

## Enterohemorrhagic *Escherichia coli* Biofilms Are Inhibited by 7-Hydroxyindole and Stimulated by Isatin<sup>∇†</sup>

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Since indole is present at up to 500  $\mu\text{M}$  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 polystyrene 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 isatin represses indole synthesis by repressing *tnaABC* 7- to 37-fold in EHEC, and extracellular indole levels were found to be 20-fold lower. Furthermore, isatin repressed the AI-2 transporters *lsrABCDFGKR*, while significantly inducing the flagellar genes *flgABCDEFGHIJK* and *flhAEFGILMNO PQ* (which led to a 50% increase in motility). 7-Hydroxyindole induces the biofilm inhibitor/stress regulator *ycjR* and represses *cysADIJPU/flhC* (which led to a 50% reduction in motility) and *purBCDEFHKL MNRT*. Isogenic mutants showed that 7-hydroxyindole inhibits *E. coli* biofilm through cysteine metabolism. 7-Hydroxyindole (500  $\mu\text{M}$ ) 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 furanosyl 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 furanones 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 eukaryotic 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  $\mu\text{M}$ ) when *E. coli* is grown in rich medium (12, 71) and was identified initially as a stationary-phase signal that controls *cysK*, *astD*, *tnaB*, and *gabT* (3) as well as *tnaAL* 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 biofilms 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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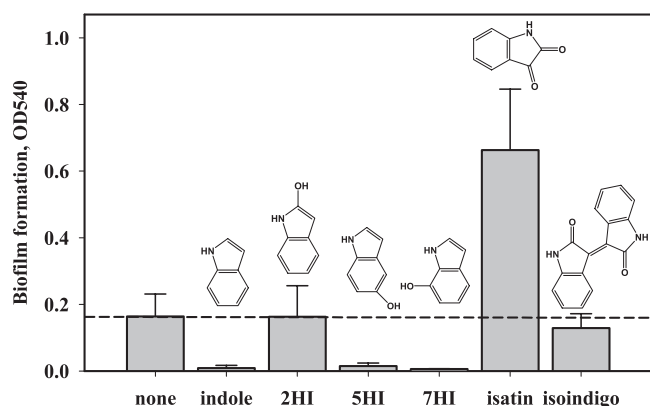


FIG. 1. Biofilm formation of EHEC in LB at 30°C after 7 h in 96-well plates with indole and hydroxyindoles. Indole, 2-hydroxyindole (2HI), 5-hydroxyindole (5HI), and 7-hydroxyindole (7HI) were used at 1,000  $\mu$ M, whereas isatin and isoindigo were used at 250  $\mu$ M. Each experiment was repeated 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.

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, an effective means of preventing EHEC biofilm formation has not been elucidated, and there is no effective treatment for EHEC infections, since antibiotic treatment increases the risk of hemolytic-uremic syndrome and renal failure (68, 73). We also tested the effect of hydroxyindoles on *P. aeruginosa* PAO1 since this strain is an opportunistic human pathogen (25).

Based on this proven competition for cell signals and given that indole controls biofilms (36a) and is present at up to 700  $\mu$ M (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. The genetic basis of EHEC biofilm formation was also investigated so that the impact of hydroxyindole signals on biofilm genes could be better interpreted.

## 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  $\mu$ M), 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  $\mu$ g/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 (BST model FC81; Biosurface Technologies Corp., Bozeman, MT), and was visualized with confocal microscopy as described

TABLE 1. Strains and plasmids used in this study<sup>a</sup>

Strain or plasmid	Description or genotype	Reference
<b>Strains</b>		
<i>E. coli</i> O157:H7	EHEC Stx1 <sup>+</sup> and Stx2 <sup>+</sup>	64 <sup>b</sup>
<i>E. coli</i> K-12 BW25113	<i>lacI</i> <sup>q</sup> <i>rnnB</i> <sub>T14</sub> $\Delta$ <i>lacZ</i> <sub>W116</sub> <i>hsdR514</i> $\Delta$ <i>araBAD</i> <sub>AH33</sub> $\Delta$ <i>rhaBAD</i> <sub>LD78</sub>	2
<i>E. coli</i> K-12 BW25113 $\Delta$ <i>cysB</i>	K-12 BW25113 $\Delta$ <i>cysB</i> $\Omega$ Km <sup>r</sup>	2
<i>P. aeruginosa</i> PAO1	Wild type	63
<i>Vibrio harveyi</i> BB170	BB120 <i>luxN::Tn5</i> (AHL sensor negative, AI-2 sensor positive)	66
<i>E. coli</i> UT481/pCX39	$\Delta$ <i>lac-pro met pro zzz::Tn10 thy supD</i> r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> <i>ftsQ2p::lacZ</i> Amp <sup>r</sup>	19
<b>Plasmids</b>		
pCM18	Em <sup>r</sup> ; pTRKL2-P <sub>CP25</sub> -RBSII- <i>gfpmut3*</i> -T <sub>0</sub> -T <sub>1</sub> (GFP plasmid for visualizing biofilm)	24
pCA24N- <i>cysB</i> <sup>+</sup>	Cm <sup>r</sup> ; <i>lacI</i> <sup>q</sup> pCA24N P <sub>T5-lac</sub> :: <i>cysB</i> <sup>+</sup>	35

<sup>a</sup> Km<sup>r</sup>, Amp<sup>r</sup>, Em<sup>r</sup>, and Cm<sup>r</sup> are the antibiotic resistance phenotypes for kanamycin, ampicillin, erythromycin, and chloramphenicol, respectively, and Stx is the Shiga toxin.

<sup>b</sup> 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 1,000  $\mu$ M in the continuous feed upon inoculation; DMF was added as the negative control to the no-hydroxyindole flow chamber. The initial inoculum was  $1.9 \times 10^8$  to  $4.7 \times 10^8$  cells/ml. Each flow chamber experiment was repeated two times (six experiments). A dispersion experiment was also performed with 7-hydroxyindole by adding 1,000  $\mu$ M 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 SP5 scanning confocal laser microscope with a 63 $\times$  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 microscopic 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, isatin (50  $\mu$ M) and 7-hydroxyindole (1,000  $\mu$ M) dissolved in DMF were added to the motility agar. DMF (0.1%) was added as the negative control. Each experiment was performed two times using two independent cultures, with each culture evaluated in triplicate.

**Biofilm total RNA isolation for DNA microarrays.** For the microarray experiments, 10 g glass wool (Corning Glass Works, Corning, NY) was used to form biofilms (51) in 250 ml of LB in 1-liter Erlenmeyer shake flasks which were inoculated with overnight cultures that were diluted 1:167. 7-Hydroxyindole (1,000  $\mu$ M) in 250  $\mu$ l DMF, isatin (250  $\mu$ M) in 250  $\mu$ l DMF, or 250  $\mu$ l DMF alone was added. The cells were shaken at 250 rpm and 30°C for 7 h to form biofilms on the glass wool. The glass wool was removed from the culture quickly and gently washed two times in 100 ml 0.85% NaCl buffer at 0°C (less than 30 seconds). Biofilm cells were removed from glass wool by sonication for 2 min in 200 ml of 0.85% NaCl buffer at 0°C, and cell pellets were stored at  $-80^\circ\text{C}$  until used. RNA was isolated from the planktonic and biofilm cells as described previously (51).

**DNA microarrays.** An *E. coli* GeneChip 2.0 genome array (product no. 900551; Affymetrix, Santa Clara, CA) was used to study the differential gene expression profile of the EHEC biofilm after addition of 7-hydroxyindole and isatin as described in the gene expression technical manual; the array contains 10,208 probe sets for open reading frames, rRNA, tRNA, and intergenic regions for four *E. coli* strains: MG1655, CFT073, O157:H7-Sakai, and O157:H7-EDL933 (EDL933 was used in this study). Hybridization was performed for 16 h, and the total cell intensity was scaled automatically in the software to an average value of 500. The data were inspected for quality and analyzed according to the procedures described in Data Analysis Fundamentals, which includes use of premixed polyadenylated transcripts of the *Bacillus subtilis* genes (*lys*, *phe*, *thr*, and *dap*) at different concentrations. Genes were identified as differentially expressed if the change in *P* value was less than 0.05 and the expression ratio was greater than threefold for biofilm versus planktonic cells, twofold for 7-hydroxyindole, or fourfold for isatin, since the standard deviations were 2.3 for biofilm versus planktonic cells, 1.5 for 7-hydroxyindole addition, and 3.9 for isatin addition. Gene functions were obtained from the Affymetrix-NetAffx analysis center.

**Real-time PCR.** To corroborate the DNA microarray data, the transcription 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  $\mu$ l, and 50 ng of total RNA was used for each reaction, with the final forward and reverse primer concentrations at 0.5  $\mu$ M each. After amplification, template specificity was ensured by using melting-curve analysis. *rnsG* was used as the housekeeping gene for normalizing the data.

TABLE 2. Primers used for RT-PCR

Gene	Forward primer	Reverse primer
<i>lsrA</i>	5'-AACATCCTGTTTGG GCTGGCAA-3'	5'-AAACAAGCGTTCGGTT TCCGCA-3'
<i>lsrB</i>	5'-AGCATCCTGGCTG GGAAATTGT-3'	5'-AAATTCITTCACCGTG CCGCGT-3'
<i>lsrC</i>	5'-ACAGCGTTTGAC GCAGTTT-3'	5'-ACGCAGGCTGCAATTG CTTT-3'
<i>cysP</i>	5'-AACCCGCGTTTGA ACAGCAAT-3'	5'-TGGATATTCTTCGGGT TGCCCT-3'
<i>rnsG</i>	5'-TATTGCACAATGG GCGCAAG-3'	5'-ACTTAACAACCGCCT GCGT-3'

**AI-2, indole, and  $\beta$ -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  $\mu$ M 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  $\text{H}_2\text{O}$ -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 (*ftsQ2p::lacZ*) (19) was grown at 30°C with 100  $\mu$ g ampicillin/ml from diluted overnight cultures to a turbidity of 2 at 600 nm. The  $\beta$ -galactosidase activities were calculated based on a protein concentration of 0.31 mg protein/ml/OD<sub>600</sub>. 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  $\mu$ M 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 (OD<sub>600</sub> ~2.0) were harvested and resuspended in 50 mM Tris-HCl buffer (pH 7.4; OD<sub>600</sub> ~10.0) and were contacted with 1,000  $\mu$ M 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* PAO1 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  $1.43 \pm 0.09/\text{h}$ , whereas the growth rates were  $1.38 \pm 0.01/\text{h}$  with 1,000  $\mu$ M indole,  $1.48 \pm 0.08/\text{h}$

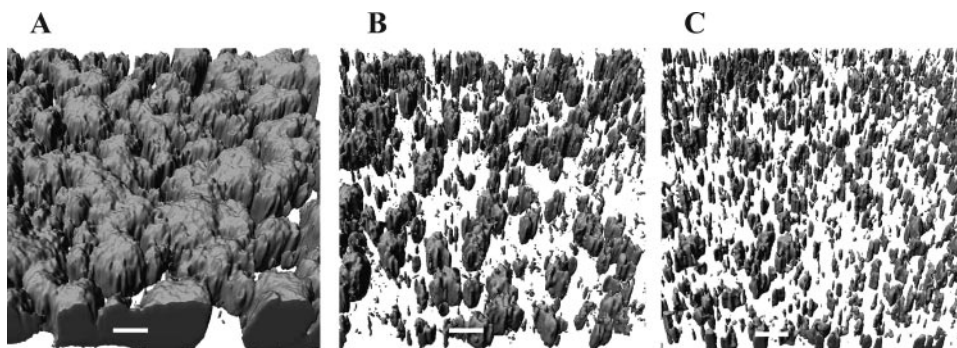


FIG. 2. EHEC biofilm formation in LB at 30°C after 24 h in flow cells without an indigoid compound but with the diluent DMF (A), with 1,000  $\mu\text{M}$  indole (B), and with 1,000  $\mu\text{M}$  7-hydroxyindole (C). Each experiment was repeated two times, and one representative image is shown. The scale bar indicates 20  $\mu\text{m}$ .

with 1,000  $\mu\text{M}$  5-hydroxyindole,  $1.14 \pm 0.03/\text{h}$  with 1,000  $\mu\text{M}$  7-hydroxyindole, and  $1.44 \pm 0.04/\text{h}$  with 250  $\mu\text{M}$  isatin. Hence, these indigoids do not significantly change the specific growth rate of EHEC. For 5-hydroxyindole, concentrations as high as 2,000  $\mu\text{M}$  did not affect cell growth ( $1.40 \pm 0.05/\text{h}$ ). In addition, indole, 5-hydroxyindole, 7-hydroxyindole, and isatin at 500  $\mu\text{M}$  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  $\mu\text{M}$ , and indigo, isoindigo, and indirubin were screened at 250  $\mu\text{M}$  since they were insoluble in water above 250  $\mu\text{M}$ . We chose concentrations of 250 to 1,000  $\mu\text{M}$  since EHEC produced  $362 \pm 3 \mu\text{M}$  indole after 8 h and K-12 produced  $403 \pm 8 \mu\text{M}$ , 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  $\mu\text{M}$  concentrations of indole, 2-hydroxyindole, 5-hydroxyindole, and 7-hydroxyindole as well as 250  $\mu\text{M}$  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  $\mu\text{M}$  stimulated biofilm formation in a dose-dependent manner, and 7-hydroxyindole from 0 to 2,000  $\mu\text{M}$  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 interspersed with water channels in rich medium at 30°C at 24 h. To our knowledge, this is the first EHEC biofilm image in a continuous-flow chamber. Upon addition of indole at 1,000  $\mu\text{M}$ , biofilm formation was reduced and consisted of individual slender microcolonies (Fig. 2B). Upon addition of 7-hydroxyindole at 1,000  $\mu\text{M}$ , 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 ( $7.9 \pm 3$  versus  $46 \pm 6 \mu\text{m}^3/\mu\text{m}^2$ ) and reduced mean biofilm thickness 4-fold ( $14 \pm 5$  versus  $55 \pm 5 \mu\text{m}$ ), and addition of 7-hydroxyindole reduced biofilm mass 10-fold ( $4.5 \pm 3$  versus  $46 \pm 6 \mu\text{m}^3/\mu\text{m}^2$ ) and reduced mean biofilm thickness 5.5-fold ( $10 \pm 5$  versus  $55 \pm 5 \mu\text{m}$ ). 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  $\mu\text{M}$  at 24 h, while the level of indole was maintained above 185  $\mu\text{M}$  for 24 h upon addition of indole (1,000  $\mu\text{M}$ ), and the level of 7-hydroxyindole was maintained above 540  $\mu\text{M}$  for 24 h upon addition of 7-hydroxyindole (1,000  $\mu\text{M}$ ). 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  $0.60 \pm 0.03 \text{ nmol/mg protein/min}$  (from 1,100  $\mu\text{M}$  to 380  $\mu\text{M}$  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  $\mu\text{M}$ ) 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).

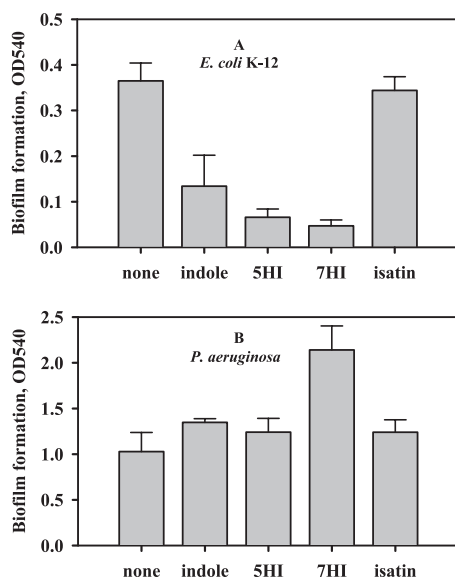


FIG. 3. Biofilm formation in LB at 30°C after 24 h in 96-well plates for *E. coli* K-12 BW25113 with 1,000  $\mu$ M indole, 1,000  $\mu$ M 5-hydroxyindole (5HI), 1,000  $\mu$ M 7-hydroxyindole (7HI), and 250  $\mu$ M isatin (A) and for *P. aeruginosa* PAO1 with 500  $\mu$ M indole, 500  $\mu$ M 5-hydroxyindole (5HI), 500  $\mu$ M 7-hydroxyindole (7HI), and 250  $\mu$ M isatin (B). Each experiment was repeated two to four times with six wells each, and 1 standard deviation is 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 formation 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  $\mu$ M) (12, 71), the levels of extracellular indole were measured from *E. coli* K-12 and EHEC shake flasks at 30°C for 24 h. *E. coli* K-12 produced  $401 \pm 11$   $\mu$ M indole, and EHEC produced  $308 \pm 12$   $\mu$ M indole at 24 h. The lower concentrations of extracellular indole for EHEC than for *E. coli* K-12 may partially explain the higher level of biofilm formation for EHEC than for *E. coli* K-12 (crystal violet density,  $1.76 \pm 0.15$  versus  $0.37 \pm 0.04$  at 30°C at 24 h).

To determine if hydroxyindoles are signals for pseudomonads too, we added indole, 5-hydroxyindole, 7-hydroxyindole, and isatin to *P. aeruginosa* PAO1. There was a significant increase in biofilm formation for *P. aeruginosa* PAO1 with 7-hydroxyindoles (Fig. 3B). Indole, 5-hydroxyindole, and isatin at 500  $\mu$ M increased *P. aeruginosa* PAO1 biofilm formation by 20 to 30%, and 7-hydroxyindole (500  $\mu$ M) increased biofilm formation twofold (Fig. 3B). It is notable that EHEC produces indole but *P. aeruginosa* alone does not produce indole or any hydroxyindoles in LB after 24 h and that indole and 7-hydroxyindole inhibit EHEC biofilm but enhance *P. aeruginosa* biofilm without cell growth inhibition. Also, it has been previously shown that *Pseudomonas* cells engineered to express toluene *o*-monooxygenase increase *E. coli* biofilm formation by de-

creasing indole concentration (36a). Therefore, we suggest that indole and hydroxyindoles are interspecies biofilm signals.

**Differential gene expression in 7-h EHEC biofilms.** To investigate the global genetic basis of hydroxyindole regulation of EHEC biofilm formation, DNA microarrays were first used to determine differential gene expression for EHEC biofilm cells versus planktonic cells. It was found that 402 genes were regulated significantly (more than threefold) in biofilms; 119 genes were induced, and 283 were repressed. Most noticeable was that in biofilm cells, 20 pathogenic genes of the locus of enterocyte effacement (LEE) operon (a total of 54 genes in EDL933) (48) were highly repressed (3- to 11-fold) (see Table S1 in the supplemental material). Also, 13 genes of the *wca* operon (*wcaABCDEFGHIJKL* and *rcaA*) that are involved in colanic acid synthesis and biofilm formation (50) and 6 fimbrial genes (*fimB*, *ycbQRS*, *ybgD*, and Z1538) were repressed in the biofilm cells (see Table S1 in the supplemental material). Nitrate reductase genes (*narGHIJK*) and the galactitol phosphotransferase (PTS) family of genes (*agaVWYZ*) 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  $\mu$ M) 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 (*purBCDEFHKL MNRT*) were repressed 3- to 12-fold; previously, we found that AI-2 repressed the *purDEFHKMT* operon in K-12 (52). In addition, genes related to cysteine biosynthesis (*cysADIIPU*) were repressed 4- to 11-fold, and previously, we found that the nontoxic biofilm inhibitor ursolic acid repressed *cysDJK* (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 *ftsQ2p* (19) was investigated using a  $\beta$ -galactosidase reporter; unlike indole (36a), neither 7-hydroxyindole nor isatin altered *ftsQ2p* expression significantly. Hence, these hydroxyindoles do not appear to work through SdiA.

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

To confirm the presence of 7-hydroxyindole, the extracellu-

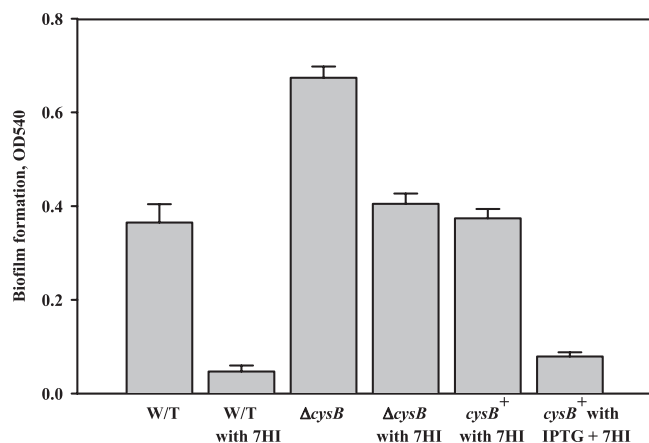


FIG. 4. Biofilm formation in LB at 30°C after 24 h in 96-well plates with expression of  $cysB^+$  (via 1 mM IPTG) and addition of 7-hydroxyindole (7HI, 1,000  $\mu$ M) with *E. coli* K-12 BW25113  $\Delta cysB/pCA24N-cysB^+$ . Each experiment was repeated two times with six wells each, and 1 standard deviation is shown. The wild type (WT) is BW25113,  $\Delta cysB$  is BW25113  $\Delta cysB$ , and  $cysB^+$  is BW25113  $\Delta cysB/pCA24N-cysB^+$ .

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  $\mu$ M in a shake flask at 7 h, which matches well with the extracellular 7-hydroxyindole (540  $\mu$ M) 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 *E. coli* biofilm formation (49) and since the microarray data showed that several flagellar genes (*c1936*, *fliC*, and *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% ( $2.3 \pm 0.5$  cm without hydroxyindole versus  $1.1 \pm 0.1$  cm with 1,000  $\mu$ M 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.

**Cysteine metabolism and 7-hydroxyindole.** Since 7-hydroxyindole repressed the cysteine synthesis operon *cysADEIJP* 4- to 11-fold (see Table S1 in the supplemental material), we tested the effect of overproducing CysB via *pCA24N-cysB^+* in an *E. coli* K-12 *cysB* mutant while adding hydroxyindoles. CysB positively regulates the biosynthesis of cysteine in *E. coli* (46), and deletion of *cysB* increased biofilm formation (Fig. 4), as has been previously reported (54). Addition of 1,000  $\mu$ M 7-hydroxyindole to the *cysB* mutant resulted in a 1.8-fold reduction of biofilm (Fig. 4), while addition of 1,000  $\mu$ M 7-hydroxyindole to the K-12 wild type resulted in a 7.7-fold reduction of biofilm (Fig. 4); hence, 7-hydroxyindole is less effective in the absence of CysB. Also, induction of *cysB* (via addition of 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) to induce *cysB^+* from *pCA24N-cysB^+*) in the presence of 1,000  $\mu$ M 7-hydroxyindole resulted in a 4.7-fold greater reduction in biofilm (Fig. 4). Similar results were found with EHEC that overproduces CysB from *pCA24N-cysB^+*. Expression of *cysB* with 1 mM IPTG made 1,000  $\mu$ M 7-hydroxyindole 10-fold  $\pm$  3-fold more effective in reducing biofilm formation (note that these experiments

differ from those for Fig. 1, which lacked the *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  $\mu$ M) significantly regulated (more than fourfold) 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 (*lsrABCDFGKR*), indole biosynthesis genes (*tnaABC* and *yliH*), cysteine desulfurase genes (*sufABCDE*), nitrate reductase genes (*narHIJUVWYZ*), hydrogen uptake genes (*hyaABCDEF*), propionate metabolism genes (*prpBCDE*), and acid resistance-related genes (*gadABC*) (see Table S1 in the supplemental material).

The genes most significantly induced by isatin were flagellar genes (*flgABCDEFGHIJK* and *fliAEFGILMNOPQ*), 44 various transport genes (e.g., *proVWX* and *cirA*), and *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  $\mu$ M) on motility and found that it was increased by  $47\% \pm 8\%$ ; 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 (*fliC*, *c1936*, and *motAB*), nitrate reductase genes (*narUVWYZ*), propionate metabolism genes (*prpBCDE*), carbamoyl phosphate synthetase genes (*carAB*), transport genes (*yieG*, *lldP*, *uraA*, and *sdaC*) and *phoH*, *ompF*, and *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  $\mu$ M isatin since *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  $\pm$  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  $\pm$  0.5-fold, while 7-hydroxyindole had no effect on intracellular indole concentration.

Since the AI-2 transporter genes (*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  $\mu$ M AI-2 increased EHEC bottom biofilm formation in LB medium 3.0-fold  $\pm$  0.7-fold after 15 h and 10-fold  $\pm$  2.5-fold after 24 h; total biofilm was increased 1.6-fold  $\pm$  0.2-fold after 24 h. Therefore, AI-2 increases EHEC liquid/solid biofilm formation as it does that of K-12 (20).

**Verification of microarray results by RT-PCR.** RT-PCR was used to verify gene expression for *lsrA*, *lsrB*, and *lsrC* for the isatin microarray experiments and for *cysP* for the 7-hydroxyindole microarray experiment. The results showed comparable changes in expression for the isatin arrays for *lsrA* ( $-37$ -fold in microarrays versus  $-72$ -fold with RT-PCR), for *lsrB* ( $-64$ -fold in microarrays versus  $-110$ -fold with RT-PCR), and for *lsrC*

(−18-fold in microarrays versus −41-fold with RT-PCR) as well as for *cysP* for the 7-hydroxyindole arrays (−11.8 in microarrays versus −17.3 with RT-PCR).

## 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 (*wcaABCDEFGHIJKL* and *rcsA*) are down-regulated in young (7-h) EHEC biofilm cells, as seen previously with asymptomatic bacteriuria *E. coli* (*galEKTU*) (23) and with nonpathogenic *E. coli* (*wcaJ*) (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 biphosphate 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 (*narIGHK* are induced in biofilm cells, and *narUZYWW* are repressed with isatin), and *narVW* 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 hydroxyindoles 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 hydroxyindoles 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 hydroxyindoles (36a). For example, wild-type toluene *o*-monooxygenase 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  $\mu$ M) do not cause toxicity or induce a stress response (unpublished data).

Previously, we found that cysteine biosynthetic genes (*cysPUWA*) 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 *cysDJK* (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 (*cysPJUIDA*) and isatin repressed cysteine desulfurase genes (*sufABCDSE*) that convert cysteine into alanine and sulfane-sulfur (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

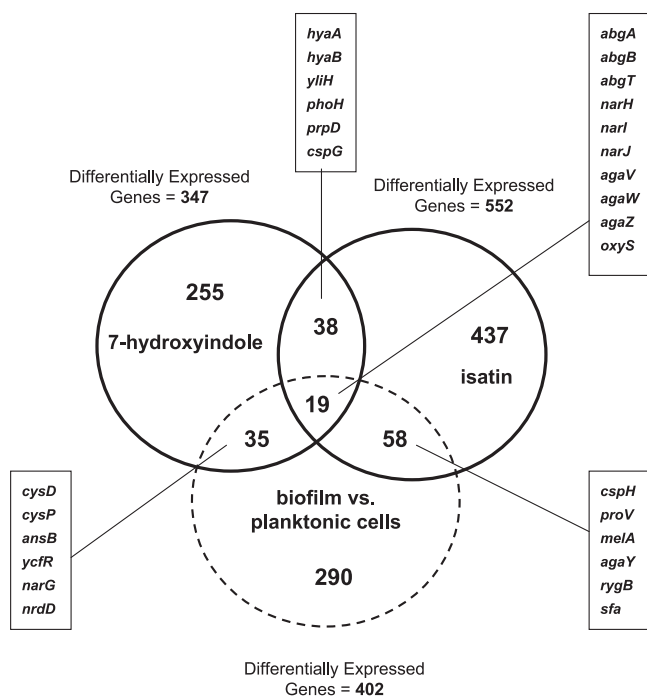


FIG. 5. Common differentially expressed genes between isatin (250  $\mu$ M), 7-hydroxyindole (1,000  $\mu$ M), and biofilm cells grown in LB medium at 30°C at 250 rpm for 7 h.

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 (*flgABCDEFGHIJK* and *fliAEFGILMNOPQ*) and repressed the AI-2 importers *lsrABCD*, *GKR* (see Table S1 in the supplemental material). In an analogous fashion, AI-2 induced the same flagellar genes (*flgABCDEFGHIJKLMN* and *fliACDFHIKLMNOPQ*) 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 *tnaA* (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:

*abgABT*, involved in *p*-aminobenzyl-glutamate utilization (31), *agaVWZ*, part of the *N*-acetylgalactosamine PTS system (8), and *narIJH*, associated with nitrate reductase (13), were all repressed in the presence of isatin and 7-hydroxyindole, whereas they were up-regulated in biofilm cells. Fifty-seven genes were common between isatin and 7-hydroxyindole cells, and the most prominent was the *hyaABCDE* operon, associated with hydrogenase (42), which was highly repressed in isatin biofilm cells. The hydrogenase genes *hyaABDEF*, as well as other hydrogenase genes (*hybAC* and *hycABF*), were significantly repressed in biofilm cells of *E. coli* K-12 MG1655 (59). The hydrogenases are probably used to regulate pH by removing the acid formate by converting it to CO<sub>2</sub> and H<sub>2</sub> (46), and hydrogenase 1 (*hya* operon) serves only to remove hydrogen (17), so if isatin represses these genes, then perhaps this is a result of internal pH changes induced by isatin. Furthermore, *yliH* was also repressed 9.8-fold by isatin, and since YliH represses biofilm formation (12), it is consistent that isatin repressed this gene while increasing biofilm formation. *oxyS* RNA is involved in detoxifying oxidative damage (21); this locus was induced 4.6-fold by isatin, and this indicates that isatin may also cause oxidative stress, as has been seen with K-12 biofilms previously (51). Seventy-seven genes were common between isatin-treated cells and biofilm cells, and 54 genes were common between 7-hydroxyindole-treated cells and biofilm cells.

In this study, we verified the hypothesis that hydroxyindoles may be signals, based on our recognition of the large extracellular indole concentrations and the prevalence of relatively nonspecific oxygenases that can hydroxylate indole, such as monooxygenases and dioxygenases in *Pseudomonas*, *Ralstonia*, and *Burkholderia* species (15, 16, 56). It was found that indole, 5-hydroxyindole, and 7-hydroxyindole are potent inhibitors of EHEC biofilm formation (which suggests their use as potential therapeutics), whereas isatin stimulates biofilm formation. Along with brominated furanones (52) and ursolic acid (54), 7-hydroxyindole becomes one of the few known nontoxic biofilm inhibitors.

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