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Curcumin, a Known Phenolic from *Curcuma longa*, Attenuates the Virulence of *Pseudomonas aeruginosa* PAO1 in Whole Plant and Animal Pathogenicity Models

THIMMARAJU RUDRAPPA AND HARSH P. BAIS*

Department of Plant and Soil Sciences, University of Delaware, Delaware Biotechnology Institute, Newark, Delaware 19711

The effect of curcumin on the virulence of *Pseudomonas aeruginosa* (PAO1) using whole plant and animal pathogenicity models was investigated. The effect of curcumin on PAO1 virulence was studied by employing in vitro assays for virulence factor production, *Arabidopsis thaliana*/*Caenorhabditis elegans* pathogenicity models, and whole genome microarray analysis. It is shown that the curcumin inhibits PAO1 virulence factors such as biofilm formation, pyocyanin biosynthesis, elastase/protease activity, and acyl homoserine lactone (HSL) production. As a consequence of this, curcumin treatment resulted in the reduced pathogenicity of *P. aeruginosa*−*C. elegans* and *P. aeruginosa*−*A. thaliana* infection models. In addition, transcriptome analysis of curcumin-treated PAO1 revealed down-regulation of 31 quorum sensing (QS) genes, of which many have already been reported for virulence. The supplementation of HSLs along with the curcumin treatment resulted in increased pathogencity and recovery of higher bacterial titers in a plant pathogenecity model. These data reveal the involvement of curcumin in QS interruption to reduce pathogenicity. Curcumin attenuates PAO1 virulence by down-regulation of virulence factors, QS, and biofilm initiation genes. The effect of curcumin on multiple targets such as virulence, QS, and biofilm initiation makes curcumin a potential supplemental molecule for the treatment of *P. aeruginosa* infections.

KEYWORDS: Curcumin; biofilm; quorum sensing; microarray; virulence

INTRODUCTION

For over 50 years, a handful of antibiotics have been the sole agents used in the treatment of most bacterial infections (*1*). This has led researchers to search for alternative natural chemicals and to re-evaluate previously characterized natural plant products for their potential use in treating infectious diseases. *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen commonly found in soil and water (*2*), capable of infecting immunocompromised patients, including individuals with cystic fibrosis and extensive burn wounds (*3*). The production of an impressive array of both cell-associated and extracellular virulence factor by *P. aeruginosa* is regulated by a cell-to-cell communication signaling mechanism known as quorum sensing (QS) (*4*). In *P. aeruginosa*, and most Gram-negative bacteria, these signal molecules are *N*-acyl-homoserinelactones (AHLs) (*5*). *P. aeruginosa* possesses two known AHL-dependent QS systems: *lasR*−*lasI* and *rhlR*−*rhlI*, with the cognate signal molecules *N*-(3-oxododecanoyl)-L-homoserinelactone (3-oxo-C_{12}-HSL) and *N*-butyryl-L-homoserinelactone (C_4-HSL), respectively. It is known that the *rhl* system (RhlR/C_4-HSL) is important for optimal biofilm formation, whereas the *las* system (LasR/3-oxo-C_{12}-HSL) is essential for biofilm differentiation and plays a role during the irreversible attachment stage (*6*). These signals mediate the synthesis of a variety of virulence factors such as exoproteases, siderophores, exotoxins, and several secondary metabolites (*7*). Research has suggested that QS circuits play an important role in *P. aeruginosa* biofilm development (*8, 9*). Initial studies have demonstrated that biofilm maturation in *P. aeruginosa* is dependent upon the *las*, but not the *rhl*, QS system (*5, 10*).

Until 1995, it was generally believed that human pathogens were incapable of infecting plants. This view changed when Rahme et al. (*2*) reported that *P. aeruginosa* PA14 was a potent foliar pathogen in a variety of plants. Recent work has demonstrated that *P. aeruginosa* (PAO1) is a successful root pathogen of *Arabidopsis* and *Caenorhabditis elegans* (*11, 12*). In addition, the antimicrobial compounds in root exudates influenced PAO1 biofilm formation on roots, possibly by targeting QS (*11*). Given that plant-derived compounds have been used to treat microbial infections for centuries (*1*), screening of plant-derived compounds may facilitate the discovery of antibacterials that attenuate bacterial pathogenesis by interfering with QS systems.

* Corresponding author [e-mail hbais@udel.edu; fax (970) 831-3447].

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The role of QS circuits in *P. aeruginosa*'s pathogenesis is still unsettled. Previous work has shown that QS mutants are attenuated in virulence in a *C. elegans* model, but they exhibit full virulence on the *Arabidopsis thaliana* root model system (*11, 13–15*). Although some studies suggest biofilms produced by QS mutants of *P. aeruginosa* are not fully developed, leaving bacterial cells more susceptible to antimicrobials (*16, 17*), Heydorn et al. (*16*) reported that biofilm formation in a *lasI* mutant is nearly indistinguishable from that in wild type. Our studies using plant root infection model also show that the QS mutants show similar root infectivity against *A. thaliana* compared to the wild type *P. aeruginosa* (*11*). These variable results suggest that QS-mediated cell-to-cell communication may not be necessary for *P. aeruginosa* biofilm formation under all experimental conditions. The efforts to understand the involvement of QS and biofilm formation in *P. aeruginosa*'s pathogenicity should be rigorously tested by evaluating the effect of various known and unknown compounds that attenuate either QS or biofilm formation. The testing of natural products that target functions important for bacterial virulence or activators of host innate responses should be given preference compared to the ones that inflict direct antimicrobial effect.

Curcumin is a major constituent of turmeric (*Curcuma longa* L.) roots/rhizomes. Turmeric has traditionally been used as an insect repellent, and the antimicrobial activities of its extract are well-established (*18, 19*). In contrast, curcumin shows weak antimicrobial activity against *P. aeruginosa* (*20*). In this paper, we report that curcumin at sub-minimum inhibitory concentration (MIC) level reduced *P. aeruginosa* pathogenicity by down-regulating virulence factors through the reduction of QS signals. Additionally, we also show the use of curcumin as a potential candidate to minimize *P. aeruginosa* pathogenesis in *C. elegans* and *Arabidopsis* systems. These effects were corroborated by microarray analysis data of the curcumin-treated *P. aeruginosa* transcriptome. Our work reveals the importance of curcumin as a major anti-infective compound against *P. aeruginosa*.

MATERIALS AND METHODS

Chemicals. Curcumin and rifampicin were purchased from Sigma-Aldrich (St. Louis, MO). Ciprofloxacin was obtained from Bayer Inc. (Morristown, NJ). The 3-oxo-C_{12}-HSL and C_4-HSLs were procured from Cayman Chemical Co., Burlington, Ontario, Canada. Stock solutions of all the chemicals were prepared with HPLC grade methanol except 3-oxo-C_{12}-HSL and C_4-HSLs, which were prepared in analytical grade distilled ethanol.

Bacterial Strain, Plant Material, and Growth Conditions. *P. aeruginosa* clinical isolate PAO1 was used and maintained as described (*2*). Seeds of wild-type *A. thaliana* ecotype Columbia (Col-0) were purchased from Lehle Seeds (Round Rock, TX). Seeds were surface sterilized using commercial sodium hypochlorite (0.3% v/v) for 10–12 min and washed four times in sterile double -distilled water. Surface-sterilized seeds were placed on Murashige and Skoog (MS) (*21*) basal solid media in Petri dishes for germination and incubated in a growth chamber illuminated with cool white fluorescent light (45 μmol m^{-2} s^{-1}). Twenty-day-old seedlings were transferred to six-well plates containing 4 mL of liquid MS basal media. Plant cultures were maintained on an orbital platform shaker (Laboratory-Line Instruments) set at 90 rpm with a photoperiod of 16 h of light and 8 h of darkness at 25 ± 2 °C.

Effect of Curcumin on Growth and biofilm Formation in *P. aeruginosa* PAO1. For growth assays, PAO1 was grown in LB medium in the presence or absence of curcumin (0.5– 30.0 μg mL^{-1} or ciprofloxacin (3 μg mL^{-1}) using an initial OD_{600} of 0.02. Curcumin and ciprofloxacin were initially dissolved in ethanol and methanol separately (maximum concentration of the solvent used was 0.02% v/v) and supplemented to the LB/MS medium. Curcumin supplementation to the liquid LB media did not change the pH of the media. Bacterial growth was measured at 600 nm at 5 h intervals up to 25 h of the growth period. Bacterial number was enumerated using the colony counts post curcumin treatment.

The effect of curcumin (1.5–3.0 μg mL^{-1}) on *P. aeruginosa* PAO1 biofilm formation on abiotic surfaces was studied using a crystal violet assay as previously described (*22*), using polypropylene tubes. Biofilms were quantified by crystal violet staining followed by ethanol solubilization and measuring the optical density (OD) at 600 nm (*22*). For biofilm quantification, curcumin was added at less than MIC levels (1.5–3.0 μg mL^{-1}) to LB medium at the onset of incubation period. The volume of ethanol added was adjusted such that all tubes contained equal concentration of the solvent. All treatments had three replicates each.

Effect of Curcumin on Virulence Factors Production in *P. Aeruginosa* PAO1. To determine the effect of curcumin on pyocyanin biosynthesis, PAO1 was grown in LB medium in the presence or absence of curcumin (1.5–3 μg mL^{-1}) and harvested as previously described (*23, 24*). Pyocyanin was estimated and normalized against live cell numbers following the method described in the literature (*23, 24*). Briefly, to determine pyocyanin concentrations, PAO1 was grown in LB medium in the presence or absence of curcumin (1.5—5 μg mL^{-1}) and harvested as described previously (*16, 17*). Pyocyanin was estimated following the method described by Mahajan-Miklos et al. (*23*). Plate assays for total protease and elastase activities were performed as described by Brint and Ohman (*25*). Protease activity was estimated using the method described by Brint and Ohman (*25*), with slight modifications. The bacterial culture was grown with and without curcumin in 50 mL of 5% peptone and 0.25% tryptic soy broth (PTSB) at 37 °C for 24 h. The supernatant was collected and filter-purified using a 0.22 μm nylon filter. A 50 μL aliquot of the supernatant was then added to the reaction mixture containing 0.8% azocasein (Sigma Chemical Co.) in 500 μL of 50 mM K_2HPO_4, pH 7. The reaction mixture was incubated at 25 °C for 3 h. The reaction was stopped by adding 0.5 mL of 1.5 M HCl and placed on ice for 30 min and centrifuged. After adding 0.5 mL of 1 N NaOH, the optical density was recorded at 440 nm. To determine elastase activity, 50 μL of supernatant from the 24 h PTSB cultures was added to the tubes containing 1 mL of 10 mM Na_2HPO_4, pH 7, and 20 mg of elastin−congo red (Sigma). The reaction mixture was incubated for 4 h at 37 °C in an incubator shaker. Tubes were then centrifuged, and the optical density at 495 nm was recorded.

Effect of Curcumin on the Pathogenicity of *P. Aeruginosa* PAO1 on *Arabidopsis*. Ten-day-old *A. thaliana* plants ($n = 20$ per treatment) were used for the in vitro root pathogenicity assays. PAO1 was adjusted to an $OD_{600} = 0.02–0.04$ (~2.8×10^8 cells mL^{-1}) by adding into 2 mL of liquid MS medium holding *A. thaliana* plants. A noninoculated control was maintained under similar conditions. To assess the effect of curcumin on PAO1 pathogenicity, curcumin (3.0 μg mL^{-1}) was added to the MS (*21*) medium along with the bacteria. Each experiment was conducted in triplicate. Plant mortality was monitored after inoculation with *P. aeruginosa* in the presence and absence of curcumin. *Arabidopsis* plants showing severe disease symptoms in the form of root rotting and chlorosis were considered to be dead. Data are the average of two independent experiments. Bars show one standard error. Two-way ANOVA for plant mortality: $F_{\text{treatment}} = 12.30$, df = 1, 31, $P < 0.005$.

The biofilm experiment on *Arabidopsis* root surface was performed as described previously (*11, 26*). A similar experiment was also conducted to estimate the in root bacterial count as colony forming units (CFU) per gram of fresh weight as described previously (*11, 20*).

Effect of Curcumin on the Pathogenicity of *P. aeruginosa* PAO1 in *C. elegans*. PAO1 was grown overnight at 37 °C and supplemented in the nematode growth medium (NGM) as per the published protocol (*27*). The NGM plates were incubated at 37 °C for 4−6 h and allowed to equilibrate to room temperature before seeding with the adult worms (N2 Bristol). Before solidification, all of the experimental plates were supplemented with 40 μmol 5-fluorodeoxyuridine, which was used to prevent progeny development. The anti-infective property of

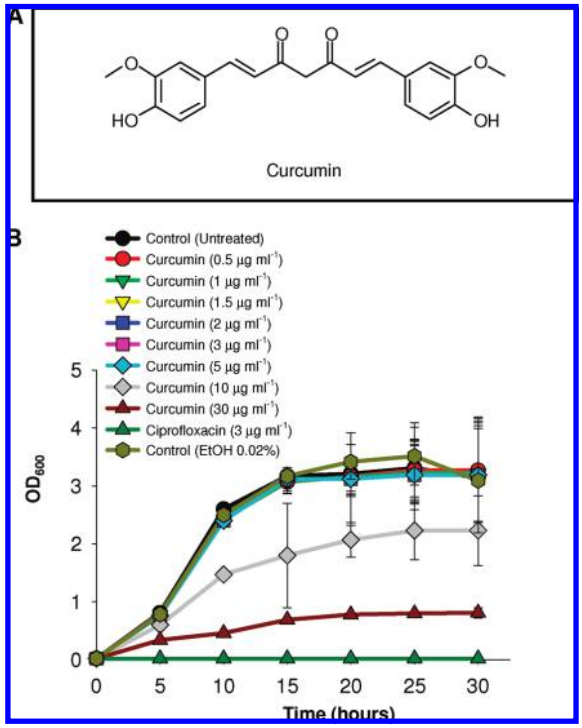

Figure 1. (**A**) Chemical structure of curcumin. (**B**) Effect of curcumin on planktonic growth of *P. aeruginosa* PAO1. Cells were grown in LB medium containing different concentrations of curcumin; growth was assessed by reading the absorbance at 600 nm at indicated time points. (Values ± standard error; $n = 6$.)

curcumin was tested by using the *P. aeruginosa* kill assay protocol described by Tan et al. (*27*). In each assay, 20–30 adult nematodes were transferred per plate, conducted in triplicates. The bacterial population inside the nematode guts was determined as described by Garsin et al. (*28*).

Extraction and Quantification of Total AHLS and 3-Oxo-C_{12}-HSL and C_4-HSLs. Acyl-HSLs were extracted from curcumin-treated PAO1 cultures and a control (without curcumin treatment). Solutions of 1 μg μL^{-1} of the three acyl-HSL standards (*N*-hexanoyl-DL-homoserinelactone, *N*-octonoyl-DL-homoserinelactone, and *N*-dodecanoyl-DL-homoserinelactones from Fluka, Mannheim, Germany) were prepared in methanol and injected for HPLC testing. 3-Oxo-C_{12}-HSL and C_4-HSL, the HSL molecules implicated in *P. aeruginosa* QS, were extracted as described previously (*29*). Furthermore, the samples were subjected to electrospray ionization mass spectrometry (ESI-MS) to determine the molecular ion traces in the extracts from curcumin-treated PAO1 culture. The values were expressed as micromoles of total HSLs produced. The experiment had three replicates each and was repeated twice. The data were analyzed by pairwise *t* test and considered to be significant at $P = 0.05$.

Cotreatment of AHL/3-Oxo-HSL and Curcumin on *A. thaliana* Plants Infected with PAO1. *A. thaliana* plants infected with PAO1 were cotreated with AHL/3-oxo-HSL and curcumin. *A. thaliana* plants were infected with PAO1 following a similar protocol as per the above-mentioned description. We added curcumin (3 μg mL^{-1}) and AHL/3-oxo-HSL (100 μmol each) in the medium carrying PAO1 infected *A. thaliana* plants. Post-7-day plant roots were harvested, and bacterial enumeration was performed and represented as CFU per gram of fresh weight as described previously (*11, 26*). Each experiment was conducted in triplicate.

Microscopy. To view the adherent PAO1 cells by confocal microscopy, the samples were stained with the *Bac*Light Kit (Molecular

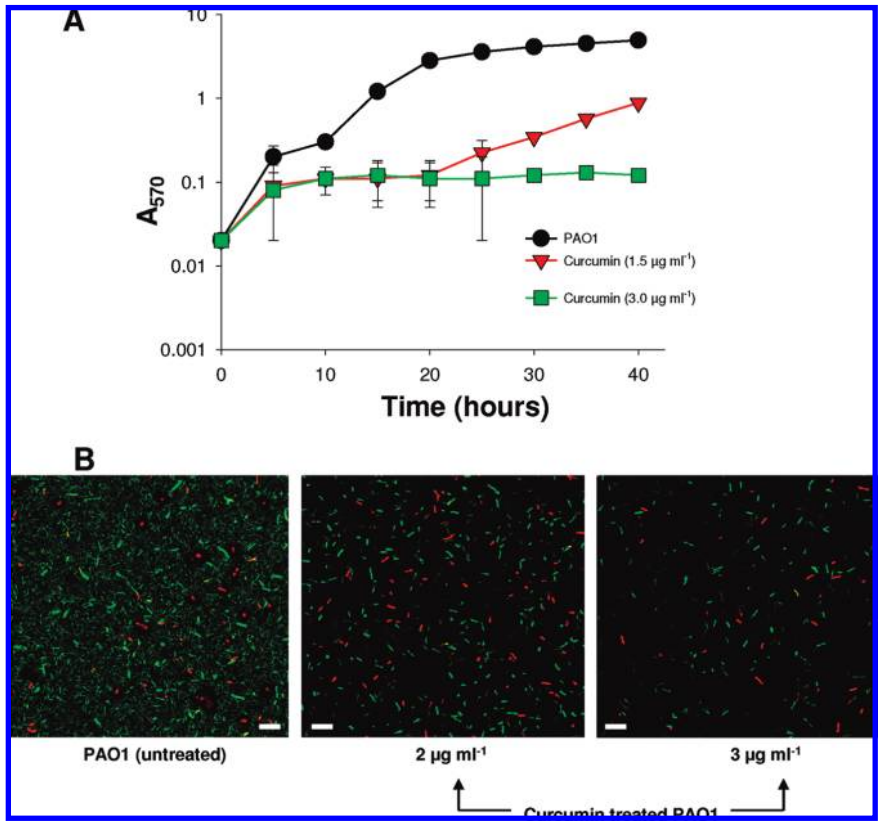

Figure 2. Curcumin inhibits biofilm formation. (**A**) Optical density (OD) readings from microtiter plate assays of biofilm formation by PAO1 untreated and curcumin-treated PAO1. OD_{570} of solubilized crystal violet from microtiter assays over time for curcumin-treated PAO1 is shown (mean ± SD; $n = 5$). (**B**) Confocal scanning laser microscopy of untreated PAO1 and curcumin-treated PAO1 (post 48 h) grown on two-well Permanox sunken glass slides. PAO1 untreated slide in panel **B** shows a continuous biofilm compared to curcumin-treated PAO1. PAO1 in the absence and presence of curcumin was stained with a LIVE/DEAD *Bac*Light Bacterial Viability Kit. The green fluorescence represented by SYTO 9 stain shows live bacteria compared to red-fluorescent propidium iodide exhibiting dead bacteria. Bars = 5 μm.

Probes, Eugene, OR) as described earlier (*11, 26*). The *Bac*Light live dead kit stains viable bacterial cells exhibiting green fluorescence, compared to dead cells showing red fluorescence. Images were captured with a 10× objective on a Zeiss 5 LSM 510 NLO attached to an Axiovert 200 M with an automated stage microscope equipped with Zeiss LSM 510 software. CSLM was performed 48 h postinoculation and post-treatment with curcumin (2–3 μg mL^{-1}). Each experiment was repeated twice with three replicates each.

Microarray Gene Expression Analysis of PAO1 Treated with Curcumin. PAO1 was grown in triplicates in LB medium supplemented with 3 μg mL^{-1} of curcumin with the initial OD_{600} adjusted to ~0.02 at 37 °C on an incubator shaker set at 200 rpm for 10 h. At the end of the incubation period (~2 OD_{600}), 5 mL of the culture was removed and RNA was isolated using a Ribopure RNA isolation kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions in triplicates separately. The microarray analysis was performed at Ambion Inc. Ten micrograms of the RNA was amplified using a MessageAmp-II Bacteria kit (Ambion Inc.) according to the manufacturer's instructions. The data were normalized per chip by dChip invariant set normalization, and each gene was normalized to the median taken for that gene across all samples. For data analysis, the average intensity of >200 at least in one of the comparison conditions was used. A two-sample *t* test using a *P* value cutoff of 0.05 was applied to identify genes that were statistically differentially expressed.

Statistical Analysis. All of the data were analyzed for variance using Microsoft Excel XP version, followed by a pairwise Student's *t* test (*30*). The data means were considered to be different at the probability of $P < 0.05$.

RESULTS

Effect of Curcumin on PAO1 Growth. To test the efficacy of curcumin (**Figure 1A**) against *P. aeruginosa*, we first investigated its effect on planktonic growth of PAO1. Whereas curcumin concentrations below 3 μg mL^{-1} did not affect the planktonic growth, concentrations greater than 5 μg mL^{-1} partially inhibited bacterial growth (**Figure 1B**). The MIC for PAO1 using curcumin was recorded at 30 μg mL^{-1} (**Figure 1B**).

Curcumin Inhibits biofilm Initiation on Abiotic Surfaces. Adherence of the bacteria to polypropylene tubes (*15*) was used to test our hypothesis that curcumin inhibits biofilm initiation in PAO1. Sub-MIC levels of curcumin (1.5–3 μg mL^{-1}) were supplemented in the media from the onset of incubation with PAO1, and the number of adherent cells was quantified. **Figure 2A** shows the inhibition of biofilm formation in PAO1 by curcumin concentrations of less than the MIC. Confocal microscopy data also suggested the inhibition of biofilm formation by curcumin (2–3 μg mL^{-1}) leading to negligible bacterial aggregations (**Figure 2B**).

Curcumin Down-regulates PAO1 Virulence Factor. We tested the effect of curcumin on pyocyanin production by PAO1. The data suggested that treatment with 1.5–3 μg mL^{-1} of curcumin reduced the production of pyocyanin by more than ~60–80% (**Figure 3A**). Apart from pyocyanin, PAO1 also produces other virulence factors, such as protease and elastase, which are regulated by the *lasI*−*lasR* and *rhlI*−*rhlR* QS systems (*18*). We investigated the effect of curcumin on the production of these virulence factors in PAO1. The data suggested that the curcumin treatment resulted in an approximately 2-fold decrease in the production of protease and elastase (**Figure 3B**,**C**).

To check whether curcumin affects the QS system, we studied the effect of curcumin on the production of HSLs. Treatment with 1 μg mL^{-1} curcumin resulted in a 25% reduction in 3-oxo-C_{12}-HSL and a >2% reduction in C_4-HSL production compared to controls ($t = 5.95$; $P = 0.004$) (**Figure 4A**,**B**). Furthermore, an approximately 3-fold reduction in total homoserine lactone synthesis was observed with 5 g mL^{-1} curcumin treatment

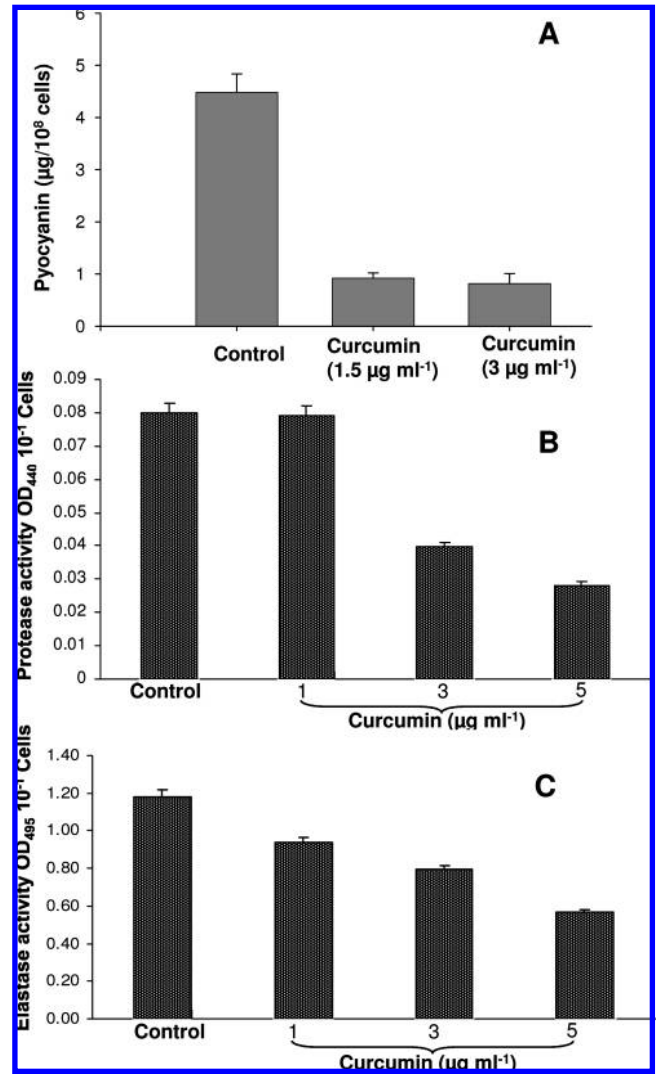

Figure 3. Effects of curcumin on virulence factors production: (**A**) levels of pyocyanin in curcumin-untreated and -treated PAO1; levels of total protease (**B**) and elastase (**C**) activity in curcumin-untreated and -treated PAO1. All virulence assays were performed as a proportion of cell density to exotoxin production to rule out the possibility of curcumin effect on PAO1 growth.

(Supporting Information Figure 1). The HPLC traces of 3-oxo-C_{12}-HSL and C_4-HSL in curcumin-treated culture filtrate extracts were confirmed using ESI-MS (data not shown). All of these data together suggest that curcumin treatment affected both virulence factors production and the QS system in PAO1.

Effect of Curcumin on PAO1 Infectivity on *A. thaliana* Plants. We hypothesized that if curcumin inhibited virulence factors of *P. aeruginosa*, treatment of *A. thaliana* with curcumin could prevent bacterial infections in planta. Therefore, we tested the effects of curcumin on the PAO1 infection of *A. thaliana* (Col-0) roots at sub-MIC levels (1–3 μg mL^{-1}). Consistent with our hypothesis, curcumin-treated plants had lower mortality rates compared to controls (**Figure 5A**,**B**). Interestingly, curcumin-treated *Arabidopsis* plants infected with PAO1 showed patchy film formation on the roots, compared to robust biofilm on untreated roots (**Figure 5C**). All of these data together indicate that curcumin treatment causes a reduction in the plant mortality by suppressing biofilm formation, a potent virulence factor of *P. aeruginosa* for its pathogenicity in plants (*11*).

Supplementation of AHL/3-Oxo-HSL along with Curcumin in PAO1-Infected *A. Thaliana* Plants. Our previous

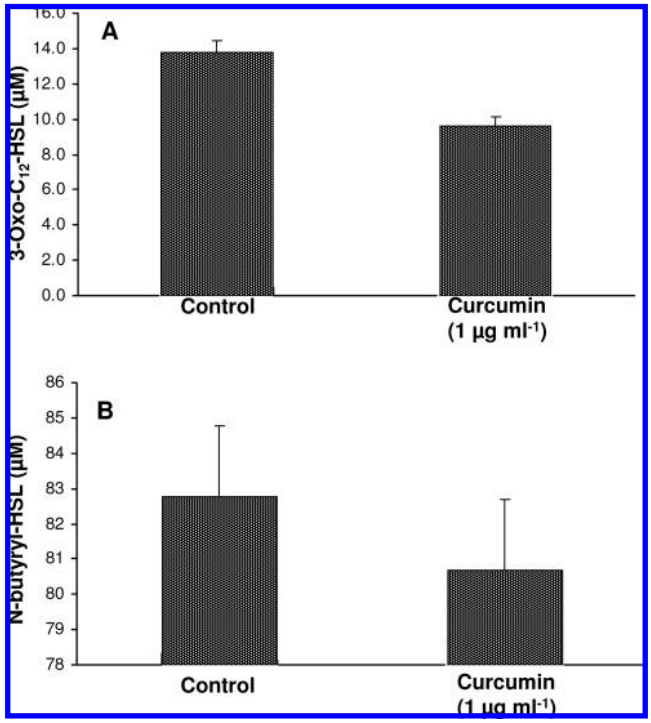

Figure 4. Effect of curcumin on homoserine lactone production. Levels of 3-oxo-*N*-dodecanoyl-L-homoserine lactone (**A**) and *N*-butyryl-L-homoserine lactones (**B**) in curcumin-untreated and -treated PAO1 were estimated by high-performance liquid chromatography.

results showed that curcumin treatment reduced the total HSL production in PAO1. In addition, we also observed that curcumin supplementation attenuates PAO1 pathogenicity on *A. thaliana* plants. To evaluate curcumin involvement in QS interference in PAO1, we exogenously supplemented AHL/3-oxo-HSL in the curcumin-treated PAO1-infected *A. thaliana* plants. Interestingly, the plants that were cotreated with AHL/3-oxo-HSL and curcumin showed higher bacterial CFUs in plants compared to the lone curcumin treatments (**Figure 6**).

Exogenous Supplementation of Curcumin Prevents PAO1 Killing of *C. elegans*. Given that the exogenous supplementation of curcumin enabled susceptible *A. thaliana* plants to resist PAO1 infection, we further investigated the anti-infective, preventative effects of curcumin on the ability of PAO1 to kill *C. elegans*. Curcumin was administered onto a PAO1 lawn on NGM agar and was seeded with adult worms ($n = 30$), and the treatment plates were then compared to controls. Our data demonstrated that following the exposure to PAO1 under standard slow killing assay conditions (*22*), the majority (~100%) of the worms were killed within 80–100 h (**Figure 7A**). No mortality was observed with heat-killed PAO1-fed nematodes (data not shown). Increasing concentrations of exogenously supplemented curcumin reduced nematode mortality, resulting in a survival rate of ~28% by 100 h (**Figure 7A**).

To determine whether PAO1-curcumin-treated cells proliferate in *C. elegans* gut, a CFU count was performed at the 40 h time point. The number of PAO1 CFU increased over the 2 day time course from undetectable levels to about $\sim 6 \times 10^4$ bacteria per worm (**Figure 7C**). Additionally, *C. elegans* fed on *Escherhica coli* OP50 did not show distention of the nematode gut, whereas those fed PAO1 showed distention, a possible indication of an infection-like process (**Figure 7B**). Interestingly, nematodes fed with curcumin-treated PAO1 also showed distention, but exhibited prolonged survival rates. Interestingly, increasing concentrations of curcumin had an

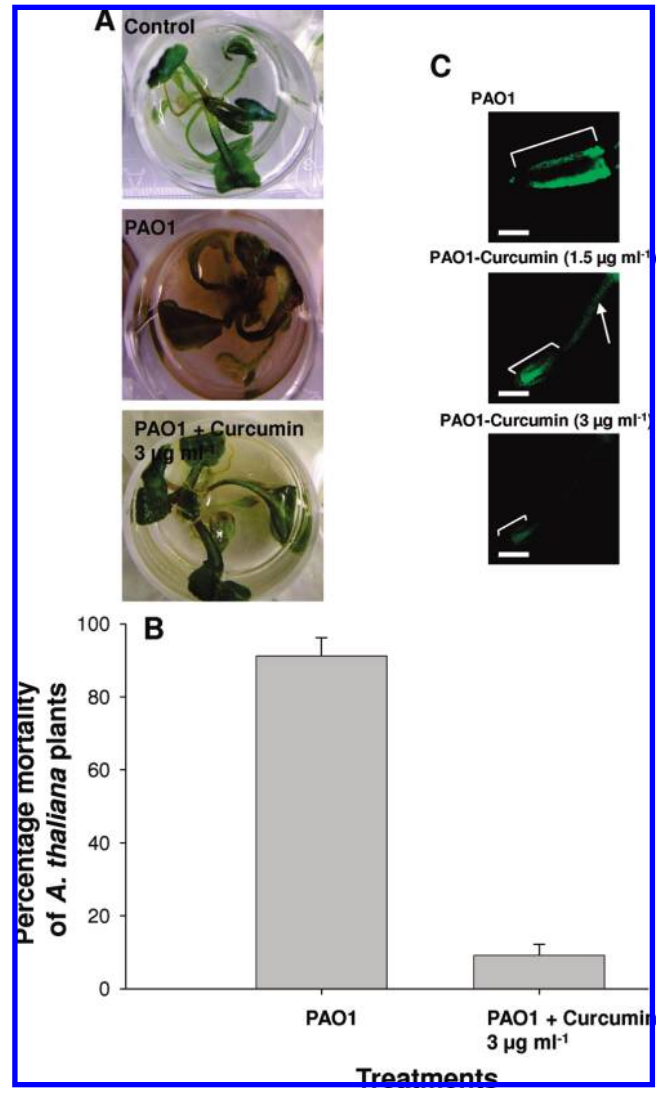

Figure 5. (**A**, **B**) Curcumin reduces *Arabidopsis* mortality caused by PAO1. Curcumin (3 μg mL^{-1}) and PAO1 were added to *Arabidopsis* growth media, and plant mortality was recorded 7 days postincubation with the PAO1. (**C**) In planta PAO1 biofilm formation in untreated and curcumin-treated *A. thaliana* roots (scale bars = 50 μm).

additive effect on the PAO1−nematode combination, resulting in the recovery of a significant amount ($P < 0.005$) of bacteria from nematode intestines (**Figure 7C**). These data suggested that curcumin acted as an anti-infective agent. Although curcumin treatment did not kill microbes, it affected their pathogenicity.

Effects of Curcumin on PAO1 Transcriptome. Having shown that curcumin acts on major virulence factors in PAO1 and prevents its pathogenicity in plant and animal models, it was necessary to check the transcriptional response in PAO1 postcurcumin treatment. Microarray data suggested that curcumin treatment significantly ($P < 0.05$) affected the expression of 716 genes (~12% of the total genome), with up-regulation of 8% of the PAO1 genome and down-regulation of 3.4% of the genes. (Supporting Information Figure 2). Several of the affected genes coded for hypothetical proteins; this result was not surprising, as almost 44% of the predicted open reading frames of *P. aeruginosa* encode hypothetical proteins (*31*).

Functional classification on the 716 genes was then performed to further elucidate how these curcumin-induced changes in transcription were distributed with regard to their function as shown in Supporting Information Fgiure 2B. Curcumin treat-

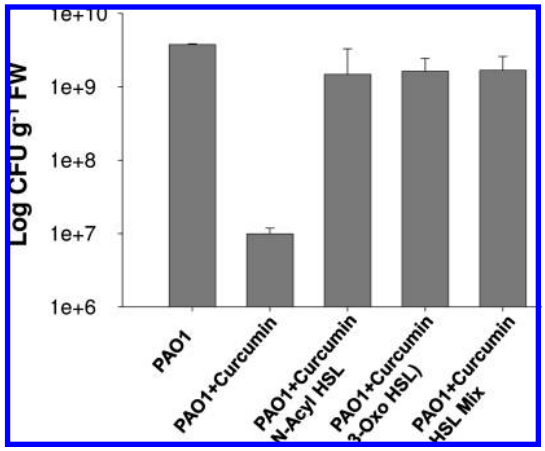

Figure 6. Effect of cotreatment of curcumin and homoserine lactone on the planktonic growth of *P. aeruginosa* PAO1 on *Arabidopsis thaliana* roots expressed as number of colony-forming units (CFUs) per gram fresh weight of roots. The data are the average of six replicates of two independent experiments (bars = ± standard error).

ment also resulted in the altered expression pattern of the genes related to QS (43 in total; 0.7% of the total genome) (Supporting Information Figure 2 and Table 1). Some of the listed QS genes are involved in virulence factor production; for example, *phzD* is significantly down-regulated ($p = 0.005$) (*26*). In addition, the curcumin also down-regulated *lasI* and *rhlA*, genes involved in QS and initiation of biofilm formation (Supporting Information Table 1). Furthermore, six type-III secretion factors genes and at least 57 genes involved in transcriptional regulation also showed altered expression patterns [Supporting Information Figure 2B; (*32*)]. Although the function of the majority of altered genes is unknown, it is tempting to speculate that the down-regulation of these genes might be the reason for reduced synthesis and secretion of virulence factors.

DISCUSSION

In the current study, we have demonstrated that curcumin treatment of PAO1 attenuated biofilm formation as well as down-regulated the production of extracellular virulence factors and AHLs. Curcumin-treated PAO1 also exhibited reduced virulence in both *A. thaliana* and *C. elegans* models. Additionally, curcumin caused a reduced expression of various genes involved in QS and biofilm initiation.

Evidence is accumulating showing that the ability to form biofilms in many organisms involves QS regulation (*8, 9*). *P. aeruginosa* has been shown to form biofilms, which has been linked to its pathogenicity in pulmonary infections of cystic fibrosis patients (*33*) and recently in plant roots (*11*). Clinical

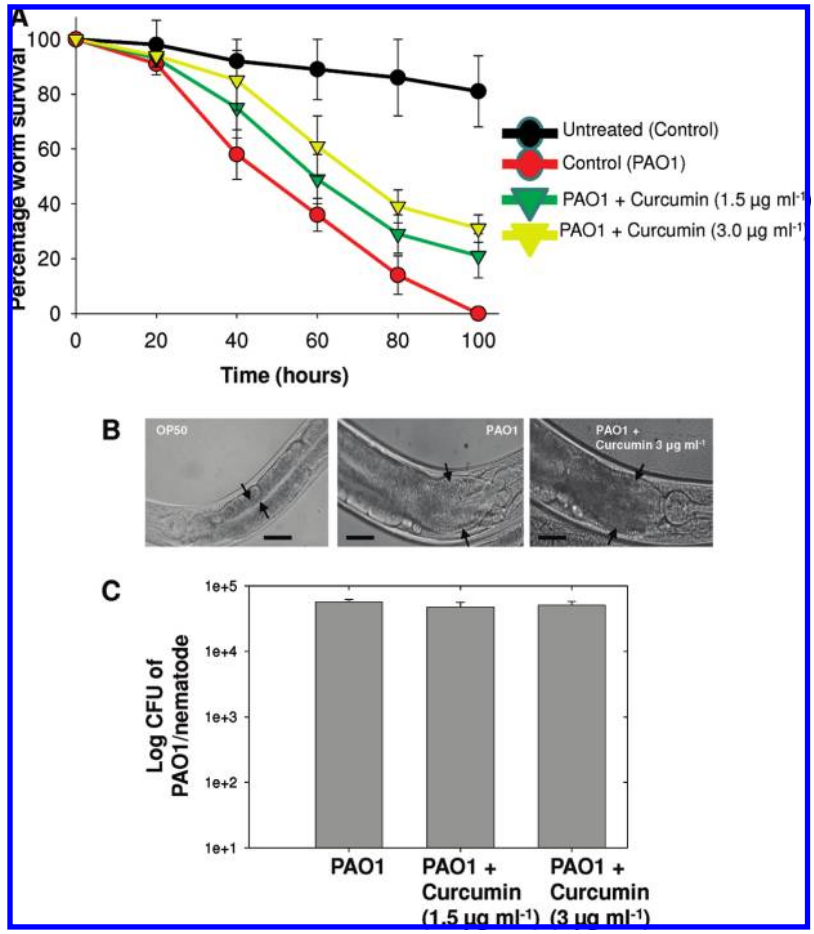

Figure 7. Effect of curcumin on *C. elegans* mortality inflicted by PAO1. (**A**) Curcumin supplementation reduced PAO1 virulence, resulting in reduced mortality of *C. elegans*. (**B**) *C. elegans* fed *Escherichia coli* OP50 did not show distention of the nematode gut, whereas those fed PAO1 showed distention, a possible indication of an infection-like process. Nematodes fed curcumin-treated (3 μg mL^{-1}) PAO1 also showed distention, but exhibited prolonged survival. Arrows indicate worm gut girth. Bar = 5 μm. (**C**) CFU of PAO1 recovered from untreated and curcumin-treated *P. aeruginosa*. *C. elegans* were placed on a lawn of untreated PAO1 and curcumin-treated PAO1. Over a 40 h, nematodes were washed to remove bacteria from their integument and ruptured to recover bacteria from digestive tracts. PAO1 CFU was determined by plating on appropriate selective media. Error bars = SD from triplicate experiments.

studies have shown that the development of resistance to antibiotics in *P. aeruginosa* is a serious side effect of current antipseudomonal treatments (*34*). This has led researchers to search for compounds that have anti-infective properties, as well as the ability to inhibit QS and generic virulence factors. Consistent with our findings, the natural compound, furanone, has been shown previously to affect the QS system of PAO1, reducing virulence in a mouse pulmonary model (*35*).

The ability of curcumin to inhibit pathogenesis of PAO1 in a plant system highlights its role as an anti-infective. Furthermore, by using a recently established *C. elegans* model for *P. aeruginosa* pathogenesis (*13, 27*), we have shown that curcumin's anti-infective property was not restricted to plant pathogenesis. Interestingly, our results revealed that curcumin attenuated total HSL production in PAO1 and that its supplementation reduced plant pathogenicity. Along similar lines addition of exogenous HSL with curcumin reverted the plant pathogenicity effect, represented as recovery of higher bacteria titers. These data point toward a hypothesis that curcumin may target the QS responses in PAO1 through retardation of HSL synthesis or by disablement to receive QS signal.

Interestingly, whereas a comparable number of CFUs was detected in the gut of *C. elegans* fed curcumin-treated and -untreated PAO1, reduced mortality was observed in nematodes fed curcumin-treated PAO1 (**Figure 7**). PAO1 cells throughout the lumen of the worm guts were observed in *C. elegans* fed untreated PAO1, which also displayed markedly distended guts (**Figure 7B**). Previously it has been shown that *P. aeruginosa* was able to proliferate within the nematode's gut in slow-killing assays, resembling an infection-like process (*21*). In the current study, nematodes infected with PAO1 and treated with increased concentrations (3 μg mL^{-1}) of curcumin also showed similar distention in the guts. However, curcumin-treated worms showed reduced worm mortality (**Figure 7A**). These observations were supported by the fact that similar numbers of bacteria were recovered from nematodes fed curcumin-treated and -untreated PAO1 (**Figure 7C**). These results were unexpected in that *P. aeruginosa*−*C. elegans* infectivity studies suggested that bacterial proliferation in the nematode gut was directly proportional to killing. We can only speculate that the expression of virulence factors in PAO1 cells treated with curcumin, present in the worm guts, was impaired in the host. Additional in vivo expression studies of virulence factors in bacteria treated with curcumin and present in the gut are needed to confirm this hypothesis.

Our data have shown that curcumin specifically down-regulated genes that are functionally implicated in virulence factor production, biofilm initiation, and QS (*36*). However, the role of QS in biofilm formation is unclear and conditional upon environment (*37*) and down-regulation of QS; suppression of biofilm initiation by curcumin also indicated its ability to target both. Our results have also shown that whereas curcumin down-regulated biofilm initiation genes, it up-regulated genes involved in mature biofilm formation (data not shown). This was not surprising, given that the microarray was timed for 12 h (log phase) and that curcumin might have impeded microcolony formation by down-regulating genes involved in biofilm initiation. Previous research has also reported that these genes were involved in mature film formation in *P. aeruginosa* and were functionally regulated after 14 h of planktonic culture initiation (*37*). Additionally, most of the biofilm initiation genes that were targeted by curcumin had no putative functions assigned to them, indicating the possibility of finding new molecular targets for controlling biofilm formation in PAO1. Previous researchers have used total root secretions from *Beta vulgaris* to investigate transcriptional profiling in PAO1 (*38*). Our study was, to our knowledge, the first to utilize an individual root-derived compound to elucidate novel molecular targets in PAO1. Future research, investigating transcriptome analysis, is needed to further elucidate the mechanism used by curcumin to down-regulate virulence factors in PAO1.

The use of natural low molecular weight compounds is novel and attractive, particularly when these virulence factors inhibitors were nontoxic to bacteria and have no adverse effect on beneficial bacterial consortia of the host at the concentrations used. Whereas most of the commonly used antibiotics target the basal bacterial life processes, there is an exciting possibility that curcumin's anti-infective property operates by blocking microcolony or biofilm initiation in *P. aeruginosa* (data not shown), which in turn controls virulence factor production, surface colonization, and biofilm formation. Surprisingly, the effects of curcumin against PAO1 differed, depending on the host system (**Figures 5** and **7**). Curcumin did not support biofilm formation on the root surface (**Figure 5C**), whereas *C. elegans* feeding on PAO1 lawns treated with curcumin showed significant bacterial counts in the worm guts. These responses suggested that different host defenses/environments influence the activity of curcumin. However, the effects of curcumin on preinfected worms have yet to be determined. Most importantly, our results have demonstrated that bacterial virulence can be controlled by substances that block biofilm formation and extracellular toxin production. Given the large number of pathogenic bacteria that utilize these mechanisms of action (*39*), natural small molecular weight compounds may find applications in drug discovery.

Supporting Information Available: Supporting Information Figures 1 and 2 and Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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