Down Regulation of Virulence Factors of *Pseudomonas aeruginosa* by Salicylic Acid Attenuates Its Virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*

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Salicylic acid (SA) is a phenolic metabolite produced by plants and is known to play an important role in several physiological processes, such as the induction of plant defense responses against pathogen attack. Here, using the *Arabidopsis thaliana*-*Pseudomonas aeruginosa* pathosystem, we provide evidence that SA acts directly on the pathogen, down regulating fitness and virulence factor production of the bacteria. *Pseudomonas aeruginosa* PA14 showed reduced attachment and biofilm formation on the roots of the *Arabidopsis* mutants *lox2* and *cpr5-2*, which produce elevated amounts of SA, as well as on wild-type *Arabidopsis* plants primed with exogenous SA, a treatment known to enhance endogenous SA concentration. Salicylic acid at a concentration that did not inhibit PA14 growth was sufficient to significantly affect the ability of the bacteria to attach and form biofilm communities on abiotic surfaces. Furthermore, SA down regulated three known virulence factors of PA14: pyocyanin, protease, and elastase. Interestingly, *P. aeruginosa* produced more pyocyanin when infiltrated into leaves of the *Arabidopsis* transgenic line *NahG*, which accumulates less SA than wild-type plants. This finding suggests that endogenous SA plays a role in down regulating the synthesis and secretion of pyocyanin in vivo. To further test if SA directly affects the virulence of *P. aeruginosa*, we used the *Caenorhabditis elegans*-*P. aeruginosa* infection model. The addition of SA to *P. aeruginosa* lawns significantly diminished the bacterium's ability to kill the worms, without affecting the accumulation of bacteria inside the nematodes' guts, suggesting that SA negatively affects factors that influence the virulence of *P. aeruginosa*. We employed microarray technology to identify SA target genes. These analyses showed that SA treatment affected expression of 331 genes. It selectively repressed transcription of exoproteins and other virulence factors, while it had no effect on expression of housekeeping genes. Our results indicate that in addition to its role as a signal molecule in plant defense responses, SA works as an anti-infective compound by affecting the physiology of *P. aeruginosa* and ultimately attenuating its virulence.

Pseudomonas aeruginosa is a prevalent opportunistic pathogen in humans, causing chronic lung infections in cystic fibrosis patients, burn victims, and other immunocompromised people (61, 62). Pathogenesis of *P. aeruginosa* is mediated by a suite of cell-associated and excreted virulence factors. The cell-associated factors include flagella that aid in motility, systems that are involved in the delivery of effector proteins into the host cells (67), and lipopolysaccharide that suppresses host immune responses as well as being involved in the establishment of persistent infections (13). Secreted factors such as elastase and protease cause the degradation of host proteins such as elastin, collagen, and transferrins, destroying the integrity of the host tissues (2, 34), while low-molecular-weight toxins such as pyocyanin affect multiple sites of the cell machinery (38, 58).

P. aeruginosa is known for its intrinsic and acquired resistance against a wide range of antimicrobial agents, which has led to difficulty in treating infections, especially in cystic fibrosis patients (47, 65). Antibiotic resistance in *P. aeruginosa* has

been partially attributed to active efflux pumps that expel antimicrobial compounds (1, 17, 52). Furthermore, several gram-negative bacteria, including *P. aeruginosa*, form structured aggregates, which under certain conditions are referred to as biofilms (50), and biofilm formation has been found to be partially responsible for the persistent *P. aeruginosa* infections in the lungs of immunocompromised cystic fibrosis patients (12, 23, 61, 62). Biofilms consist of a matrix of complex polysaccharides in which the bacteria are embedded, and the formation of this structure is controlled by quorum-sensing mechanisms (16, 23, 43, 51, 63). Biofilm confers superior survival capability on the bacteria by providing a physical barrier against the entry of antimicrobial agents (12, 50); in addition, bacteria inside the biofilm are in a quiescent state (metabolically less active) and thus relatively insensitive to antimicrobial agents (19) and environmental stress (50). Recently, lactoferrin, a component of a healthy person's innate immunity and present in the mucosa, was found to attenuate biofilm formation in *P. aeruginosa* and could protect against persistent infections (61). The emergence of *P. aeruginosa* resistance to multiple antibiotics, including ciprofloxacin, that are commonly used to treat lung infections (42) highlights the need to design more effective regimens to treat these infections. Con-

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sequently, novel strategies are being proposed, including the development of therapeutics that have the potential to disrupt biofilms, down regulate known virulence factors, and regulate genes crucial for pathogenesis and quorum sensing (26, 27, 28, 29, 74).

P. aeruginosa also infects plants, eliciting soft rot symptoms in thale cress (*Arabidopsis thaliana*) and lettuce (*Letuca sativa*) (56, 57), and recently it has been shown to be a potent root pathogen of *Arabidopsis* (71) as well as of animals such as *Caenorhabditis elegans* (44, 66), *Drosophila* (14) and *Galleria mellonella* (48). Molecular studies on the pathogenesis of *P. aeruginosa* revealed that this bacterium requires similar subsets of virulence factors for plant and animal infection (55, 56). This finding makes *Arabidopsis* a convenient model to study the molecular basis of pathogenesis, which could aid in the discovery of novel compounds for treatment of infections (53). Here, we show evidence that the plant compound salicylic acid (SA) can attenuate the infectivity of *P. aeruginosa* in the *Arabidopsis thaliana* and *Caenorhabditis elegans* infectivity models without inhibiting the growth of the bacteria, by down regulating the production of a set of virulence factors and biofilm formation.

MATERIALS AND METHODS

Plant material, bacterial culture, *Caenorhabditis elegans* strains, and chemicals. *Arabidopsis thaliana* ecotype Col-0 seeds were purchased from Lehle, Round Rock, TX. The transgenic line *NahG*, carrying the salicylic acid hydroxylase gene, and the mutant line *npr1-1* (nonexpressor-of-pathogenesis-related protein), were kind gifts from Xinnan Dong (Duke University, NC). The transgenic line 35S-LOX-2(-) (6) (ABRC accession no. CS3748) (hereafter referred to as *lox2*; defective in jasmonic acid accumulation in response to stress and hyperaccumulator of salicylic acid) and the mutant *cpr5-2* (accumulates larger amounts of salicylic acid) were obtained from the *Arabidopsis* Biological Resource Center, Ohio State University, Columbus. The seeds were surface sterilized in 2% sodium hypochlorite for 2 min, followed by three washes with sterile distilled water, and surface-sterilized seeds were placed on static Murashige and Skoog basal medium in petri dishes for germination and incubated in a growth chamber. After 2 weeks the seedlings were transferred to either 12-well plates or culture tubes containing 2 to 3 ml of MS liquid medium and grown on an orbital platform shaker (Lab-Line Instruments) set at 90 rpm with a photoperiod of 16 h light and 8 h dark at 25 ± 2°C. Cultures of *Pseudomonas aeruginosa* PA14 and *Caenorhabditis elegans* wild-type strain Bristol N2 were gifts from Frederick M. Ausubel (Department of Genetics, Harvard Medical School, Boston, MA). Salicylic acid and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Effect of salicylic acid on the growth of *Pseudomonas aeruginosa*. *P. aeruginosa* PA14 was grown overnight at 37°C in LB broth. LB broth medium (2.5 ml) was dispensed into each well of 12-well plates (Falcon, NJ). Stock solutions (100 mM) of salicylic acid were prepared in methanol, and filter-sterilized stock was added to the wells of a 12-well plate to a final concentration of 0.1, 0.25, 0.5, 1, 2, 3, or 4 mM salicylic acid. PA14 inoculum was added to give a final optical density at 600 nm (OD_{600}) of 0.04. The plates were incubated in a shaker set at 90 rpm and 37°C. The effect of SA on growth was assessed by a CFU count, using a dilution plate technique (69).

Root inoculation. Wild-type *A. thaliana* Col-0, transgenic lines *NahG* and *lox2*, and mutants *npr1-1* and *cpr5-2* were grown in 9-cm-diameter petri dishes containing 25 ml MS basal medium for 2 weeks. The plants were transferred to glass culture tubes containing 2 ml of liquid MS medium and placed in a rotary shaker set at 60 rpm under a day/light cycle of 16/8 h for 5 to 7 days, after which the plants were inoculated with appropriate aliquots of *P. aeruginosa* PA14 suspension added to the liquid medium to a final OD_{600} of 0.02. The plants were incubated at 30°C on a rotary shaker. Mortality was recorded at 5 days after inoculation. Each experiment was conducted twice with five replicates. Heat-killed bacteria (121°C for 5 min) were included as one of the control treatments; the bacterial suspension was diluted to a final OD_{600} of 0.02, and plants were incubated as described above.

Biofilm formation on *Arabidopsis thaliana* roots. Wild-type Col-0, transgenic lines (*NahG* and *lox2*), and mutants *npr1-1* and *cpr5-2* of *A. thaliana* were grown and inoculated with PA14 in culture tubes as described above. Three days after inoculation the roots were excised, stained with 10 ml of 75 μ g ml⁻¹ Calcofluor (fluorostain; Sigma-Aldrich) for 30 min, and observed under a fluorescence Olym-

pus BX60 microscope equipped with CoolSnap imaging software (Media-Cybernetics, San Diego, CA) to determine the presence of biofilm formation, as described in the literature (4, 71).

Biofilm formation assay and biofilm quantification. The ability of PA14 to form biofilm on abiotic surfaces was investigated according to a previously described crystal violet (CV) assay (50), except that polypropylene tubes were used instead of polystyrene tubes. Briefly, tubes containing 500 μ l of a 1/100 dilution of an overnight LB broth culture in BDT medium (Bushnell-Haas mineral salts medium supplemented with 0.2% dextrose and 0.5% tryptone) were incubated statically at 30°C for 24 h. The biofilm was qualitatively assayed using crystal violet staining as previously described (50). Biofilm formation was quantified using a microtiter plate assay. PA14 was grown in 96-well polyvinyl chloride (PVC) microtiter plates (Fischer Scientific) at 37°C in biofilm growth medium that consisted of LB medium plus 0.15 M ammonium sulfate, 100 mM potassium phosphate (pH 7), 34 mM sodium citrate, 1 mM MgSO₄, and 0.1% (wt/vol) glucose. The inoculum for microtiter plates was obtained by growing the cells with agitation in biofilm growth medium to mid-logarithmic phase and diluting the cells to an OD_{600} of 0.01 in fresh biofilm growth medium. One hundred microliters of the diluted cells was aliquoted to each well of 96-well PVC microtiter plates. The microtiter plates were incubated under stationary conditions. Cells that adhered to the wells were stained with 0.1% (wt/vol) CV in wash buffer (0.15 M ammonium sulfate, 100 mM potassium phosphate [pH 7], 34 mM sodium citrate, and 1 mM MgSO₄) at room temperature for 20 min. Excess CV was then removed, and the wells were rinsed with water. The CV that had stained the cells was solubilized in 200 μ l of 80% (vol/vol) ethanol and 20% (vol/vol) acetone. Biofilm formation was quantified by measuring the OD_{570} for each well, using an Opsys MR-Dynex plate reader (Chantilly, VA).

Effect of SA supplementation on biofilm formation on abiotic and biotic surfaces. The effect of SA on biofilm formation by PA14 was investigated using a crystal violet assay as described in the previous section. In the first experiment SA was added to the BDT medium from the onset of the incubation period. The concentrations of SA assessed were in the range of 0.1 to 5 mM. The volume of added dimethyl sulfoxide/ethanol was adjusted so that all tubes contained the same concentration of the solvent. Biofilm formation was quantified every 5 h during a 50-hour incubation period. Qualitative CV staining was performed using polypropylene tubes and photographed after 24 h of incubation with SA. All treatments were conducted in triplicate.

Effect of salicylic acid on pyocyanin production by *P. aeruginosa* PA14. *P. aeruginosa* PA14 was grown overnight at 37°C in LB broth. LB broth (2.5 ml) was dispensed into each well of 12-well plates. Stock solutions (100 mM) of salicylic acid and its derivatives acetyl salicylic acid, salicylamide, methyl salicylate, and benzoic acid were prepared in methanol; filter-sterilized stock was added to a 12-well plate to a final concentration of 0.1, 0.5, or 1 mM. PA14 inoculum was added to give a final OD_{600} of 0.04. The plates were incubated in a shaker set at 90 rpm. The culture was removed at 6, 12, 18, and 24 h of incubation, and pyocyanin was extracted as described in the literature (20). Briefly, the 2.5-ml culture was extracted in 1.5 ml of chloroform. The chloroform was transferred to a clean tube, and 0.8 ml of 1 N HCl was added and gently shaken to bring the pyocyanin to the pink aqueous phase (pyocyanin extracted with chloroform turns pink). The OD_{520} of the aqueous solution was measured and the pyocyanin concentration determined by multiplying this measurement by 17.07 (19). The effect of salicylic acid on pyocyanin production by PA14 was also assessed in an M9-glucose (0.2%, wt/vol) minimal medium following the same protocol as described above, except that bacteria were grown in 125-ml conical flasks containing 50 ml of the growth medium.

Effect of salicylic acid and its derivatives on elastase and protease production by *P. aeruginosa* PA14. Plate assays for total protease and elastase activity were performed as described by Brint and Ohman (8). To determine total protease activity, cultures of PA14 were stab inoculated on medium containing 0.8% nutrient broth (Luria) and 1.5% powdered skim milk amended with 1 mM or 5 mM SA and were incubated at 37°C for 12 to 24 h before visual inspection for a zone of clearing. The same procedure was followed for elastase activity except that 0.5% elastin (Sigma, St. Louis, MO) was incorporated into the medium in place of 1.5% skim milk and cultures were incubated for 48 h. Protease activity was quantified using a modification of the method described by Greene et al. (22). Bacterial cultures were grown with and without added SA in 50 ml of 5% peptone and 0.25% tryptic soy broth (PTSB) at 37°C for 24 h. The supernatants were collected and filter purified using a 0.22- μ m nylon filter. A 100- μ l aliquot of supernatant was then added to reaction mixtures containing 0.8% azocasein (Sigma) in 500 μ l of 50 mM K₂HPO₄, pH 7. Reaction mixtures were incubated at 25°C for 3 hours. The reaction was stopped by adding 0.5 ml of 1.5 M HCl, and the mixture was placed on ice for 30 min and then centrifuged. After addition of 0.5 ml of 1 N NaOH, the OD_{440} was recorded. To determine elastase activity, 100

Salicylic acid (SA) Levels

FIG. 1. *P. aeruginosa* attachment to and biofilm formation on *A. thaliana* roots. Plant roots were inoculated with PA14 cells for 5 days. Roots were excised and stained with Calcofluor, and images were taken under a microscope and fluorescent illumination. (A) *npr1-1* and (B) wild-type Col-0 plants, which produce low levels of SA; (C) *lox2* and (D) *cpr5-2* plants, which produce higher levels of salicylic acid. Bars, 1 mm.

μ l of supernatant from the 24-hour PTSE cultures was added to tubes containing 1 ml of 10 mM Na_2HPO_4 , pH 7, and 20 mg of elastin-Congo red (Sigma). Reaction mixtures were incubated with agitation for 4 hours at 37°C. Tubes were then centrifuged, and the OD_{495} was determined.

Assessing differential pyocyanin production in the leaves of *Arabidopsis* with genotypes altered in SA synthesis. Strains with three different genotypes of *Arabidopsis*, i.e., the wild-type Col-0, the transgenic line *NahG*, and the mutant *npr1-1*, were grown in solidified MS medium for 3 weeks. At this time, 1 g fully expanded leaves were excised and vacuum infiltrated with a cell suspension (OD_{600} of 0.02) of PA14. The inoculated leaves were placed in moist chambers and incubated at 30°C for 36 h. At the end of the incubation period the leaf was extracted in 3 ml chloroform, and all further steps were similar to those described in "Effect of salicylic acid on pyocyanin production by *P. aeruginosa* PA14" above. The CFU of *P. aeruginosa* PA14 in the leaves of different genotypes of *A. thaliana* were enumerated by weighing a 1-cm² leaf disk, and macerating the tissue in 1 ml of sterile distilled water in a Eppendorf tube with a tissue macerator (Kontes, size C). This was further serially diluted, and 20 μ l of the suspension was plated on a solid LB medium supplemented with 100 μ g ml⁻¹ rifampin. The number of colonies was counted after overnight incubation at 37°C.

Estimation of free salicylic acid content in *Arabidopsis*. Free SA levels in wild-type *A. thaliana* (Col-0), transgenic lines (*NahG* and *lox2*), and the mutant *npr1-1* were determined by the method of Bowling et al. (7) at 1, 2, and 3 days following infection with *P. aeruginosa* PA14. Approximately 1 g of fresh tissue was either used directly or stored at -80°C until use. The fresh tissue was ground in liquid nitrogen to a fine powder with a chilled pestle and mortar. Three milliliters of 90% methanol and 250 ng *o*-anisic acid (internal standard) were added to each sample. Samples were vortexed, sonicated for 20 min, and centrifuged for 20 min at 1,700 $\times$ g in a tabletop centrifuge. The supernatant was transferred to a new tube, and the pellet was reextracted with 2 ml 90% methanol. The two supernatants were combined, vacuum dried, and frozen at -80°C; then 2.5 ml 5% trichloroacetic acid was added, and the samples were vortexed, sonicated for 5 min, and centrifuged at 1,700 $\times$ g for 15 min. The supernatant was extracted twice with 2.5 ml of a 1:1 (vol/vol) mixture of ethyl acetate and cyclopentane. The organic phases were combined, vacuum dried, and frozen at -80°C. Just prior to loading of samples for high-pressure liquid chromatography, each was resuspended in 250 μ l of 20% methanol, vortexed, sonicated for 5 min, and filtered through a 0.22- μ m nylon filter. High-pressure liquid chromatography was performed as described earlier (7).

Microarray analysis. Batch cultures of *P. aeruginosa* (PA14) were grown in duplicates in LB medium supplemented with either 0 or 1.0 mM of salicylic acid at 37°C and 200 rpm. The initial OD_{600} was adjusted to ~0.02, and the cultures were grown for 10 h with shaking. At the end of the incubation period, 5 ml of the culture was retrieved and RNA was isolated using a Ribopure RNA isolation kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. The concentration of the RNA was assessed using a Nanodrop system (NanoDrop, Wilmington, DE) and the integrity estimated by 28S/18S ratio using an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA). The microarray analysis was performed at Ambion Inc., Austin, TX. Two micrograms of the RNA was amplified using a MessageAmp II-Bacteria kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions and used in the microarray analysis. Microarray analysis was performed by using the Affymetrix *P. aeruginosa* GeneChip. The Affymetrix instrumentation consisted of a GeneChip hybridization oven 640, a

GeneChip Fluidics Station 450, and a high-resolution GeneChip scanner 3000 (GeneChip, Santa Clara, CA). The data analysis was performed using the data analysis software GeneSpring7.2 (Silicon Genetics, Redwood City, CA) DNA-Chip Analyzer (dChip; <http://www.dchip.org>). The data were normalized per chip by dChip invariant set normalization, and each gene was normalized to the median measurement taken for that gene across all the samples. For 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.

Effect of salicylic acid on the efficacy of ciprofloxacin against *P. aeruginosa* PA14. *Pseudomonas aeruginosa* PA14 was grown overnight in Mueller-Hinton broth medium. The MIC of ciprofloxacin against PA14 was determined using a 96-well plate serial dilution method, with PA14 inoculated to a final OD_{600} of 0.02. The 96-well plate was incubated at 37°C overnight and the growth determined by measuring the OD_{600} using a microtiter plate reader (Dynex, VA). Ciprofloxacin (1 μ g ml⁻¹) significantly reduced the CFU, from 5.6×10^9 to 2.5×10^5 . We used a concentration of ciprofloxacin 10 times lower than this (0.1 μ g ml⁻¹) to check if SA potentiates the antibacterial activity of ciprofloxacin. One milliliter MH broth was dispensed into wells of 12-well cell culture plates, and ciprofloxacin was added to a final concentration of 0.1 either with or without 1.0 mM SA. The PA14 suspension was added to each of the wells to a final OD_{600} of 0.02. The plates were incubated in a shaker set at 100 rpm at 37°C for 24 h. At the end of the incubation period, a CFU count for each treatment was done by serial dilution and plating on LB agar.

Nematode-killing assay with SA supplementation. PA14 was grown overnight at 37°C in LB broth. A 1:10 dilution of the saturated culture was made in LB broth, and 10 μ l of the diluted culture was spread on 3.5-cm-diameter plates containing nematode growth medium (NGM). The plates were incubated at 30°C for 24 h and allowed to equilibrate to room temperature for 30 to 60 min before being seeded with the nematode *Caenorhabditis elegans* wild-type strain Bristol N2. Nematodes were multiplied on NGM plates with *Escherichia coli* OP50 as the food source. Adult populations were synchronized on similar plates by transferring two adult nematodes to each plate and allowing them to lay eggs overnight, after which the adult nematodes were removed and killed. The plates containing the eggs were incubated for 4 to 5 days to get a uniform adult population. Twenty to 30 adult nematodes were used for each assay. Similarly, to test the anti-infective property of SA (diluted in methanol) against the bacteria, SA was administered at different concentrations (0.1 to 2.0 mM) by dropping it on the PA14 lawn 10 to 15 min prior to worm seeding. A separate methanol control was also tested for comparison as per the description provided above. An additional control involved inoculating worm plates with heat-killed PA14. In each assay, 20 to 30 adult nematodes were added per plate, and each assay was carried out in triplicate. The plates were incubated at 20°C and scored for live and dead worms at least every 24 h for 5 days. A worm was considered dead when it failed to respond to plate tapping or a gentle touch with a platinum wire. Worms that died as a result of getting stuck to the wall of the plate were not included in the analysis.

Estimation of bacterial CFU within the *C. elegans* gut. CFU of bacteria within the nematode gut were counted by a method described in the literature (21). For each replication, 10 adult *C. elegans* worms were picked from different treatments (PA14 and PA14 treated with different concentrations of SA), transferred into a 1.5-ml Eppendorf tube containing 500 μ l of M9 buffer supplemented with 20 μ g/ml gentamicin, and washed with three changes of the above cocktail to remove surface

bacteria. The nematodes were disrupted in a 1.5-ml Eppendorf tube containing 50 μ l of M9 medium with 1% Triton X-100. The resulting slurry was serially diluted and plated on LB agar medium containing 20 μ g/ml rifampin, and the number of CFU was counted.

RESULTS AND DISCUSSION

Salicylic acid, which is produced throughout the plant kingdom, is known to be involved in modulating a number of physiological processes, including thermogenesis, tolerance of abiotic stresses (11, 31, 59), and, most importantly, defense responses. Endogenous SA in plants modulates defense response pathways by up regulating several pathogenesis-related proteins (60) that are controlled through the transcriptional regulator NPR1 (10). Several SA-dependent but NPR1-independent defense pathways have also been reported (15, 32, 41). Although possible direct effects of SA on an infecting pathogen have been proposed (18, 37), this intriguing question has not been thoroughly investigated.

Salicylic acid levels affect bacterial attachment to plant roots. We hypothesized that SA may affect the virulence of the pathogen without affecting its growth. To test this hypothesis, we used *Arabidopsis* mutants with altered abilities to accumulate SA, such as *lox2* (5) and *cpr5-2* (7), both of which accumulate elevated concentrations of SA; *npr1-1*, a mutant that has normal levels of SA but is insensitive to its activity because it lacks the transcriptional regulator needed for SA-induced defense responses (10); the transgenic line *NahG* (39), which constitutively expresses the SA hydroxylase gene of *Pseudomonas putida* and thus has depleted concentrations of SA; and the wild-type Col-0. The infectivity assays were conducted by inoculating *P. aeruginosa* (PA14) to the roots of plants growing under in vitro conditions (71). The wild-type Col-0 and the mutant *npr1-1* supported attachment and biofilm community formation by PA14 (Fig. 1A and B). However, the mutants *lox2* and *cpr5-2*, which accumulate larger amounts of SA, showed reduced attachment and/or formation of biofilm communities on the roots (Fig. 1C and D).

Endogenous salicylic acid levels affect bacterium-induced plant mortality. The ability of PA14 to colonize and form biofilm communities on the roots of *Arabidopsis* correlated with plant mortality (Fig. 2A). *Arabidopsis* wild-type Col-0, the mutant *npr1-1*, and the transgenic line *NahG* were more susceptible to PA14 infection, resulting in almost 100% plant mortality at 5 days postinoculation, while *cpr5-2* and *lox2*, which supported less colonization of PA14, were resistant as evidenced by the lower plant mortality rates of ~50% and ~40%, respectively (Fig. 2A). *P. aeruginosa* PA14 inflicted typical disease-like symptoms such as black necrotic regions initially in the root tips, which later spread to the entire root system, then colonized the basal leaves, and finally resulted in the collapse of the plant (71). The enhanced susceptibility of the transgenic line *NahG* and the mutant *npr1-1* correlated with higher CFU counts of the bacteria in the roots, while the transgenic line *lox2* and the pathogen-resistant mutant *cpr5-2* were less susceptible to PA14 colonization and showed lower CFU than wild-type Col-0 plants (Fig. 2B). These results strongly suggest that SA has a role in inhibiting PA14 attachment and biofilm community formation on *Arabidopsis* roots.

Salicylic acid affects bacterial attachment and biofilm formation in vitro. To test the hypothesis that SA affects the

FIG. 2. Susceptibility of *A. thaliana* to *P. aeruginosa* and bacterial growth is dependent on endogenous salicylic acid levels. Strain PA14 was used to infect *A. thaliana* wild-type Col-0, the transgenic line *NahG*, and the mutant *npr1-1*, all of which produce low levels of SA, and the transgenic line *lox2* and mutant *cpr5-2*, which produce higher levels of SA. Five days after inoculation, plant mortality was recorded (A) and bacterial numbers on roots were determined (B). Error bars indicate standard deviations.

physiology of PA14, reducing its ability to attach to roots, we carried out an in vitro experiment in which SA was added into the culture medium containing salicylic acid at physiologically relevant concentrations (0.1 to 1 mM) (46, 64). The ability of PA14 to form biofilm was diminished in a concentration-dependent manner when exposed to SA (Fig. 3A). It is important to note that although SA completely inhibited the aerobic biofilms (shown as the ring formed in the region representing the air-liquid interphase of the tube), it did not decrease the formation of anaerobic biofilm (Fig. 3A). We quantified the effect of SA on PA14's adherent biofilm by using a microtiter plate assay described in the literature (25, 50). PA14 was grown in the wells of a PVC microtiter plate in a complex biofilm growth medium supplemented

FIG. 3. Salicylic acid inhibits *P. aeruginosa* attachment to and biofilm formation on an abiotic surface. PA14 cells were statically grown for 24 h at 30°C in polypropylene tubes containing Bushnell-Haas mineral salts medium supplemented with 0.2% dextrose and 0.5% tryptone. Attachment and biofilm formation were then quantitated by staining with crystal violet. (A) Biofilm formation in the presence of increasing SA concentrations. Probable anaerobic biofilms formed in the presence of 0.5 and 1 mM SA are circled. (B) Time course of biofilm formation by untreated PA14 and cells treated with increasing SA concentrations. Error bars indicate standard deviations.

with different concentrations of SA (0.1 to 1.0 mM). Addition of SA significantly reduced adherent cells (biofilm) over a 40-hour period compared to the untreated control (Fig. 3B).

Salicylic acid inhibits pyocyanin formation. We also observed reduced pigment accumulation in the SA-treated PA14. *P. aeruginosa* produces a number of colored secondary metabolites commonly referred to as phenazines; one of these, the blue pyocyanin, has been widely studied and is a potent virulence factor (57, 58). We tested the effect of SA on pyocyanin production by supplementing the growth medium with different concentrations of SA. Addition of SA to the bacterial growth medium significantly reduced the production of pyocyanin (Fig. 4A and 4B). Untreated PA14 produced ~4.5 μg of pyocyanin per 10^8 cells after 24 h of incubation, while the addition of 0.1 mM SA reduced pyocyanin production by ~50%, and 1.0 mM SA resulted in more than an 80% reduction of pyocyanin production with no apparent effect on the growth of the bacteria. Similar reductions in the quantity of pyocyanin were observed following the addition of SA (0.1, 0.5, and 1.0 M) to M9-glucose (<http://lamar.colostate.edu/~jvivanco>

FIG. 4. Salicylic acid and derivatives inhibit pyocyanin formation. PA14 cells were grown in LB medium in the absence or presence of SA or its derivatives acetyl salicylic acid (ASA), salicylamide (SAM), methyl salicylic acid (MeSA), and the SA metabolic precursor benzoic acid (BA). After 24 h, pyocyanin was extracted with chloroform and its concentration determined spectrophotometrically. (A) Visual depiction of green pyocyanin pigmentation, which is reduced in the SA-treated cultures; (B) pyocyanin levels in untreated and SA-treated cells; (C) pyocyanin levels in untreated cells and cells treated with the SA derivatives ASA, SAM, and MeSA or its metabolic BA precursor. Error bars indicate standard deviations.

TABLE 1. Differential pyocyanin production by *Pseudomonas aeruginosa* PA14 on the leaves of *Arabidopsis thaliana* with different genotypes varying in endogenous concentrations of salicylic acid

<i>A. thaliana</i> genotype and SA treatment	<i>In planta</i> pyocyanin concn, mean $\pm$ SD (μ g per 10^8 cells)
Col-0	6.45 $\pm$ 0.95
<i>NahG</i> (no SA accumulation)	15.92 $\pm$ 0.31
<i>npr1-1</i> (SA level lower than that of Col-0)	7.51 $\pm$ 0.14
<i>npr1-1</i> + 1mM SA	4.30 $\pm$ 0.45

/papers/IAI2029.pdf). We also evaluated the effect of SA derivatives, including acetyl salicylic acid, salicylamide, and methyl salicylate, and a precursor of SA, benzoic acid, on pyocyanin production by PA14 (Fig. 4C). All the SA derivatives led to reduced pyocyanin production, and the levels of reduction caused by methyl salicylate, salicylamide, and benzoic acid were comparable to that caused by SA. Interestingly, when PA14 was infiltrated into the leaves of *Arabidopsis* genotypes that accumulate different concentrations of SA, the production of pyocyanin by PA14 was inversely correlated with the concentration of SA found in the plant leaves (Table 1). PA14 when infiltrated into the leaves of the mutant *npr1-1*, which accumulates wild-type levels of SA but is truncated in an upstream signaling event that leads to synthesis of pathogenesis-related protein, accumulated 7.5 μ g pyocyanin. Addition of 1.0 mM SA with the bacterial suspension resulted in the reduction of pyocyanin production to 4.3 μ g per 10^8 cells. These results indicate that the endogenous concentration of SA in the leaves of *Arabidopsis* is sufficient to down regulate pyocyanin production in the bacteria. Using random transposon mutagenesis, Rahme et al. (57) found that mutants of PA14 that were defective in pyocyanin production showed attenuated pathogenicity in the *Arabidopsis* and mouse models, suggesting a main role for pyocyanin in the virulence of *P. aeruginosa*, and later studies have supported this observation (44). Recently, the molecular mechanism by which pyocyanin exerts its cytotoxic effect has been unraveled (58). Using *Saccharomyces cerevisiae*, Ran et al. (58) have identified a number of cellular targets of pyocyanin that encompass major cellular pathways involved in the cell cycle, electron transport and respiration, epidermal cell growth, protein sorting, and vesicle transport, and have determined that pyocyanin inactivates vacuolar ATPase, ultimately resulting in cytotoxicity.

Salicylic acid affects exoenzyme production in *P. aeruginosa* PA14. *P. aeruginosa* employs a repertoire of exoenzymes to elicit disease pathology (9, 30, 44, 58). A number of studies have found that elastases and proteases are potent virulence factors of *P. aeruginosa*. Accordingly, *P. aeruginosa* mutants *pho34B12* and *pho15*, which are defective in the synthesis of elastases and proteases, showed moderate pathogenicity on *Arabidopsis* and were also attenuated in pathogenicity in the mouse model and inflicted 56 and 62% mortality, respectively (9, 57). We hypothesized that the reduced mortality observed in SA-treated *Arabidopsis* might partly be due to down regulation of virulence factors other than pyocyanin, such as elastases and proteases. Addition of 1 mM SA to PA14 growth medium resulted in a 50% reduction of both elastase and protease activities (Fig. 5). Acetyl salicylic acid, salicylamide, methyl salicylate, and benzoic acid also caused similar reductions in elastase and

FIG. 5. Exposure of *P. aeruginosa* to salicylic acid, its derivatives, and a metabolic precursor inhibits protease production. PA14 cells were grown for 24 h in peptone-tryptic soy broth medium in the absence or presence of SA or its derivatives acetyl salicylic acid (ASA), salicylamide (SAM), methyl salicylic acid (MeSA), and the SA metabolic precursor benzoic acid (BA). Spectrophotometric assays of total protease and elastase activities were performed using the substrates azocasein and elastin-Congo red, respectively. The activity was measured as OD_{440/495}/10⁸ cells. Error bars indicate standard deviations.

protease activities (Fig. 5). These results suggest that SA affects the production of several virulence-related exoenzymes in PA14.

Global effects of salicylic acid on gene transcription. To study the effect of SA on genome-wide changes in gene expression, we used the Affymetrix GeneChip microarray technology. The microarray data suggest that SA treatment significantly ($P < 0.05$) affected the expression of 331 genes (~5% of the total genome). A total of 3.0% of the genome of PA14 was induced while 2.7% of the genes were repressed by SA (Fig. 6A and B; <http://lamar.colostate.edu/~jvivanco/papers/IAI2029.pdf>). Several of the genes whose expression was affected by SA coded for hypothetical proteins; this result was not surprising, as almost 44% of the predicted open reading frames of *P. aeruginosa* encode hypothetical proteins (73). SA treatment, however, did not affect housekeeping genes that are required for the growth and normal metabolism of the bacteria, a finding which lends mechanistic support to our data that SA, even at the highest concentration used in this study (1.0 mM), did not affect the growth of *P. aeruginosa* PA14.

Some of the genes that were down regulated by SA were related to quorum sensing, such as *rhIR* and *lasA*. These genes have been previously implicated in the synthesis of several virulence factors, including pyocyanin, protease, and elastase (29). Further, 12 genes that are involved in protein secretion and export apparatus were significantly repressed by SA treatment; for example, *exoT*, *exsB*, and *exsC* were repressed by SA treatment (Fig. 6B; <http://lamar.colostate.edu/~jvivanco/papers/IAI2029.pdf>). Nineteen transcriptional regulators (data not shown) were affected by 1.0 mM SA treatment, and although the functions of many of these genes are not known, it is plausible that these genes might affect the synthesis and secretion of a number of virulence factors of PA14.

Salicylic acid potentiates antimicrobial activity of ciprofloxacin. Salicylates have been shown to alter the susceptibility

FIG. 6. Microarray analysis of *P. aeruginosa* transcripts from cells treated with salicylic acid. PA14 cells were grown for 10 h in the absence or presence of 1 mM SA. Total RNA was then extracted, labeled, and hybridized to Affymetrix *P. aeruginosa* GeneChips. (A) Total number of genes either affected or unaffected by treatment with SA, which significantly ($P < 0.05$) affected the expression of 361 genes. (B) Functional categories of genes that are either up or down regulated in PA14 in response to SA treatment. LPS, lipopolysaccharide; RND, resistance-modulation-cell division; MFS, major facilitator superfamily.

FIG. 7. Salicylic acid potentiates ciprofloxacin activity against *P. aeruginosa*. PA14 cells were grown in Mueller-Hinton broth with ciprofloxacin (Cipro) and in the presence or absence of 1 mM SA. After 24 h, viable counts were determined in the respective cultures. Error bars indicate standard deviations.

of *Helicobacter pylori* to antibiotics, such as amoxicillin, clarithromycin, and metronidazole, by enhancing their antibacterial activity (72). Similarly, salicylate enhanced the susceptibility of *E. coli* to kanamycin (2). Therefore, it is reasonable to postulate that SA treatment could alter the susceptibility of PA14 to commercial antibiotics such as ciprofloxacin, an antibiotic used to treat *P. aeruginosa* infections in cystic fibrosis patients (42). We found that the addition of SA in conjunction with ciprofloxacin significantly enhanced the efficacy of the antibiotic (Fig. 7). In the presence of 1 mM SA the antimicrobial activity of ciprofloxacin was potentiated about threefold, suggesting that the production of virulence factors by PA14 may play an additional role in antibiotic resistance. Based on these results, we propose that endogenous SA in plants may play an important role in making pathogenic bacteria more susceptible to preformed plant antimicrobial compounds (phytoanticipins) (70), as well as to those induced compounds that are synthesized as a result of infection (phytoalexins) (24, 70).

Salicylic acid attenuates nematode killing by *P. aeruginosa* PA14. Our results demonstrate that SA affects PA14 by down regulating a number of virulence factors produced by the bacteria, thus affecting its virulence on *Arabidopsis*. However, an intrinsic problem with using plant models to study the direct effect of SA on the bacteria is the existence of SA-dependent plant defense responses that are NPR1 dependent (10) or NPR1 independent (15, 32, 41). Essentially it is very difficult to isolate the direct effect of SA on the microbe from the effect of SA on induction of plant defense responses. To overcome this obstacle, we used a *Caenorhabditis elegans*-*P. aeruginosa* infection model (44), as SA is not known to induce a defense response in nematodes. A slow killing assay was performed to measure the effect of SA on the ability of *P. aeruginosa* to infect the worm. SA was added directly to the PA14 lawn, resulting in attenuated nematocidal activity of PA14 (Fig. 8A), but with no apparent reduction in bacteria accumulated in the nematode gut as reflected by the recovered bacterial CFU (Fig. 8B).

FIG. 8. Salicylic acid attenuates nematode killing by *P. aeruginosa* but does not reduce bacterial counts in the gut. (A) Nematode growth medium agar plates containing lawns of bacteria used for *C. elegans* feeding either contained no SA (PA14) or contained increasing SA concentrations. *E. coli* strain OP50 grown on NGM served as a control. Twenty to 30 nematodes were added to each plate at time zero, and worm survival was monitored every 20 h. (B) NGM plates containing the indicated SA concentrations were seeded with PA14. *C. elegans* adult worms were transferred to the plates, and CFU were determined in the guts of 10 live worms removed from the plates at the indicated time points. Error bars indicate standard deviations.

PA14 (with no SA treatment) caused 100% mortality of adult *C. elegans* after 100 h, while the addition of 0.1 mM SA to PA14 lawns increased the time required for complete mortality to 120 h. At higher concentrations of SA, an increasing number of nematodes survived for over 120 h. For example, ~20% of the animals survived at 120 h when treated with 1.0 mM SA, while ~40% of the animals survived when treated with 2.0 mM SA. It should be noted that neither of these SA levels affects the growth rate of PA14 (data not shown). Antibiotics usually target specific metabolic events and have been routinely used to treat bacterial infections. However, the widespread emergence of antibiotic-resistant strains of pathogenic bacteria has resulted in problems in treating certain infectious diseases and necessitates the development of alternate strategies of treatment (40). SA effectively stems the infectivity of PA14 as evidenced by reduced *C. elegans* mortality after PA14 infection,

possibly due to the down regulation of virulence factors. These results suggest that in addition to its traditional role as an analgesic, SA has the potential to be used alone or in conjunction with lower doses of antibiotics to treat bacterial infections. Such an approach could slow the development of antibiotic-resistant strains of bacteria.

***P. aeruginosa* PA14 infection affects *Arabidopsis* salicylic acid levels.** Since the defense-response-altered *A. thaliana* plants used in this study varied in their susceptibility to PA14 infection and because some of these plants were affected in the salicylic acid signaling pathway, we postulated that the susceptibility of these plants to PA14 might be related to SA levels in the plant. Therefore, we analyzed the endogenous accumulation of SA in whole plants of wild-type *A. thaliana* (Col-0), transgenic lines (*NahG* and *lox2*), and the mutant *npr1-1* upon PA14 challenge at two different time points (day 1 and day 3). Although some of our infection assays were conducted on roots, we choose to sample whole plants for SA analyses due to root material limitation in some of the mutants. As expected and reported earlier (49), both *NahG* and *npr1-1* revealed depleted SA levels compared to wild-type plants (<http://lamar.colostate.edu/~jvivanco/papers/IAI2029.pdf>). At days 1 and 3 after inoculation with PA14, the total salicylic acid concentrations in the tissues were essentially the same in wild-type *A. thaliana* Col-0, transgenic line *NahG*, and the mutant *npr1-1*, while the transgenic line *lox-2* showed a higher concentration of SA (54) (<http://lamar.colostate.edu/~jvivanco/papers/IAI2029.pdf>). Collectively, these results show that SA may account for reduced PA14 infectivity when higher levels of this metabolite accumulate in the plant tissues by negatively influencing the virulence of the pathogen.

Conclusions. SA is omnipresent in the plant kingdom, and its concentration in tissue is highly regulated by abiotic and biotic factors (59). Microbial infection causes the accumulation of high levels of SA at the site of pathogen infection and, to a lesser extent, also in uninfected tissues by a process known as systemic acquired resistance (45), either by de novo synthesis or by the release of bound SA into free SA from *o*-glucosides by the action of glucosidases (45). Therefore, it is plausible that some nonhost microbes (3, 68) may be exposed to a high concentration of SA in infected plants, a concentration sufficient to affect their virulence and pathogenicity. The results presented in this study suggest that SA, besides triggering defense responses, could also act on the pathogen by disruption of aggregate/biofilm formation on biotic and abiotic surfaces and by repression of a number of virulence factors. Consistent with our results, previous studies have shown that SA affects the α -hemolysin secretion and fibronectin-binding capacities of *Staphylococcus aureus* and also that the expression of the global regulatory genes *agr* and *sarA* is attenuated by SA treatment (35, 36). Further, salicylic acid has been shown to inhibit fimbria-mediated Hep-2 cell adherence of and hemagglutination by enteroaggregative *Escherichia coli* (33) and also to inhibit attachment and colonization of contact lenses by a number of pathogenic bacteria, including *P. aeruginosa* (5). The results presented in this report largely support this hypothesis and warrant an in-depth study of the direct role of SA in plant defense, as well as its role as a potential anti-infective compound for the treatment of human diseases.

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REFERENCES

- Aeschlimann, J. R. 2003. The role of multidrug efflux pumps in the antibiotic resistance of *Pseudomonas aeruginosa* and other Gram-negative bacteria: insights from the Society of Infectious Diseases Pharmacists. *Pharmacotherapy* 23:916–924.
- Aumercier, M., D. M. Murray, and J. L. Rosner. 1990. Potentiation of susceptibility to aminoglycosides by salicylates in *Escherichia coli*. *Antimicrob. Agents Chemother.* 23:835–845.
- Bais, H. P., B. Prithiviraj, A. K. Jha, F. M. Ausubel, and J. M. Vivanco. 2005. Mediation of pathogen resistance by exudation of antimicrobials from roots. *Nature* 434:217–221.
- Bais, H. P., R. Fall, and J. M. Vivanco. 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiol.* 134:307–319.
- Bandara, B. M. K., P. R. Sankaridurg, and M. D. P. Willcox. 2004. Non-steroidal anti inflammatory agents decrease bacterial colonization of contact lenses and prevent adhesion to human corneal epithelial cells. *Curr. Eye Res.* 29:245–251.
- Bell, E., R. A. Creelman, and J. E. Mullet. 1995. A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 92:8675–8679.
- Bowling, S. A., A. Guo, H. Cao, A. S. Gordon, D. F. Klessig, and X. Dong. 1994. A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. *Plant Cell* 6:1845–1857.
- Brint, J. M., and D. E. Ohman. 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J. Bacteriol.* 177:7155–7163.
- Cao, H., R. L. Baldini, and L. G. Rahme. 2001. Common mechanisms for pathogens of plants and animals. *Annu. Rev. Phytopathol.* 39:259–284.
- Cao, H., J. Glazebrook, J. Clarke, S. Volko, and X. Dong. 1997. The *Arabidopsis npr1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88:57–63.
- Chini, A., J. John, J. J. Grant, M. Seki, K. Shinozaki, and G. J. Loake. 2004. Drought tolerance established by enhanced expression of the CC-NBS-LRR gene, ADR1, requires salicylic acid, EDS1 and ABI1. *Plant J.* 38:810–822.
- Costerton, J. W. 2001. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. *Trends Microbiol.* 9:50–52.
- Cryz, S. J., Jr., T. L. Pitt, E. Furer, and R. Germanier. 1984. Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* 44:508–513.
- D'Argenio, D. A., L. A. Gallagher, C. A. Berg, and C. Manoil. 2001. *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. *J. Bacteriol.* 183:1466–1471.
- Darrell, D., R. Subramaniam, M. Despres, Jean-Nicholas, C. Levesque, P. R. Fobert, J. L. Dangl, and N. A. Brisson. 2004. The “whirly” transcription factor is required for salicylic acid-dependent disease resistance in *Arabidopsis*. *Dev. Cell* 6:229–240.
- Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280:295–298.
- De Kievit, T. R., M. D. Parkins, R. J. Gillis, R. Srikumar, H. Ceri, K. Poole, B. H. Iglewski, and D. G. Storey. 2001. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* 45:1761–1770.
- Delaney, T. P., S. Uknes, B. Vernooij, L. Friedrich, K. Weymann, D. Negrotto, T. Gaffney, M. Gut-Rella, H. Kessmann, E. Ward, and J. Ryals. 1994. A central role of salicylic acid in plant disease resistance. *Science* 266:1247–1250.
- Drenkard, E. 2003. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microb. Infect.* 5:1213–1219.
- Essar, D. W., L. Eberly, A. Hadero, and I. P. Crawford. 1990. Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J. Bacteriol.* 172:884–900.
- Garsin, D. A., C. D. Sifri, E. Mylonakis, X. Qin, K. V. Singh, B. E. Murray, S. B. Calderwood, and F. M. Ausubel. 2001. A simple model host for identifying Gram-positive virulence factors. *Proc. Natl. Acad. Sci. USA* 98:10892–10897.
- Greene, R. V., M. A. Cotta, and H. L. Griffin. 1989. A novel, symbiotic bacterium isolated from marine shipworm secretes proteolytic activity. *Curr. Microbiol.* 19:353–356.
- Hall-Stoodley, L., J. W. Costerton, and P. Stoodley. 2004. Bacterial biofilms:

- from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2:95–108.
24. Hammond-Kosack, K. E., and J. D. G. Jones. 1996. Resistance gene-dependent plant defense responses. *Plant Cell* 8:1773–1791.
 25. Hamon, M. A., and B. A. Lazazzera. 2001. The sporulation transcription factor Spo0A is required for biofilm development in *Bacillus subtilis*. *Mol. Microbiol.* 42:1119–1209.
 26. Hentzer, M., and M. Givskov. 2003. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J. Clin. Investig* 112:1300–1307.
 27. Hentzer, M., L. Eberl, J. Nielsen, and M. Givskov. 2003. Quorum sensing: a novel target for the treatment of biofilm infections. *BioDrugs* 17:241–250.
 28. Hentzer, M., M. Givskov, and L. Eberl. 2004. Quorum sensing in biofilms: gossip in slime city. *Microb. Biofilms* 1:118–140.
 29. Hentzer, M., H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, N. Kumar, M. A. Schembri, Z. Song, P. Kristoffersen, M. Manefield, J. W. Costerton, S. Molin, L. Eberl, P. Steinberg, S. Kjelleberg, N. Hoiby, and M. Givskov. 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J.* 22:3803–3815.
 30. Holder, I. A., and A. N. Neely. 1991. The role of proteases in *Pseudomonas* infections in burns: a current hypothesis. *Antibiot. Chemother.* 44:99–105.
 31. Janda, T., G. Szalai, I. Tari, and E. Paldi. 1999. Hydrophonic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. *Planta* 208:175–180.
 32. Kachroo, P., K. Yoshioka, J. Shah, H. K. Dooner, and D. F. Klessig. 2000. Resistance to turnip crinkle virus in *Arabidopsis* is regulated by two host genes and is salicylic acid dependent but NPR1, ethylene, and jasmonate independent. *Plant Cell* 12:677–690.
 33. Kang, G., A. Balasubramanian, A. R. Koshi, M. M. Mathan, and V. I. Mathan. 2004. Salicylate inhibits fimbriae mediated HEp-2 cell adherence and haemagglutination by enteroaggregative *Escherichia coli*. *FEMS Microbiol. Lett.* 166:257–265.
 34. Kawaharajo, K., J. Y. Homma, Y. Aoyama, K. Okada, K. Morihara. 1975. Effects of protease and elastase from *Pseudomonas aeruginosa* on skin. *Jpn. J. Exp. Med.* 45:79–88.
 35. Kupferwasser, L. I., M. R. Yeaman, C. C. Nast, D. Kupferwasser, Y. Q. Xiong, M. Palma, A. L. Cheung, and A. S. Bayer. 2003. Salicylic acid attenuates virulence in endovascular infections by targeting global regulatory pathways in *Staphylococcus aureus* J. Clin. Investig 112:222–233.
 36. Kupferwasser, L. I., M. R. Yeaman, S. M. Shapiro, C. C. Nast, P. M. Sullam, S. G. Filler, and A. S. Bayer. 1999. Acetylsalicylic acid reduces vegetation bacterial density, hematogenous bacterial dissemination, and frequency of embolic events in experimental *Staphylococcus aureus* endocarditis through antiplatelet and antibacterial effects. *Circulation* 99:2791–2797.
 37. Kus, J. V., K. Zaton, R. Sarkar, and R. K. Cameron. 2002. Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. *Plant Cell* 14:479–490.
 38. Lau, G. W., H. Ran, F. Kong, D. J. Hassett, and D. Mavrodi. 2004. *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect. Immun.* 72:4275–4278.
 39. Lawton, K., K. Weymann, L. Friedrich, B. Vernooij, S. Uknes, and J. Ryals. 1995. Systemic-acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. *Mol. Plant-Microbe Interact.* 8:863–870.
 40. Li, X. Z., and H. Nikaido. 2004. Efflux-mediated drug resistance in bacteria. *Drugs* 64:159–204.
 41. Li, X., J. D. Clarke, Y. Zhang, and X. Dong. 2001. Activation of an EDS1-mediated R-gene pathway in the *sncl* mutant leads to constitutive, NPR1-independent pathogen resistance. *Mol. Plant-Microbe Interact.* 14:1131–1139.
 42. Lyczak, J. B., C. L. Cannon, and G. B. Pier. 2002. Lung infections associated with cystic fibrosis. *Clin. Microbiol. Rev.* 15:194–222.
 43. Mah, T.-F., B. Pitts, B. Pellock, G. C. Walker, P. S. Stewart, and G. A. O'Toole. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426:306–310.
 44. Mahajan-Miklos, S., M. W. Tan, L. G. Rahme, and F. M. Ausubel. 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell* 96:47–56.
 45. Malamy, J., J. Henning, and D. F. Klessig. 1992. Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. *Plant Cell* 4:359–366.
 46. Martinez, C., E. Pons, G. Prats, and J. Leon. 2004. Salicylic acid regulates flowering time and links defense responses and reproductive development. *Plant J.* 37:209–217.
 47. Mirakhor, A., M. J. Gallagher, M. J. Ledson, C. A. Hart, and M. J. Walshaw. 2003. Fosfomycin therapy for multiresistant *Pseudomonas aeruginosa* in cystic fibrosis. *J. Cystic Fibrosis* 2:19–24.
 48. Miyata, S., M. Casey, D. W. Frank, F. M. Ausubel, and E. Drenkard. 2003. Use of the *Galleria mellonella* caterpillar as a model host to study the role of the type III secretion system in *Pseudomonas aeruginosa* pathogenesis. *Infect. Immun.* 71:2404–2413.
 49. Mou, Z., W. Fan, and X. Dong. 2003. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes *Cell* 113:935–944.
 50. O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter. 1999. Genetic approaches to study of biofilms. *Methods Enzymol.* 310:91–109.
 51. Parsek, M. R., and P. K. Singh. 2003. Bacterial biofilms: an emerging link to disease pathogenesis. *Annu. Rev. Microbiol.* 57:677–701.
 52. Poole, K. 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J. Mol. Microbiol. Biotechnol.* 3:255–264.
 53. Prithiviraj, B., T. Weir, H. P. Bais, and J. M. Vivanco. 2005. Plant models for animal pathogenesis. *Cell. Microbiol.* 7:315–324.
 54. Prithiviraj, B., H. P. Bais, A. K. Jha, and J. M. Vivanco. 2005. *Staphylococcus aureus* pathogenicity on *Arabidopsis thaliana* is mediated either by a direct effect of salicylic acid on the pathogen or by SA-dependent, NPR1 independent host responses. *Plant J.* 42:417–432.
 55. Rahme, L. G., F. M. Ausubel, H. Cao, E. Drenkard, B. C. Goumnerov, G. W. Lau, S. Mahajan-Miklos, J. Plotnikova, M. W. Tan, J. Tsongalis, C. L. Walendziewicz, and R. G. Tompkins. 2000. Plants and animals share functionally common bacterial virulence factors. *Proc. Natl. Acad. Sci. USA* 97:8815–8821.
 56. Rahme, L., E. Stevens, S. Wolford, J. Shao, R. Tompkins, and F. M. Ausubel. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 268:1899–1902.
 57. Rahme, L. G., M.-W. Tan, L. Le, S. M. Wong, R. G. Tompkins, S. B. Calderwood, and F. M. Ausubel. 1997. Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. *Proc. Natl. Acad. Sci. USA* 94:13245–13250.
 58. Ran, H., D. J. Hassett, and G. W. Lau. 2003. Human targets of *Pseudomonas aeruginosa* pyocyanin. *Proc. Natl. Acad. Sci. USA* 100:14315–14320.
 59. Raskin, I. 1992. Role of salicylic acid in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:439–463.
 60. Shah, J. 2003. The salicylic acid loop in plant defense. *Curr. Opin. Plant Biol.* 6:365–371.
 61. Singh, P. K., M. R. Parsek, E. P. Greenberg, and M. J. Welsh. 2002. A component of innate immunity prevents bacterial biofilm development. *Nature* 417:552–555.
 62. Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh, and E. P. Greenberg. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407:762–764.
 63. Smith, R. S., and B. H. Iglewski. 2003. *P. aeruginosa* quorum sensing systems and virulence. *Curr. Opin. Microbiol.* 6:56–60.
 64. Soheila, K. M., A.-H. Mackerness, T. Page, C. F. John, A. M. Murphy, J. P. Carr, and V. Buchanan-Wollaston. 2000. Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant J.* 23:677–685.
 65. Spencker, F. B., L. Staber, T. Lietz, R. Schille, and A. C. Rodloff. 2003. Development of resistance in *Pseudomonas aeruginosa* obtained from patients with cystic fibrosis at different times. *Clin. Microbiol. Infect.* 9:370–379.
 66. Tan, M. W., L. G. Rahme, J. A. Sternberg, R. G. Tompkins, and F. M. Ausubel. 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proc. Natl. Acad. Sci. USA* 96:2408–2413.
 67. Tang, H. B., E. DiMango, R. Bryan, M. Gambello, B. H. Iglewski, J. B. Goldberg, and A. Prince. 1996. Contribution of specific *Pseudomonas aeruginosa* virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. *Infect. Immun.* 64:37–43.
 68. Thordal-Christensen, H. 2003. Fresh insights into processes of nonhost resistance. *Curr. Opin. Plant Biol.* 6:351–357.
 69. Tuite, J. 1969. Plant pathological methods, fungi and bacteria. Burgess Publishing Company, Minneapolis, Minn.
 70. VanEtten, H. D., J. W. Mansfield, J. A. Bailey, and E. E. Farmer. 1994. Two classes of plant antibiotics: phytoalexins versus “phytoanticipins.” *Plant Cell* 6:1191–1192.
 71. Walker, T. S., H. P. Bais, E. Déziel, H. P. Schweizer, L. G. Rahme, R. Fall, and J. M. Vivanco. 2004. *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. *Plant Physiol.* 134:320–331.
 72. Wang, W. H., W. M. Wong, D. Dailidienė, D. E. Berg, Q. Gu, K. C. Lai, S. K. Lam, and B. C. Wong. 2003. Aspirin inhibits the growth of *Helicobacter pylori* and enhances its susceptibility to antimicrobial agents. *Gut* 52:490–495.
 73. Whiteley, M., M. G. Banger, R. E. Bungarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413:860–864.
 74. Wu, H., Z. Song, M. Hentzer, J. B. Andersen, S. Molin, M. Givskov, and N. Hoiby. 2004. Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice. *J. Antimicrob. Chemother.* 53:1054–1061.