Enterohemorrhagic *Escherichia coli* Biofilms Are Inhibited by 7-Hydroxyindole and Stimulated by Isatin\(^\dagger\)

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Since indole is present at up to 500 μM in the stationary phase and is an interspecies biofilm signal (J. Lee, A. Jayaraman, and T. K. Wood, BMC Microbiol. 7:42, 2007), we investigated hydroxyindoles as biofilm signals and found them also to be nontoxic interspecies biofilm signals for enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), *E. coli* K-12, and *Pseudomonas aeruginosa*. The genetic basis of EHEC biofilm formation was also explored, and notably, virulence genes in biofilm cells were repressed compared to those in planktonic cells. In *Luria-Bertani* medium (LB) on polyostyrene with quiescent conditions, 7-hydroxyindole decreased EHEC biofilm formation 27-fold and decreased K-12 biofilm formation 8-fold without affecting the growth of planktonic cells. 5-Hydroxyindole also decreased biofilm formation 11-fold for EHEC and 6-fold for K-12. In contrast, isatin (indole-2,3-dione) increased biofilm formation fourfold for EHEC, while it had no effect for K-12. When continuous-flow chambers were used, confocal microscopy revealed that EHEC biofilm formation was reduced 6-fold by indole and 10-fold by 7-hydroxyindole in LB. Whole-transcriptome analysis revealed that indole represses indole synthesis by repressing *tnaABC* 7- to 37-fold in EHEC, and extracellular indole levels were found to be 20-fold lower. Furthermore, indole repressed the AI-2 transporters *isrABCDFGKR*, while significantly inducing the flagellar genes *flgABCDCEFGHIJK* and *fliAEGFILMNPQ* (which led to a 50% increase in motility). 7-Hydroxyindole induces the biofilm inhibitor/stress regulator *ycfR* and represses *cysADIJPU/fliC* (which led to a 50% reduction in motility) and *purBCDEFHKLMNRT*. Isogenic mutants showed that 7-hydroxyindole inhibits *E. coli* biofilm through cysteine metabolism. 7-Hydroxyindole (500 μM) also stimulates *P. aeruginosa* PAO1 biofilm formation twofold; therefore, hydroxyindoles are interspecies bacterial signals, and 7-hydroxyindole is a potent EHEC biofilm inhibitor.

Prokaryotes and eukaryotes signal not only themselves but also one another; hence, there is competition and interference of cell signals. For example, interference of acylhomoserine lactones (AHLs) is manifested by AHL lactonases and acylases, which are present in both gram-positive and gram-negative bacteria (79). Similarly, autoinducer-2 (AI-2), a furanoyl borate diester or derivative (39), may be manipulated by *Escherichia coli* and *Vibrio harveyi* to interfere with the ability of each species to assess changes in cell population (75). This competition extends beyond prokaryotes, as eukaryotes manipulate the quorum-sensing signals of bacteria, too. For example, algae block bacterial biofilm formation via furonanes by controlling both AHL signaling (25) and AI-2 signaling (52), and mammals (including humans) block AHL signaling via lactonase in sera (76). Analogously, bacteria take advantage of eucaryotic signals since enterohemorrhagic *E. coli* O157:H7 (EHEC) bacteria utilize the human hormones epinephrine and norepinephrine to activate virulence genes (33), and bacteria interfere with plant indole 3-acetic acid-based signaling by using this signal as a source of carbon, nitrogen, and energy (37). Further evidence of this intense competition is that *E. coli* senses signals (AHLs) that it cannot synthesize to control biofilm formation (36a).

Other examples that confirm the importance of cell signaling for bacterial biofilm formation include the control of exopolysaccharide synthesis by quorum-sensing signals in *Vibrio cholerae* (22). In addition, AHLs control biofilm formation in *Pseudomonas aeruginosa* (10) and *Serratia liquefaciens* (36), and we have found that in vitro-synthesized AI-2 directly stimulates *E. coli* biofilm formation (20, 26). AI-2 has also been shown to control mutualistic biofilm formation (41, 55).

Extracellular indole is found at high concentrations (over 600 μM) when *E. coli* is grown in rich medium (12, 71) and was identified initially as a stationary-phase signal that controls *cysK, astD, mnaB*, and *gabT* (3) as well as *maAL* and *phoABU* (53). Recently, indole signaling has been shown to link plasmid multimerization and cell division (9), and indole was found to be a nontoxic interspecies signal that decreases biofilm formation in *E. coli* and increases the biosynthesis of *P. aeruginosa* and *P. fluorescens* (even though these pseudomonads do not produce this signal) (36a). We found that, in a manner analogous to that in which AHL signals bind SdiA (43, 78) and control biofilm formation in *E. coli* (36a), indole controls biofilms by inducing the AHL sensor of *E. coli*, SdiA, which influences cell motility and acid resistance (36a), even though *E. coli* does not produce AHL signals (43). Beyond biofilms, indole has been shown to control multidrug exporters (e.g., *acrDE* and *cusB*) in *E. coli* K-12 (28), to regulate the pathogenicity island of en-

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teropathogenic *E. coli* (EPEC) (1), and to repress *gadX* of *E. coli* K-12 (36a), which activates virulence in EHEC (44).

Here, we focus on EHEC since it colonizes the large intestine, where its Shiga toxins cause hemorrhagic colitis and hemolytic-uremic syndrome (61). There are over 73,000 EHEC infections annually in the United States, which leads to 2,000 hospitalizations and 60 deaths, the economic cost of which is $405 million (18). It has been reported that EHEC forms biofilm on various surfaces (57, 69), and sloughing of the biofilm may cause contamination (57); however, there is no effective treatment for EHEC infection. The genetic basis of EHEC biofilm formation was also investigated so that the impact of hydroxyindole signals on biofilm genes could be better interpreted.

Based on this proven competition for cell signals and given that indole controls biofilms (36a), and is present at up to 700 μM (12), we hypothesized that hydroxylated indoles (Fig. 1) may play a role in biofilm formation since many bacterial oxygenases, such as *Pseudomonas putida* PpG7 (15), *Ralstonia pickettii* PKO1 (16), *Pseudomonas mendocina* KR1 (67), and *Burkholderia cepacia* G4 (56), readily convert indole to oxidized compounds, such as 2-hydroxyindole, 3-hydroxyindole, 4-hydroxyindole, isatin, indigo, isoindigo, and indirubin (56). Using crystal violet staining and a continuous-flow chamber to study biofilm formation along with DNA microarrays to investigate the global transcriptome response, we show here that hydroxyindoles and isatin are interspecies biofilm signals that affect EHEC, *E. coli* K-12, and *P. aeruginosa* PAO1 by controlling biofilm-related genes, including those for AI-2 transport, indole synthesis, flagellar synthesis, and cysteine regulation.

### Materials and Methods

**Bacterial strains, materials, and growth rate measurements.** The strains and plasmids used are listed in Table 1. The EHEC strain used in this study was obtained from the American Type Culture Collection (ATCC 43895) and was a Centers for Disease Control isolate (EDL933) that was implicated in two outbreaks of hemorrhagic colitis in the United States during 1982; this strain produces Shiga toxins 1 and 2 (64). The *P. aeruginosa* PAO1 strain used in this study was the sequenced Holloway strain (63). LB (58) was used for all the experiments except for the motility assay. Indole, 4-hydroxyindole, 5-hydroxyindole, 7-hydroxyindole, isatin, indigo, and indirubin were purchased from Fisher Scientific (Pittsburgh, PA). 2-Hydroxyindole (oxindole) was purchased from Sigma Chemical (St. Louis, MO), and 6-hydroxyindole was obtained from Matrix Scientific (Columbia, SC). Isoindigo was prepared as described by Hoessel et al. (29, 56). To determine the toxicities of hydroxyindoles, the specific growth rates of EHEC planktonic cells were measured in LB medium at 37°C in the presence of hydroxyindoles (0, 250, 500, 1,000, and 2,000 μM), with dimethylformamide (DMF) added at 0.1 vol% to all samples; the experiments were performed twice using independent cultures. Purified AI-2 was synthesized as described previously (20).

**Crystal violet biofilm assay.** The crystal violet biofilm assay was adapted (49); overnight cultures were diluted to a turbidity of 0.05 at 600 nm and were incubated in polystyrene 96-well plates at 30°C for 7 h or 24 h without shaking in LB medium. Each data point was averaged from at least 12 replicate wells (6 wells from two independent cultures). The experiments were performed two or four times using independent cultures.

**Flow chamber biofilm experiments.** The inoculum and biofilm growth medium was LB supplemented with 300 μg/ml erythromycin to maintain pCM18 (24) and thus to retain the constitutive green fluorescent protein (GFP) vector for visualizing the biofilm. The biofilm was formed at 30°C in a continuous-flow chamber that consisted of a standard glass microscope slide on one side and a plastic coverslip on the other side, with dimensions of 47.5 mm by 12.7 mm and a 1.6-mm gap between the surfaces (BET model FC81; Biosurface Technologies Corp., Bozeman, MT), and was visualized with confocal microscopy as described.

![Biofilm formation of EHEC in LB at 30°C after 7 h in 96-well plates with indole and hydroxyindoles. Indole, 2-hydroxyindole (2HI), 5-hydroxyindole (5HI), and 7-hydroxyindole (7HI) were used at 1,000 μM, whereas isatin and isoindigo were used at 250 μM. Each experiment was repeated two to four times with six wells each, and 1 standard deviation is shown. The structures of indole, 2HI, 5HI, 7HI, isatin, and isoindigo are shown.](image-url)

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description or genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>E. coli</em> O157:H7</td>
<td>EHEC S1x1” and S1x2”</td>
<td>64b</td>
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<tr>
<td><em>E. coli</em> K-12 BW25113</td>
<td>lac4 mB114 ΔlacZ_W316 hsdR514 ΔaraBAD ΔaiI Δo1BAD ΔhacBAD Δll1 Δll78</td>
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<tr>
<td><em>E. coli</em> K-12 BW25113 ΔcysB</td>
<td>K-12 BW25113 ΔcysB ΔKm”</td>
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<tr>
<td><em>P. aeruginosa</em> PAO1</td>
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<td><em>Vibrio harveyi</em> BB170</td>
<td>BB120 lacNc: Tn5 (AHL sensor negative, AI-2 sensor positive)</td>
<td>66</td>
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<tr>
<td><em>E. coli</em> UT481/pCX39</td>
<td>Δlac-pro met pro zzz: Tn10 thy supD tkC mKc ftsQ2p: lacZ Amp”</td>
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<td><strong>Plasmids</strong></td>
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<td>pCM18</td>
<td>Em” pTRK2-P_cysB-RBSH-gfpmut3”-Tc-T1 (GFP plasmid for visualizing biofilm)</td>
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</tr>
<tr>
<td>pCA24N-cysB”</td>
<td>Cm” lacP pCA24N P_Ts-lac-cysB”</td>
<td>35</td>
</tr>
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</table>

* a Km”, Amp”, Em”, and Cm” are the antibiotic resistance phenotypes for kanamycin, ampicillin, erythromycin, and chloramphenicol, respectively, and S1x is the Shiga toxin.

b ATCC 43895.
previously (74). The inocula were diluted to a turbidity of 0.2 at 600 nm and used to seed the flow cell for 2 h at 10 ml/h before fresh LB erythromycin medium was added at the same flow rate. To study the effect of indole and 7-hydroxyindole on biofilm formation, the compounds were dissolved in DMF and added at 1.00 μM in the continuous feed upon inoculation; DMF was added as the negative control to the no-hydroxyindole flow chamber. The initial inoculum was 1.9 × 10^6 to 4.7 × 10^6 cells/ml. Each flow chamber experiment was repeated twice (six experiments). A dispersion experiment was also performed with 7-hydroxyindole by adding 1,000 μM in the 10-ml/h feed after biofilm formation for 24 h in LB erythromycin medium in the absence of hydroxyindole. GFP allowed visualization of the EHEC biofilm by excitation with an Ar laser at 488 nm (emission, 500 to 600 nm), using a TCS SPS confocal scanning laser microscope with a 63× HCX PL FLUOTAR L dry objective, a correction collar, and a numerical aperture of 0.7 (Leica Microsystems, Mannheim, Germany).

Color confocal flow cell images were converted to gray scale (74), and biomass, substratum coverage, surface roughness, and mean thickness were determined using COMSTAT image-processing software (27) as described previously (74). For each experiment, nine different random positions were chosen for microscope analysis, and 25 images were processed for each point. Values are means of data from the different positions at the same time point, and standard deviations were calculated based on these mean values for each position. Simulated three-dimensional images were obtained using IMARIS (Bitplane, Zurich, Switzerland). Twenty-five pictures were processed for each three-dimensional image.

Motility assay. LB overnight cultures were used to assay motility in plates containing 1% tryptone, 0.25% NaCl, and 0.3% agar (62) by measuring halos at 20 h at 37°C. When the effect of isatin and 7-hydroxyindole on motility was tested, 10,208 probe sets for open reading frames, rRNA, tRNA, and intergenic regions were obtained from the Affymetrix-NetAffx analysis center (14). Microarray experiments were performed using a single-color real-time PCR detection system (Bio-Rad Laboratories). The one-step RT-PCR kit with SYBR green (Bio-Rad Laboratories, CA) on a MyiQ real-time PCR system (Bio-Rad Laboratories, CA) was used to determine the relative changes between the samples. The levels of four genes were quantified using real-time reverse transcription-PCR (RT-PCR) (70). The same total RNA used for the Affymetrix microarrays was used for these studies. The primers were designed using PrimerQuest online software (Table 2). The real-time RT-PCR was performed using an iScript one-step RT-PCR kit with SYBR green (Bio-Rad Laboratories, CA) on a MyIQ single-color real-time PCR detection system (Bio-Rad Laboratories). The threshold cycles, as calculated by the MyIQ optical system software (Bio-Rad Laboratories), were used to determine the relative changes between the samples. The experiments were run in triplicate in 20 μl, and 50 ng of total RNA was used for each reaction, with the final forward and reverse primer concentrations at 0.5 μM each. After amplification, template specificity was ensured by using melting-curve analysis. mrgG was used as the housekeeping gene for normalizing the data.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>lasA</td>
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<td>5′-AAACAGGGCTTGGTT</td>
</tr>
<tr>
<td>lasB</td>
<td>5′-AGCATCTGCGGTC</td>
<td>5′-AAATCTTTTACGGT</td>
</tr>
<tr>
<td>lasC</td>
<td>5′-ACACGCTTGGAC</td>
<td>5′-ACGGAGCTGCAATG</td>
</tr>
<tr>
<td>cysP</td>
<td>5′-ACACCCGGTGTGGA</td>
<td>5′-TGATATCTTGGG</td>
</tr>
<tr>
<td>rrsG</td>
<td>5′-TATGTCACATAGG</td>
<td>5′-ACTTACACAGGGCCT</td>
</tr>
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</table>

TABLE 2. Primers used for RT-PCR

AI-2, indole, and β-galactosidase assays. The activity of the synthesized AI-2 was assayed as described previously (66). Briefly, the reporter strain V. harveyi BB170 was grown in AB medium overnight and diluted 1:5,000 in fresh AB medium, and then supernatants from E. coli LB cultures with 250 μM isatin (harvested at 7 h) were added to the AI-2 reporter strain. Bioluminescence was measured with a 20/20 luminometer (Turner Design, Sunnyvale, CA) and reported as relative light units. The cell density of the V. harveyi reporter strain was measured by spreading the cells on LM plates and counting CFU after 24 h. The experiment was performed in duplicate. Extracellular and intracellular indole concentrations from cells in LB medium were measured spectrophotometrically in duplicate as described previously (12), by modifying the protocol of Kawamura-Sato et al. (34). Also, the spectrophotometric indole assay was corroborated with reverse-phase high-performance liquid chromatography (HPLC) using a 100- by 4.6-mm Chromolith Performance RP-18e column (Merck KGaA, Darmstadt, Germany) and gradient elution, with H₂O-0.1% formic acid and acetonitrile as the mobile phases, at a flow rate of 1 ml/min (65:35 for 0 to 5 min, 35:65 for 5 to 12 min, and 65:35 at 12 min). Under these conditions, the retention times and the absorbance maxima were 3.6 min/264 nm for 7-hydroxyindole and 5.9 min/271 nm for indole.

E. coli UT481 harboring pCX39 (ftsQ:p:lacZ) (19) was grown at 30°C with 100 μg ampicillin/ml from diluted overnight cultures to a turbidity of 2 at 600 nm. The β-galactosidase activities were calculated based on a protein concentration of 0.31 mg protein/ml/OD600. A modified Lowry protein assay kit from Pierce Biotechnology (Rockford, IL) was used to measure the total protein content.

7-Hydroxyindole degradation. For the degradation of 7-hydroxyindole, overnight EHEC cultures grown in LB were diluted to a turbidity at 600 nm of 0.05 and were regrown in LB with 1,000 μM 7-hydroxyindole for 24 h at 30°C at 250 rpm. As a negative control, autoclaved cells at a turbidity of 0.05 were contacted with 7-hydroxyindole under the same conditions to confirm that there was no evaporation or adsorption. To confirm the lack of hydroxyindole production from either indole or 7-hydroxyindole by EHEC, exponentially grown cells (OD600 ~ 2.0) were harvested and resuspended in 50 mM Tris-HCl buffer (pH 7.4; OD600 ~ 10.0) and were contacted with 1,000 μM indole or 7-hydroxyindole for 1 h. Each experiment was performed two times using two independent cultures.

Microarray data accession number. The expression data for the biofilm samples were deposited in the NCBI Gene Expression Omnibus and are accessible through accession number GSE6195 (4, 14).

RESULTS

The goals of this research were to determine if hydroxyindoles influence biofilm formation of EHEC, E. coli K-12, and P. aeruginosa PA01 and then to explore the genetic basis of this control. The genetic basis for EHEC biofilm formation was also evaluated using DNA microarrays since this has not been previously investigated and this facilitates gauging the impact of hydroxyindoles on biofilm genes.

Toxicity. To test the toxicities of hydroxyindoles on EHEC, indole, 5-hydroxyindole, 7-hydroxyindole, and isatin were tested in LB at 37°C. In the absence of hydroxyindoles, the specific growth rate was 1.43 ± 0.09/h, whereas the growth rates were 1.38 ± 0.01/h with 1,000 μM indole, 1.48 ± 0.08/h with 7-hydroxyindole, and 0.91 ± 0.02/h with 5-hydroxyindole.
FIG. 2. EHEC biofilm formation in LB at 30°C after 24 h in flow cells without an indigoid compound but with the diluent DMF (A), with 1,000 μM indole (B), and with 1,000 μM 7-hydroxyindole (C). Each experiment was repeated two times, and one representative image is shown. The scale bar indicates 20 μm.

with 1,000 μM 5-hydroxyindole, 1.14 ± 0.03/h with 1,000 μM 7-hydroxyindole, and 1.44 ± 0.04/h with 250 μM isatin. Hence, these indigoids do not significantly change the specific growth rate of EHEC. For 5-hydroxyindole, concentrations as high as 2,000 μM did not affect cell growth (1.40 ± 0.05/h). In addition, indole, 5-hydroxyindole, 7-hydroxyindole, and isatin at 500 μM were not toxic to P. aeruginosa PAO1.

**Hydroxyindoles inhibit and stimulate EHEC biofilm formation.** In the absence of hydroxyindoles, it was observed that EHEC formed robust biofilms at 30°C and at 37°C after 24 h although those at 30°C were larger; hence, all the biofilm results here are reported at 30°C. Since indole (36a) and hydroxyindoles were not toxic to EHEC, we screened various hydroxyindoles (Fig. 1) for their abilities to inhibit EHEC in 96-well plates in LB for 9 h at 30°C (similar results were found at 37°C): 2-hydroxyindole, 4-hydroxyindole, 5-hydroxyindole, 6-hydroxyindole, 7-hydroxyindole, and isatin were screened at 500 μM, and indigo, isoindigo, and indirubin were screened at 250 μM since they were insoluble in water above 250 μM. We chose concentrations of 250 to 1,000 μM since EHEC produced 362 ± 3 μM indole after 8 h and K-12 produced 403 ± 8 μM, as found through two independent assays. Indole, 5-hydroxyindole, and 7-hydroxyindole were the most effective at reducing EHEC biofilm formation (more than 50%), 4-hydroxyindole decreased biofilm by about 50%, and isatin increased EHEC biofilm (data not shown). After the preliminary screening, 1,000 μM concentrations of indole, 2-hydroxyindole, 5-indroxyindole, and 7-hydroxyindole as well as 250 μM isatin and isoindigo were tested for their impacts on biofilm formation of EHEC in LB at 30°C for 7 h. Biofilm formation was clearly inhibited by indole (18-fold), 5-hydroxyindole (11-fold), and 7-hydroxyindole (27-fold), whereas biofilm formation was stimulated by isatin (4-fold) and was not affected by 2-hydroxyindole and isoindigo (Fig. 1). Furthermore, isatin from 0 to 1,000 μM stimulated biofilm formation in a dose-dependent manner, and 7-hydroxyindole from 0 to 2,000 μM inhibited biofilm formation in a dose-dependent manner (data not shown). Therefore, use of double hydroxylation of indole to form isatin increased biofilm formation whereas the position of the single hydroxyl group had a profound effect on the efficacies of these biofilm inhibitors.

The effects of 7-hydroxyindole, the most potent biofilm inhibitor out of the 9 tested hydroxyindoles, and indole were investigated using a more rigorous continuous-flow chamber with EHEC in LB medium at 30°C. As shown in Fig. 2A, EHEC forms robust biofilms with undulating towers interdispersed with water channels in rich medium at 30°C for 24 h. To our knowledge, this is the first EHEC biofilm image in a continuous-flow chamber. Upon addition of indole at 1,000 μM, biofilm formation was reduced and consisted of individual slender microcolonies (Fig. 2B). Upon addition of 7-hydroxyindole at 1,000 μM, the microcolonies became significantly smaller, scattered towers (Fig. 2C). These visual results were quantified using COMSTAT, indicating that addition of indole reduced biofilm mass 6-fold (7.9 ± 3 versus 46 ± 6 μm³/m²) and reduced mean biofilm thickness 4-fold (14 ± 5 versus 55 ± 5 μm), and addition of 7-hydroxyindole reduced biofilm mass 10-fold (4.5 ± 3 versus 46 ± 6 μm³/m²) and reduced mean biofilm thickness 5.5-fold (10 ± 5 versus 55 ± 5 μm). Hence, 7-hydroxyindole was more effective than indole in decreasing EHEC biofilms. The results for the two independent experiments using the flow chamber for each application of DMF alone, indole, and 7-hydroxyindole were consistent.

To confirm the presence of hydroxyindoles, the extracellular indole and 7-hydroxyindole concentrations were measured in the effluent of the flow chamber by using HPLC. The level of indole without indole addition ranged from 1 to 8 μM at 24 h, while the level of indole was maintained above 185 μM for 24 h upon addition of indole (1,000 μM), and the level of 7-hydroxyindole was maintained above 540 μM for 24 h upon addition of 7-hydroxyindole (1,000 μM). Note that extracellular concentrations of indole are complex since EHEC both synthesizes and degrades indole via its reversible tryptophanase (TnaA) (47) and since it imports indole via Mtr (77). Also, it was found here that EHEC degrades 7-hydroxyindole at a rate of 0.60 ± 0.03 nmol/mg protein/min (from 1,100 μM to 380 μM for 24 h). Furthermore, added indole and 7-hydroxyindole were not converted into other hydroxyindoles in EHEC. Hence, the changes in EHEC biofilm formation are due to the presence of indole and 7-hydroxyindole.

To investigate whether 7-hydroxyindole disperses the existing strong biofilm, 7-hydroxyindole (1,000 μM) was added after biofilm formation for 24 h in LB. During the following 24 h, there was no new biofilm formed; however, no significant dispersion was observed (data not shown).
Hydroxyindoles inhibit *E. coli* K-12 biofilm and stimulate *P. aeruginosa* PAO1 biofilm. Along with the result for EHEC, the effect of hydroxyindoles on the biofilm formation of *E. coli* K-12 BW25113 was evaluated using the crystal violet assay. 5-Hydroxyindole and 7-hydroxyindole significantly inhibited *E. coli* K-12 biofilm formation, as with EHEC (Fig. 3A). However, isatin did not affect *E. coli* K-12 biofilm significantly (Fig. 3A), whereas isatin increased EHEC biofilm. Since *E. coli* K-12 produces high concentrations of extracellular indole when *E. coli* is grown in rich medium (over 600 μM) (12, 71), the levels of extracellular indole were measured from *E. coli* indole when indole inhibit EHEC biofilm but enhance hydroxyindoles in LB after 24 h and that indole and 7-hydroxyindole twofold (Fig. 3B). It is notable that EHEC produces *purDEFHKMT* (52) and *BCDEFHKLMNRT* (54). Carbamoyl phosphate synthetase genes (*narGHIJK*) were repressed 4- to 11-fold, and previously, we found that the nontoxic biofilm inhibitor ursolic acid repressed *narGHIJK* and the galactitol phosphotransferase (PTS) family of genes (*agaVWXYZ*) that are involved in the synthesis of dihydroxyacetone phosphate (8) were both induced in the biofilm cells (see Table S1 in the supplemental material). Nitrate reductase genes (*narGHIJK*) and the galactitol phosphotransferase (PTS) family of genes (*agaVWXYZ*) that are involved in the synthesis of dihydroxyacetone phosphate (8) were both induced in the biofilm cells (see Table S1 in the supplemental material).

Since EHEC from an aggregative colony forms more biofilm than cells from a smooth colony (69), aggregation of EHEC and *E. coli* K-12 was also measured in LB at 30°C for 24 h. EHEC formed sevenfold more aggregates than *E. coli* K-12, which suggests that the highly aggregative behavior of EHEC may be related to its ability to form robust biofilms.

**Differential gene expression with 7-hydroxyindole in 7-h EHEC biofilms.** Since hydroxyindoles affected biofilm formation (Fig. 1 and 2), we focused on differential gene expression in the biofilm itself rather than on planktonic cells. 7-Hydroxyindole (1,000 μM) significantly regulated (more than twofold) 347 genes, with 93 genes induced and 254 genes repressed (see Table S1 in the supplemental material). Among the repressed genes, 12 genes related to purine nucleotide biosynthesis (*purBCDEFHJKLMNRT*) were repressed 3- to 12-fold; previously, we found that *AI-2* repressed the *purDEFHJKLMNRT* operon in K-12 (52). In addition, genes related to cysteine biosynthesis (*cysADIJPU*) were repressed 4- to 11-fold, and previously, we found that the nontoxic biofilm inhibitor ursolic acid repressed *cysDIJK* (54). Carbamoyl phosphate synthetase genes (*carAB*) were also repressed (see Table S1 in the supplemental material).

Although indole inhibits *E. coli* K-12 biofilm formation through *sdiA* (36a), 7-hydroxyindole did not change *sdiA* expression (via microarrays) in EHEC biofilm. To investigate this further, transcription of the *sdiA*-controlled promoter *fbsQ2p* (19) was investigated using a β-galactosidase reporter; unlike indole (36a), neither 7-hydroxyindole nor isatin altered *fbsQ2p* expression significantly. Hence, these hydroxyindoles do not appear to work through *sdiA*.

Among the induced genes were ribonucleoside-diphosphate reductase operon genes (*mdHIIEF*), propionate metabolism genes (*pppCDE*), nitrate reductases (*narZ*), the biofilm stress regulator *ycfR* (80), and a hypothetical gene (*yheN*). Also repressed were hydrogen uptake genes (*hyaABCDEF*) and motility-related genes (*fliC*, c1936) (see Table S1 in the supplemental material).

To confirm the presence of 7-hydroxyindole, the extracellular
Similar results were found with EHEC that overproduces CysB, resulting in a 4.7-fold greater reduction in biofilm (Fig. 4). The wild type (WT) is BW25113, and 1 standard deviation is shown. The level of 7-hydroxyindole was 550 μM after 20 h). Therefore, the reduction in cell motility upon addition of 7-hydroxyindole may contribute to the inhibition of EHEC biofilm formation.

**Cysteine metabolism and 7-hydroxyindole.** Since 7-hydroxyindole repressed the cysteine synthesis operon \( \text{cysADEIJP} \) (via 1 mM IPTG) and addition of 7-hydroxyindole (7HI, 1,000 μM) with \( \text{E. coli} \) K-12 BW25113 Δ\( \text{cysB}\) [pCA24N-\( \text{cysB}^+ \)]. Each experiment was repeated two times with six wells each, and 1 standard deviation is shown. The wild type (WT) is BW25113, Δ\( \text{cysB} \) is BW25113 Δ\( \text{cysB} \), and \( \text{cysB}^+ \) in BW25113 Δ\( \text{cysB}\) [pCA24N-\( \text{cysB}^+ \)].

lar 7-hydroxyindole concentration was measured using HPLC when the biofilm cells were prepared for the microarray experiment. The level of 7-hydroxyindole was 550 μM in a shake flask at 7 h, which matches well with the extracellular 7-hydroxyindole (540 μM) concentration found in the flow chamber at 24 h.

**Motility and 7-hydroxyindole.** Since it has been reported that motility is essential for initial cell attachment in \( \text{E. coli} \) biofilm formation (49) and since the microarray data showed that several flagellar genes (c1936, \( \text{flc} \), and \( \text{motB} \)) were repressed by 7-hydroxyindole (see Table S1 in the supplemental material), the motility of EHEC was tested using a swimming assay (62). 7-Hydroxyindole decreased motility by 53% (2.3 ± 0.5 cm without 7-hydroxyindole versus 1.1 ± 0.1 cm with 1,000 μM 7-hydroxyindole after 20 h). Therefore, the reduction in cell motility upon addition of 7-hydroxyindole may contribute to the inhibition of EHEC biofilm formation.

**Verification of microarray results by RT-PCR.** RT-PCR was used to verify gene expression for \( \text{isrA} \), \( \text{lsrB} \), and \( \text{lsrC} \) for the \( \text{isatin} \) microarray experiments and for 7-hydroxyindole microarray experiment. The results showed comparable changes in expression for the \( \text{isatin} \) arrays for \( \text{lsrA} \) (−37-fold in microarrays versus −72-fold with RT-PCR), for \( \text{lsrB} \) (−64-fold in microarrays versus −110-fold with RT-PCR), and for \( \text{lsrC} \) differ from those for Fig. 1, which lacked the \( \text{cysB}^+ \) plasmid and were conducted at 7 h). Hence, overproducing CysB dramatically improved the effectiveness of 7-hydroxyindole, which implies that this biofilm inhibitor works through the cysteine biosynthesis pathway.

**Differential gene expression with isatin in 7-h EHEC biofilms.** Isatin (250 μM) significantly regulated (more than four-fold) 552 genes, with 151 genes induced and 401 genes repressed (see Table S1 in the supplemental material). Among the repressed genes were eight genes related to the AI-2 transporters (\( \text{lsrABCDFGKR} \), indole biosynthesis genes (\( \text{tnaABC} \) and \( \text{yihH} \)), cysteine desulfurase genes (\( \text{sufABCDES} \)), nitrate reductase genes (\( \text{narHIUVWYZ} \)), hydrogen uptake genes (\( \text{hyaABCDEF} \), propionate metabolism genes (\( \text{pppBCDE} \)), and acid resistance-related genes (\( \text{gadABC} \)) (see Table S1 in the supplemental material).

The genes most significantly induced by isatin were flagellar genes (\( \text{flgABCDFGEHIJK} \) and \( \text{flaAEFGILMNOPQ} \)), 44 various transport genes (e.g., \( \text{provWX} \) and \( \text{cirA} \)), and \( \text{treBC} \), which encode the trehalose PTS permease and trehalose-6-phosphate hydrolase (6). Based on the induction of the flagellar genes, we investigated the effect of isatin addition (50 μM) on motility and found that it was increased by 47% ± 8%; therefore, the increase in cell motility upon addition of isatin may contribute to the stimulation of EHEC biofilm formation. Note that among the genes differentially expressed in response to isatin (increased biofilm) and 7-hydroxyindole (decreased biofilm), the motility-related genes (\( \text{flc} \), c1936, and \( \text{motAB} \)), nitrate reductase genes (\( \text{narUVWYZ} \)), propionate metabolism genes (\( \text{pppBCDE} \)), carbamoyl phosphate synthetase genes (\( \text{carABC} \)), transport genes (\( \text{vieG} \), \( \text{ldlP} \), \( \text{unaA} \), and \( \text{salCD} \)) and \( \text{phoH} \), \( \text{ompF} \), and \( \text{fis} \) genes were oppositely regulated (see Table S1 in the supplemental material).

**Isatin, indole, and AI-2.** Based on the DNA microarray data with isatin, we investigated indole concentrations with 250 μM isatin since \( \text{tnaA} \) was repressed sevenfold with this compound (see Table S1 in the supplemental material). It was found that isatin represses extracellular indole concentrations 20-fold ± 13-fold in EHEC. Since extracellular indole represses biofilms (11, 12, 36a, 80), it appears that isatin increases EHEC biofilm formation by dramatically decreasing indole concentrations. It was also found that isatin reduced intracellular indole concentration 2.6-fold ± 0.5-fold, while 7-hydroxyindole had no effect on intracellular indole concentration.

Since the AI-2 transporter genes (\( \text{lsrABCDFGKR} \)) were highly regulated by the addition of isatin (see Table S1 in the supplemental material), we investigated the impact of purified AI-2 on EHEC biofilm formation. We found that 50 μM AI-2 increased EHEC bottom biofilm formation in LB medium 3.0-fold ± 0.7-fold after 15 h and 10-fold ± 2.5-fold after 24 h; total biofilm was increased 1.6-fold ± 0.2-fold after 24 h. Therefore, AI-2 increases EHEC liquid/solid biofilm formation as it does that of K-12 (20).

**Indole and isatin control E. coli O157:H7 biofilms.** FIG. 4. Biofilm formation in LB at 30°C after 24 h in 96-well plates with expression of \( \text{cysB}^+ \) (via 1 mM IPTG) and addition of 7-hydroxyindole (7HI, 1,000 μM) with \( \text{E. coli} \) K-12 BW25113 Δ\( \text{cysB}\) [pCA24N-\( \text{cysB}^+ \)]. Each experiment was repeated two times with six wells each, and 1 standard deviation is shown. The wild type (WT) is BW25113, Δ\( \text{cysB} \) is BW25113 Δ\( \text{cysB} \), and \( \text{cysB}^+ \) in BW25113 Δ\( \text{cysB}\) [pCA24N-\( \text{cysB}^+ \)].
DISCUSSION

EHEC serotype O157:H7 is a human pathogen responsible for outbreaks of hemorrhagic colitis, causing bloody diarrhea that can lead to hemolytic-uremic syndrome, which affects mostly children around the world (7). However, to date, no effective therapy has been found (7, 68). Antibiotics, antimotility agents, narcotics, and nonsteroidal anti-inflammatory drugs are not usually provided, as they increase the risk of developing hemolytic-uremic syndrome, a major cause of acute renal failure in children (68). The LEE operon genes in a pathogenicity island are required for forming the attaching and effacing lesion to the host epithelial cells (45), and quorum-sensing signals (AI-2 and an unidentified AI-3) are involved in the quorum-sensing regulation of the LEE genes (60, 61).

Bacterial biofilms are ubiquitous, and they have been extensively investigated under a variety of conditions. While there is increasing interest in bacterial biofilm formation in the gastrointestinal (GI) tract (30, 40), the genetic basis of EHEC biofilm formation is not well studied, whereas there are four single-time-point microarray studies of E. coli K-12 biofilm formation (5, 32, 51, 59) and one temporal study (11).

In this study, we found that EHEC serotype EDL933 forms robust biofilms under static conditions and a continuous-flow system. Surprisingly, biofilm cells formed on glass wool significantly repressed (3- to 11-fold) 20 pathogenic genes from the LEE island, compared to planktonic cells, which indicates that at 7 h, the virulence genes are not utilized yet. However, it is possible that the biofilm on glass wool lacks cues present for a biofilm on epithelial cells and that these cues may be necessary for induction of the virulence genes. It has been reported that the EPEC espA mutant formed more biofilms than wild-type EPEC (44). In our microarray data, espA was highly repressed (11-fold), along with repression of all the LEE operon genes, in the 7-h biofilm cells (see Table S1 in the supplemental material); however, EHEC biofilm formation in the GI tract may be different due to the presence of many signals from other GI bacteria and the host epithelial cells, unlike in the axenic glass wool culture used here. For example, we have determined that indole produced by E. coli K-12 and other commensal bacteria in the intestine affects EHEC chemotaxis and attachment (unpublished data), so indole is important for pathogenesis with EHEC. In addition, yceK, a predicted lipoprotein-encoding gene, is down-regulated eightfold in 7-h E. coli JM109 biofilms formed on glass wool (51) but was up-regulated eightfold in the EHEC biofilm; hence, this gene may play a different role in pathogenic E. coli or this may be an artifact of only a single time point used here. Also, colanic acid biosynthesis genes (wcaABCDEFGHIJKLMNOPQRST) and rcsA are down-regulated in young (7-h) EHEC biofilm cells, as seen previously with asymptomatic bacteruria E. coli (galEKTU) (23) and with nonpathogenic E. coli (wcd) (32). The fimbria-like gene ycbQ is down-regulated in E. coli K-12 BW25113 15-h biofilm cells (11) and was also repressed 14-fold in the EHEC biofilm. Also, agaY, a tagatose biphosphatase aldolase gene, was induced fivefold in our data, which agrees well with earlier studies (11). Furthermore, nitrate reductase genes were significantly changed in this study (narGHK are induced in biofilm cells, and narUZYWW are repressed with isatin), and narW were induced in biofilm cells of E. coli K-12 PHL628 (32); hence, our data indicate that nitrate metabolism may play a role in the E. coli biofilm formation during the transition from aerobiosis to anaerobiosis (32).

For compounds to be called cell-to-cell signals, they must satisfy four criteria (72): (i) the putative signal must be produced during a specific stage (indole is produced primarily in the stationary phase (71)), (ii) the putative signal must accumulate extracellularly and be recognized by a specific receptor (indole is a known extracellular signal [28, 53, 71] that is exported by AcrEF [34] and is imported by Mtr [77]), (iii) the putative signal must accumulate and generate a concerted response (indole has been shown to delay cell division [9]), and (iv) the putative signal must elicit a response that extends beyond the physiological changes required to metabolize or detoxify the signal (indole has been shown to control biofilms [36a] and cell division [9], which are not related to indole metabolism). Therefore, indole is a cell-to-cell signal molecule.

In this study, we demonstrate that hydroxylated indoles may be used to increase or decrease biofilm formation of pathogenic EHEC and that hydroxindoles and isatin are interspecies biofilm signals. These results are important in that they indicate that the signal indole may undergo interference via oxygenase attack (by cells which do not synthesize it), with the result that the altered molecule influences the same phenotype (biofilm formation) in a different manner due to the presence of an unrelated strain. For example, unoxidized indole inhibits biofilm formation in EHEC while oxidized indole (isatin) stimulates it (Fig. 1). Therefore, this is another example where cells can detect the presence of other bacteria by the way their signals are manipulated (75). Furthermore, since hydroxindoles are signals, this gives credence to our idea that some bacterial oxygenases may have evolved to regulate the concentrations of the interspecies signal indole by forming hydroxindoles (36a). For example, wild-type toluene o-monoxygenase of Burkholderia cepacia G4 (56) hydroxylates indole to form primarily 7-hydroxyindole in E. coli (unpublished data), so 7-hydroxyindole is available for signaling. We are currently studying the mechanism by which indole and 7-hydroxyindole affect biofilm formation of P. aeruginosa PAO1 and have found that indole and 7-hydroxyindole (1,000 μM) do not cause toxicity or induce a stress response (unpublished data).

Previously, we found that cysteine biosynthetic genes (cysPUW4) were induced in biofilm cells at 7 and 15 h and in young biofilm cells at 4 h (11) and that a plant extract biofilm inhibitor, ursolic acid, repressed cysDK (54). Also, deletion of cysE (65) or cysB (54) enhances biofilm formation in E. coli. Here, cysteine biosynthetic genes (cysPD) in biofilm cells were induced compared to those in planktonic cells, while 7-hydroxyindole most significantly repressed cysteine biosynthetic genes (cysPUIA4) and isatin repressed cysteine desulfurase genes (sufABCDE) that convert cysteine into alanine and sulfane-sulfane (38) (see Table S1 in the supplemental material). It appears that isatin and 7-hydroxyindole both work through sulfur metabolism but do so in an opposite manner. Therefore, these results confirm that sulfur metabolism plays an important role in E. coli biofilm formation. It also appears...
that 7-hydroxyindole and ursolic acid share a common mechanism of biofilm inhibition through sulfur metabolism. Unlike indole, 7-hydroxyindole does not work through SdiA in EHEC (no change of sdiA expression) and does not alter sdiA transcription in K-12, while indole inhibits E. coli K-12 biofilm formation through SdiA (36a).

In our temporal E. coli K-12 biofilm study, we found that motility and flagellar genes were induced in E. coli K-12 biofilm cells at 7 h and in planktonic cells or young biofilm cells at 4 h (11). In the current study, addition of isatin significantly induced 22 flagellar genes (flgABCFGHIJKLMN and flkACDFHJKLMNQPQ) in E. coli K-12 (52), and addition of AI-2 enhanced E. coli K-12 biofilm (20). Hence, isatin mimics AI-2 for stimulating biofilm formation. AI-2 concentrations were not altered by isatin (data not shown). Therefore, EHEC biofilm stimulation by isatin is not directly caused by AI-2 accumulation but is probably caused by the increased motility as a result of flagellar synthesis (74), the 20-fold reduction in indole concentration as a result of repression of imad (11, 12, 36a, 80), the changes in AI-2 transport, and the repression of sulfur metabolism (54, 65).

We determined the common differentially expressed genes between cells exposed to isatin and 7-hydroxyindole and for biofilm versus planktonic cells (Fig. 5). Totals of 347 and 552 genes were differentially expressed in the presence of 7-hydroxyindole and isatin, respectively, and for biofilm versus planktonic cells, the number of genes was 402. Of these, 19 genes were differentially expressed under all three conditions: 

**FIG. 5.** Common differentially expressed genes between isatin (250 μM), 7-hydroxyindole (1,000 μM), and biofilm cells grown in LB medium at 30°C at 250 rpm for 7 h.

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