negative bacteria. Therefore, *E. coli* may have evolved to utilize alternative signals, such as the accumulation of certain metabolites. Signaling via metabolites may allow cells to fine-tune the regulation of target genes in response to changing environmental conditions. In addition, signaling by indole may not be limited to *E. coli*, as indole induces spore formation in the myxobacterium *Stigmatella aurantiaca* (14).

In *E. coli*, the addition of synthetic indole activated the *astD*, *tnaB*, and *gabT* fusions but did not activate *cysK::lacZ* or several control *lacZ* fusions. The use of a *tnaA* null mutant demonstrated that conditioned medium lacking indole exhibited a reduced ability to activate these fusions, relative to wild-type-conditioned medium (Table 1). In the case of *cysK::lacZ*, which is not activated by indole, it is unclear why conditioned medium from a *tnaA* mutant supported a lower level of activation. One possibility is that production of the signal for *cysK* activation is indirectly coupled to the activity of tryptophanase.

For the *tnaB* fusion, indole appears to be the primary extracellular signal required for activation, as conditioned medium lacking indole exhibited a 70% reduction in *tnaB* activation. Previous studies by Yanofsky et al. reported that indole was not able to induce expression of the *tna* operon (42). We have obtained SVS1144 *tnaA'*-*lacZ*, used by Yanofsky et al., and found that it is induced by indole during growth in LB. The use of different media and/or indole concentrations could account for the differences in our results.

With the *astD* and *gabT* fusions, there was significant residual activation with conditioned medium lacking indole (Table 1). In addition, the concentration of synthetic indole required for activation of the *astD* and *gabT* fusions in LB only was

above 500 μM , a concentration higher than the 340 μM that we have observed in stationary-phase *E. coli* supernants. However, when indole was added back to conditioned medium from a *tnaA* mutant lacking indole, the level of *gabT::lacZ* activation could be restored to wild-type levels with physiologically relevant concentrations of indole (300 μM). In light of these results, we propose that a second extracellular signal is produced by *E. coli* and that the combination of both signals is required for full activation of *gabT* and possibly *astD*.

In Fig. 4, a model that represents a possible physiological role for signaling by indole is presented. The initial component of this model is the *tnaAB* operon, which is activated by cyclic AMP receptor protein-cyclic AMP complex (CRP-cAMP) (6). We hypothesize that nutrient depletion during the increase in cell density is the initial trigger that activates *tnaAB* via CRP-cAMP. This activation is predicted to result in an increase in indole production. In support of this model, our preliminary studies indicate that the concentration of extracellular indole increases when cells are starved at low cell density. The intracellular indole is then exported by the AcrEF efflux system (21) to the outside of the cell, where it accumulates in the growth medium. At this time, the components of the indole response pathway are unknown. The signaling pathway may involve the Mtr permease, which transports indole into the cell (42). The net result of this putative signaling pathway is predicted to be a positive amplifying loop for indole production via the *tnaAB* operon.

Two targets of indole-mediated signaling are the *astD* and *gabT* genes. These genes function in pathways that degrade amino acids to pyruvate or succinate (1). Furthermore, tryptophanase enzyme (TnaA) is able to catabolize tryptophan, cysteine, and serine to pyruvate (35). Studies by Zinser and Kolter have shown that the ability to catabolize amino acids is an important parameter in the ability to persist and compete in stationary phase (44). This raises the possibility that signaling by indole may play a role in a pathway which prepares the cells for a nutrient-poor environment when the catabolism of amino acids becomes important for energy production.

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