A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation

Colleen T. O'Loughlin[a], Laura C. Miller[b], Albert Siryaporn[a], Knut Drescher[a,c], Martin F. Semmelhack[b], and Bonnie L. Bassler[a,d,1]

Departments of [a]Molecular Biology, [b]Chemistry, and [c]Mechanical and Aerospace Engineering, Princeton University, Princeton, NJ 08544; and [d]Howard Hughes Medical Institute, Chevy Chase, MD 20815

Contributed by Bonnie L. Bassler, September 18, 2013 (sent for review July 23, 2013)

Quorum sensing is a chemical communication process that bacteria use to regulate collective behaviors. Disabling quorum-sensing circuits with small molecules has been proposed as a potential strategy to prevent bacterial pathogenicity. The human pathogen *Pseudomonas aeruginosa* uses quorum sensing to control virulence and biofilm formation. Here, we analyze synthetic molecules for inhibition of the two *P. aeruginosa* quorum-sensing receptors, LasR and RhlR. Our most effective compound, meta-bromo-thiolactone (mBTL), inhibits both the production of the virulence factor pyocyanin and biofilm formation. mBTL also protects *Caenorhabditis elegans* and human lung epithelial cells from killing by *P. aeruginosa*. Both LasR and RhlR are partially inhibited by mBTL in vivo and in vitro; however, RhlR, not LasR, is the relevant in vivo target. More potent antagonists do not exhibit superior function in impeding virulence. Because LasR and RhlR reciprocally control crucial virulence factors, appropriately tuning rather than completely inhibiting their activities appears to hold the key to blocking pathogenesis in vivo.

Quorum sensing is a process of bacterial communication used to collectively control group behaviors (1, 2). This process relies on the production, release, and group-wide detection of signal molecules called autoinducers, which in gram-negative bacteria are typically homoserine lactones (HSLs) (1, 2). HSLs are produced by LuxI-type enzymes, and cytoplasmic LuxR-type proteins function as HSL receptors (1, 2). Apo–LuxR-type proteins are insoluble (3, 4). Binding to autoinducers stabilizes LuxR-type receptors, enabling dimerization, DNA binding, and transcription of quorum-sensing target genes (3, 4). LuxI/R-signaling cascades are essential for virulence in many pathogens, and disabling these circuits with small molecules could prevent virulence (2). *Pseudomonas aeruginosa* is a pathogen important in cystic fibrosis, burn units of hospitals, and in implanted medical devices including intubation tubes and stents (5). To orchestrate synchronous production of virulence factors and biofilm formation, *P. aeruginosa* relies on two major LuxI/R quorum-sensing systems, the Las and Rhl systems (6–8). In *P. aeruginosa*, LasI produces and LasR responds to the autoinducer 3OC12–HSL (Fig. 1*A*) (5, 7, 10, 11). The LasR:3OC12–HSL complex activates transcription of many genes including *rhlR,* which encodes a second quorum-sensing receptor (Fig. 1*B*) (11, 12). RhlR binds to the autoinducer C4–HSL (Fig. 1*A*), the product of RhlI (6, 7, 13, 14). RhlR:C4–HSL also directs a large regulon of genes, some of which are also members of the LasR regulon (Fig. 1*B*) (6, 15, 16). This tandem regulatory arrangement allows LasI/R to control the first wave of quorum-sensing–controlled gene expression and RhlI/R to control the second (6, 11, 16, 17). Because LasR activates expression of *rhlR*, deletion of *lasR* reduces expression of both LasR- and RhlR-regulated target genes (11, 17). Previous screens have identified small-molecule inhibitors of the *P. aeruginosa* quorum-sensing receptors; however, none delivered a molecule capable of influencing virulence in tissue culture or animal models (18–20).

Results

We previously reported the molecule chlorolactone (CL) (Fig. 1*A*) as an inhibitor of the LasR/RhlR homolog CviR from *Chromobacterium violaceum* (21). Based on the CL structure, we designed and synthesized a focused library of ∼30 molecules (21) (*SI Appendix*, Fig. S3*A*). Here, we present our analyses for CL and three additional molecules [chloro-thiolactone (CTL), meta-chloro-thiolactone (mCTL), and meta-bromo-thiolactone (mBTL); Fig. 1*A*]. First, we tested the molecules for inhibition of quorum sensing in WT *P. aeruginosa* PA14 using the quorum-sensing–regulated production of the virulence factor pyocyanin as the readout (Fig. 1*C*). Following activation by LasR:3OC12–HSL, the RhlR:C4–HSL complex activates expression of the pyocyanin operon (15, 22–24) (Fig. 1*B*). Therefore, *P. aeruginosa* PA14 produces pyocyanin at high cell density, and limited pyocyanin production occurs in a *lasR* or a *rhlR* null mutant and in the *lasR*, *rhlR* double mutant (Fig. 1*C*).

Neither CL nor CTL inhibited pyocyanin production in vivo (Fig. 1*C*). By contrast, mCTL and mBTL showed potent inhibition of pyocyanin (Fig. 1*C*) while not affecting *P. aeruginosa* PA14 growth. We calculated 50% inhibitory concentration (IC_{50}) values of 8 μM (±2) for mBTL and 9 μM (±2) for mCTL (Fig. 1*D*). Inhibition was not due to interaction of these molecules with pyocyanin, as incubation of pyocyanin-containing cell-free culture fluids from untreated *P. aeruginosa* PA14 with 100 μM mBTL or mCTL did not alter pyocyanin levels (*SI Appendix*, Fig. S1). We synthesized four previously reported LasR inhibitors for comparison in our pyocyanin assay (*SI Appendix*, Fig. S2*A*) (18–20). Two compounds, itc-13 and V-06–018, inhibited pyocyanin production in *P. aeruginosa* PA14 with higher IC_{50} values [56 μM (±10) and 18 μM (±2), respectively] than mBTL and mCTL (*SI Appendix*, Fig. S2 *B* and *C*). The other molecules (PD-12 and B7) showed limited or no inhibition in vivo. (*SI Appendix*, Fig. S2*B*).

Significance

In this study, we prepare synthetic molecules and analyze them for inhibition of the *Pseudomonas* quorum-sensing receptors LasR and RhlR. Our most effective compound, meta-bromo-thiolactone, not only prevents virulence factor expression and biofilm formation but also protects *Caenorhabditis elegans* and human A549 lung epithelial cells from quorum-sensing–mediated killing by *Pseudomonas aeruginosa*. This anti–quorum-sensing molecule is capable of influencing *P. aeruginosa* virulence in tissue culture and animal models. Our findings demonstrate the potential for small-molecule modulators of quorum sensing as therapeutics.

Author contributions: C.T.O., L.C.M., A.S., K.D., M.F.S., and B.L.B. designed research; C.T.O., L.C.M., A.S., and K.D. performed research; C.T.O., L.C.M., A.S., and K.D. contributed new reagents/analytic tools; C.T.O., L.C.M., A.S., K.D., M.F.S., and B.L.B. analyzed data; and C.T.O., L.C.M., A.S., K.D., M.F.S., and B.L.B. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The microarray intensity data have been deposited in the Princeton University MicroArray database, http://puma.princeton.edu (PUMA db, http://puma.princeton.edu/cgi-bin/publication/viewPublication.pl?pub_no=558).

[1]To whom correspondence should be addressed. E-mail: bbassler@princeton.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1316981110/-/DCSupplemental.

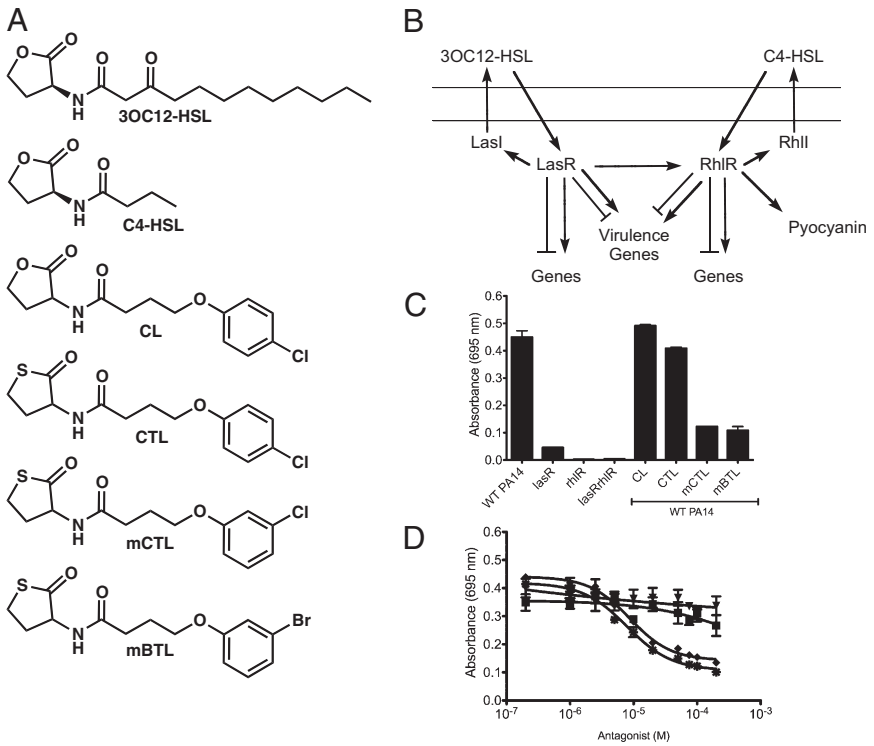

Fig. 1. Small-molecule control of pyocyanin production in *P. aeruginosa* PA14. (*A*) Structures of autoinducers and inhibitors discussed in this study. (*B*) A simplified schematic of the major components of the *P. aeruginosa* quorum-sensing circuit. (*C*) Pyocyanin production was measured at OD = 695 nm in cell-free culture fluids prepared from WT *P. aeruginosa* PA14, *lasR*, and *rhlR* single and double mutants and in WT treated with 100 μM CL, CTL, mCTL, and mBTL. Error bars represent SD for two replicates. (*D*) Pyocyanin inhibition titrations were performed with WT *P. aeruginosa* PA14 in triplicate with CL (inverted triangles), CTL (squares), mCTL (diamonds), and mBTL (asterisks). Error bars represent SD of three replicates.

Thus, mBTL is more potent than mCTL and these previously identified inhibitors.

To determine the enantiomer of mBTL responsible for inhibition, we performed a chiral separation. The *S* enantiomer is active (IC_{50} = 4 μM), whereas the *R* enantiomer displays residual activity (IC_{50} = 100 μM) (*SI Appendix*, Fig. S3 *A* and *B*). Because the racemic mixture has an IC_{50} value only twofold higher than the isolated *S* enantiomer, we conclude that the *R* enantiomer does not influence inhibition. For ease of synthesis, we use racemic mixtures for the remainder of the work.

mBTL contains a four-carbon linker (Fig. 1*A*). We synthesized and tested mBTL derivatives with two- to six-carbon linkers and lactone versions of mBTL harboring four- or six-carbon linkers, containing or lacking a 3-oxo-moiety. None of these compounds exhibited increased potency (*SI Appendix*, Fig. S3 *A*, *C*, and *D*). Thus, mBTL remains the most potent in vivo inhibitor in our collection.

We investigated whether mBTL interacts with LasR, RhlR, or both receptors using recombinant *Escherichia coli* strains producing the receptor proteins and containing target *gfp* reporter fusions (*rsaL–gfp* for LasR and *rhlA–gfp* for RhlR). In the absence of ligand, neither receptor activates expression of the target–*gfp* fusion (Fig. 2 *A* and *B*). When autoinducer is supplied at 95% maximal effective concentration (EC_{95}), LasR and RhlR activate gene expression. mBTL partially antagonizes this effect (22 ± 4% and 43 ± 10% inhibition, respectively; Fig. 2 *A* and *B*). In the absence of autoinducer, mBTL, when provided at the EC_{95} of the native ligand for each receptor, acts as a partial agonist of both LasR and RhlR (72 ± 3% and 80 ± 25%, respectively). As we have previously reported (21), molecules such as mBTL that resemble autoinducers induce conformational changes in LuxR-type receptors that impair their ability to interact with RNA polymerase which lowers their transcriptional activation potential. In the presence of autoinducer, this reduction in transcription potential manifests as antagonism. In the absence of autoinducer, some transcription activation can still occur. Thus, without autoinducer present, molecules such as mBTL appear as partial agonists.

LuxR-type proteins require cognate autoinducers to fold (3, 4). Consistent with this, LasR and RhlR are insoluble in the absence of autoinducer and are present in the whole cell (WC) fraction but not the soluble (S) fraction following SDS/PAGE (Fig. 2 *C* and *D*). Addition of 100 μM 3OC12–HSL or mBTL solubilizes LasR (Fig. 2*C*). mBTL also solubilizes RhlR; surprisingly, C4–HSL, its native autoinducer, does not (Fig. 2*D*). We suspect that autoinducer-bound RhlR is not particularly soluble when overproduced in *E. coli*, and the protein likely aggregates. mBTL appears to protect the protein from aggregation. We have previously demonstrated that similar protection occurs with CviR bound to CL (21).

To verify that mBTL functions by inhibiting quorum sensing in vivo, we used microarrays to examine the consequences of administering mBTL to WT *P. aeruginosa* and to the *lasR* and *rhlR* mutants. Treatment of WT *P. aeruginosa* PA14 caused alterations in expression of many of the known quorum-sensing targets (*SI Appendix*, Table S1). For example, LasR–regulon genes including *rpoS* and *nor* were down-regulated (*SI Appendix*, Tables S1 and S2). RhlR-controlled virulence genes, for example, those encoding rhamnolipids (*rhlA* and *rhlB*), and phenazines (*phzA2, phzB1, and phzB2*) were also repressed (*SI Appendix*, Table S1 and S3). Indeed, the profile of WT cells treated with mBTL matches well with the combined profiles of the *lasR* and *rhlR* mutants (*SI Appendix*, Table S1). However, the fold activation and fold repression is not as dramatic in the mBTL-treated WT as in the mutants, confirming that mBTL does not fully inhibit either regulator (*SI Appendix*, Table S1–S3). Thirteen genes are activated twofold or more in WT *P. aeruginosa* PA14 when treated with mBTL (*SI Appendix*, Table S4). By contrast, 213 genes are down-regulated twofold or more when WT is treated with

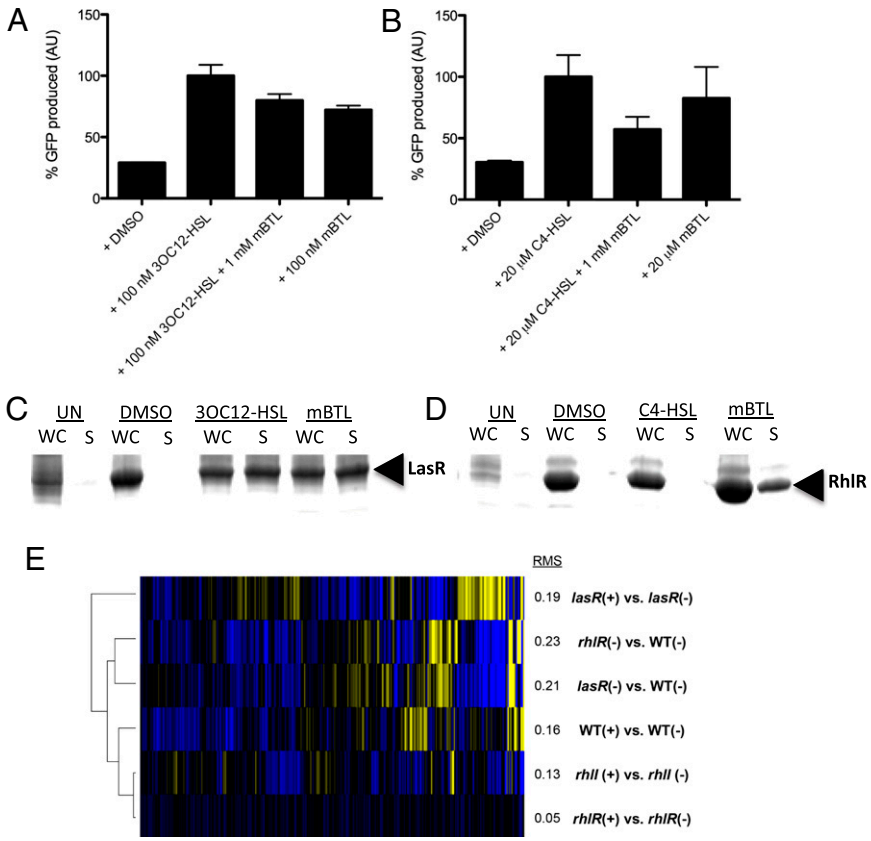

Fig. 2. mBTL binds and inhibits LasR and RhlR, and the primary in vivo target of mBTL is RhlR. (*A*) LasR activation of expression of *rsaL–gfp* in *E. coli*. (*B*) RhlR activation of expression of *rhlA–gfp* in *E. coli*. In each panel, *gfp* expression in the presence of the cognate autoinducer (100 nM 3OC12–HSL or 20 μM C4–HSL) is set to 100%. mBTL was tested for inhibition at 1 mM. Agonism was examined at 100 nM for LasR and at 20 μM for RhlR. Error bars represent SD of three replicates. (*C*) SDS/PAGE analysis of whole cell (WC) and soluble (S) extracts from *E. coli* cultures expressing LasR in the presence of DMSO, 100 μM 3OC12–HSL, or 100 μM mBTL. An uninduced control (UN) is shown for comparison. (*D*) Same as *C* with RhlR in the presence of DMSO, 100 μM C4–HSL, or 100 μM mBTL. (*E*) Hierarchical clustering, heat maps, and the RMS of the fold change (log_{10}) of mBTL-treated (+) or DMSO-treated (–) WT *P. aeruginosa*, *lasR*, *rhlR*, and *rhlI* mutants. Dendrogram to the left of the map indicates average Euclidean linkage distances between the gene expression profiles. Blue and yellow indicate decreased and increased expression, respectively. Data are the average of three independent biological experiments, one in which the Cy3 and Cy5 dyes were swapped.

mBTL (*SI Appendix*, Table S1). These data indicate that the major role of mBTL in WT *P. aeruginosa* PA14 is as an antagonist that exerts control over virulence through partial inhibition of LasR and RhlR, not via up-regulation of other genes.

The most important comparisons for defining the target of mBTL are the mBTL-treated and untreated *lasR* and *rhlR* mutants (*SI Appendix*, Tables S5 and S6 and Fig. 2*E*). In the absence of LasR, mBTL treatment still alters expression of some genes. By contrast, there is essentially no difference between the untreated and mBTL-treated *rhlR* mutant profiles. Thus, although some of the mBTL effects occur through LasR, all of the mBTL effects depend on RhlR (*SI Appendix*, Tables S5 and S6 and Fig. 2*E*). Thus, mBTL functions in vivo by inhibiting quorum sensing via RhlR.

Given that mBTL acts as a partial agonist of RhlR in recombinant *E. coli* when the cognate autoinducer C4–HSL is not present (Fig. 2*B*), we explored further the extent of mBTL agonism of RhlR in vivo. To do this, we treated a *P. aeruginosa rhlI* null strain with mBTL and performed microarrays. mBTL activated twofold or higher expression of 44 genes (*SI Appendix*, Table S7). For comparison, RhlR bound to C4–HSL activated nearly 300 genes (*SI Appendix*, Table S3). In every case in which a gene could be activated by C4–HSL or by mBTL, C4–HSL was a much more potent activator. Importantly, only one gene that was activated by mBTL in the *rhlI* mutant was activated by mBTL in WT *P. aeruginosa* (*SI Appendix*, Table S7 and S4). These data indicate that mBTL can act as a weak RhlR agonist in vivo, but only when the native autoinducer is absent, a situation that is not likely to occur in WT bacteria.

To determine if mBTL can limit virulence, we used a *Caenorhabditis elegans* fast-kill infection assay (8, 25). WT *P. aeruginosa* PA14 and the *lasR* mutant rapidly kill *C. elegans*: 77 and 90% of worms die after 24 h, respectively (Fig. 3*A*), indicating that LasR is not required for pathogenesis in this assay. Much less killing occurs when the nematodes are exposed to the *rhlR* or the *lasR*, *rhlR* double null strain (31 and 17%, respectively, died in 24 h; Fig. 3*A*), showing that RhlR is required for virulence in this assay. Treatment of WT and the *lasR* mutant *P. aeruginosa* PA14 strains with 50 μM mBTL protects *C. elegans* from killing (23 and 50% death, respectively; Fig. 3*B* and *SI Appendix*, Fig. S4). Together, these results confirm that the relevant in vivo target of mBTL is RhlR and, importantly, that inhibiting RhlR could form the basis of an antibacterial therapeutic strategy.

We tested whether mBTL could improve the outcome for mammalian cells during infection using the human lung carcinoma cell line A549. mBTL at 100 μM is not toxic to A549 cells (*SI Appendix*, Fig. S5). WT, *lasR*, and *rhlR* single mutants and the *lasR*, *rhlR* double mutant *P. aeruginosa* are all capable of killing A549 cells (Fig. 3*C*). Treatment with mBTL reduces killing by the WT and the *lasR* mutant strain but not by the *rhlR* or the *lasR*, *rhlR* double mutant strains (Fig. 3*C*). These results validate the conclusion reached on the basis of the microarray data presented above. Specifically, the relevant target of mBTL is

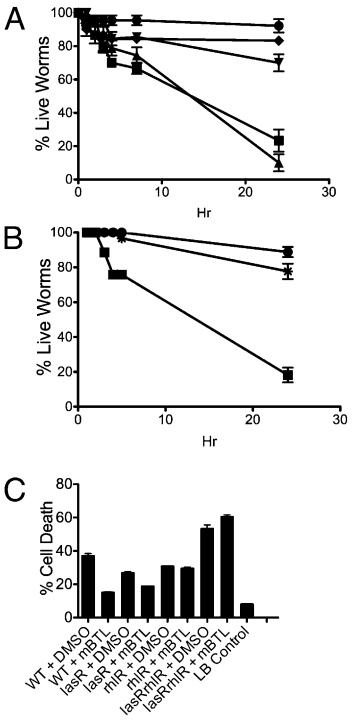

Fig. 3. mBTL inhibits *P. aeruginosa* PA14 virulence toward *C. elegans* and human A549 lung cells. (*A*) *C. elegans* were applied to lawns of *E. coli* HB101 (circles), WT *P. aeruginosa* PA14 (squares), *lasR* mutant (triangles), *rhlR* mutant (inverted triangles), and *lasR*, *rhlR* double mutant (diamonds) strains. The percent live worms was calculated every hour for the first 5 h and again at 24 h. Error bars represent SEM of three replicates. (*B*) Same as *A*. *E. coli* HB101 (circles), WT *P. aeruginosa* PA14 (squares), and WT *P. aeruginosa* in the presence of 50 μM mBTL (asterisks). (*C*) The percent cell death was calculated using propidium iodide uptake into A549 lung cells after 8 h and normalized to cells lysed with detergent. Error bars represent SEM of three replicates.

present in the WT and the *lasR* mutant but is not in the *rhlR* single and *lasR*, *rhlR* double mutant strains. Thus, RhlR appears to be the major in vivo target.

Beyond being a clinically relevant pathogen, *P. aeruginosa* is an industrial and medical nuisance because it causes blockages in filtration devices and stents. *P. aeruginosa* PA14 also clogs microfluidic chambers that model such devices (26). Clogging is due to biofilms that produce exopolysaccharide-containing streamers that act as sieves to catch passing cells (26). Compared with WT, *lasR* and *rhlR* single and double null mutants exhibit dramatically delayed clogging, demonstrating that quorum sensing is required to form blockages (Fig. 4*A*). In the presence of 100 μm mBTL, WT *P. aeruginosa* PA14 exhibits a phenotype indistinguishable from the mutants (Fig. 4*A*). We also examined the ability of mBTL to influence static biofilm formation in *P. aeruginosa* PA14. WT *P. aeruginosa* PA14 forms biofilms with an average height of 27.5 ± 11.5 μm (Fig. 4*B*). Treatment of WT with 100 μM mBTL results in a decrease in the average height to 10 ± 4 μm (Fig. 4*B*). Treatment of the *lasR* null strain with mBTL also decreases the average height of the biofilm (15.7 ± 4.9 μm to 6.6 ± 2.3 μm). However, treatment of the *rhlR* null strain (25 ± 10.6 μm untreated vs. 22.1 ± 7.5 μm treated) or the *lasR*, *rhlR* double null strain (11.9 ± 5.5 μm untreated vs. 16.2 ± 5.9 μm treated) did not influence the height of the biofilm, indicating that the action of mBTL relies on the presence of RhlR (Fig. 4*B*). Taken together these findings show that mBTL inhibits quorum-sensing–controlled biofilm formation in *P. aeruginosa* PA14.

Discussion

This work exploring interference with quorum sensing demonstrates that mBTL, an analog of the native *P. aeruginosa* autoinducers, represses expression of the genes encoding the virulence factor pyocyanin, prevents biofilm formation, and protects *C. elegans* and human lung epithelial cells from killing by *P. aeruginosa*. Both the LasR and RhlR quorum-sensing receptors are partially inhibited by mBTL; however, in the contexts that we have examined, RhlR, not LasR, is the relevant in vivo target. Much emphasis has been placed on the discovery of LasR inhibitors because LasR is situated at the top of the *P. aeruginosa* quorum-sensing cascade (27). Our results suggest that the critical in vivo target for modulation is RhlR. Experiments in *Drosophila melanogaster* also demonstrate that the *P. aeruginosa* PA14 *lasR* mutant strain is virulent, whereas the *rhlR* mutant strain is attenuated (28) suggesting, as do our results, that RhlR is the pertinent in vivo quorum-sensing regulator.

It is curious that mBTL, our most potent in vivo inhibitor, is a partial agonist/partial antagonist of RhlR and LasR in the recombinant *E. coli* assay (Fig. 2 *A* and *B*) and according to the *P. aeruginosa* microarrays (*SI Appendix*, Table S1). This feature of mBTL may be critical for its ability to function in vivo. Because LasR and RhlR act reciprocally to control key virulence genes (Fig. 1*B*), molecules that function as pure antagonists of both receptors might not be superior antivirulence therapies. Consistent with this notion, CL is a more potent inhibitor of both LasR- and RhlR-directed transcription in *E. coli* than is mBTL, and CL does not agonize either receptor (*SI Appendix*, Fig. S6). Nevertheless, CL exhibits no in vivo inhibition of pyocyanin production (Fig. 1 *B* and *C*), and it does not attenuate killing of A549 lung cells by *P. aeruginosa*. We suspect that a "sweet spot" exists in terms of antagonist potency for inhibitors of LasR and/or RhlR and that mBTL has the appropriate level of potency to be effective in vivo. Consider a case in which LasR activates and RhlR represses a particular virulence factor (see Fig. 1*B*). A highly potent, dual LasR–RhlR antagonist could result in derepression and thus production of the virulence factor. Many examples exist in which LasR and RhlR act reciprocally in their regulation of particular genes, including virulence genes (9, 11, 17).

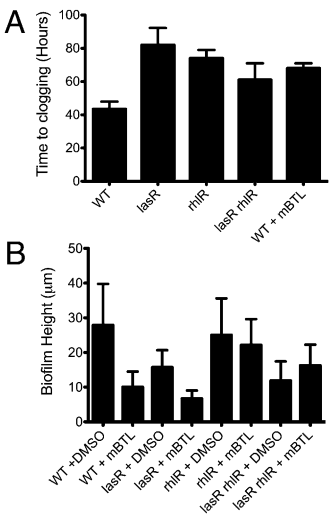

Fig. 4. mBTL inhibits quorum-sensing–regulated clogging of microfluidic chambers and biofilm formation in static cultures. (*A*) Time to clogging was measured for the designated *P. aeruginosa* PA14 strains and for the WT in the presence of 100 μM mBTL. Error bars represent SD of six replicates. (*B*) Biofilms were grown in static cultures at the base of a glass-bottom microtiter plate in the presence or absence of 100 μM mBTL. Biofilm thickness was measured using confocal microscopy. Error bars indicate SD of five to eight biological replicates.

It is noteworthy that, in the lung cell assay, the *lasR*, *rhlR* double mutant causes more cell death than does the WT (Fig. 3*C*). This result may stem from misregulation of virulence factors that are controlled in opposing directions by RhlR and LasR (Fig. 1*B*). In the preceding paragraph, we argue that potent inhibition of both LasR and RhlR could result in increased pathogenicity. The phenotype of the double mutant in the lung cell assay supports this idea. Complete inhibition of LasR and RhlR would yield the same phenotype as deletion of both receptors. Apparently, that phenotype is high virulence. Consistent with our results and interpretation, the quadruple Δ*lasI,* Δ*lasR*, Δ*rhlI*, Δ*rhlR* mutant remains virulent in a mouse lung infection model (29). Lastly, we note that the double *lasR*, *rhlR* null mutant is not hypervirulent in the nematode assay. The quorum-sensing–controlled virulence factors required for pathogenicity in mammalian cells are not precisely identical to those that are essential for virulence in nematodes which presumably accounts for this discrepancy (5, 8). What is critical is that, despite these differences, mBTL attenuates virulence of WT *P. aeruginosa* PA14 in both assays and in a RhlR-dependent manner.

In the nematode and lung cell experiments shown here, we pregrew the bacteria with mBTL and supplied a dose of inhibitor at the start of infection. Virulence was not reduced when *P. aeruginosa* PA14 was pregrown in the absence of inhibitor (*SI Appendix*, Fig. S7), indicating that mBTL functions as a prophylactic. The use of prophylactics, specifically for *P. aeruginosa*, is an established procedure, so anti–quorum-sensing prophylactic molecules could find use in the clinic. Further optimization of mBTL could tune efficacy and delivery and possibly broaden use beyond prophylaxis. An immediate focus is the head group given that thiolactones are susceptible to hydrolytic ring opening.

Our results showing that mBTL prevents biofilm formation and clogging in microfluidic devices (Fig. 4) suggest that, in addition to the implications of mBTL as an antiinfective, deploying anti–quorum-sensing molecules could prevent the failure of devices that are prone to fouling by biofilms. Thus, such molecules could be useful in industrial settings and in contexts requiring medical implants. Here we administered the inhibitor in the liquid phase; a next goal is to embed mBTL-like molecules in materials used for production of such devices, resulting in products that are innately resistant to biofilms. Taken together, our data concerning mBTL make an excellent case for the effectiveness of quorum-sensing modulators for the attenuation of quorum-sensing–controlled phenotypes in pathogenic bacteria.

Materials and Methods

Strains and Media. *E. coli* strains were grown at 37 °C in Luria broth (LB) (Fisher). Plasmid pET23b (Novagen) was used to express *lasR* and *rhlR* in *E. coli* strain BL21-Gold (DE3) (Stratagene). Plasmids were maintained with 100 μg/mL ampicillin. Plasmid pEVS141 (30) was used for *rhlA–gfp* or *rsaL–gfp* expression and maintained with 50 μg/mL of kanamycin. *P. aeruginosa* strains were grown with shaking at 37 °C in LB. *C. elegans* WT strain N2 was propagated on nematode growth media with an *E. coli* HB101 lawn as the food source at 20 °C. A549 human lung carcinoma cells (American Type Culture Collection #CCL-185) were grown in DMEM (Gibco) plus 20% fetal bovine serum and 1× PenStep (Sigma) at 37 °C.

The *P. aeruginosa rhlR* strain (*rhlR*::*MAR2xT7*) and the *rhlI* strain (*rhlI*::*MAR2xT7*) come from the *P. aeruginosa* PA14 ordered transposon library (31). The *P. aeruginosa lasR* and *lasR*, *rhlR* double mutant strains were constructed using λ Red recombination as described previously (32–34). See *SI Appendix* for complete method.

Pyocyanin Analyses. Overnight, *P. aeruginosa* cultures were subcultured 1:1,000 into 5 mL fresh medium. CL, CTL, mBTL, and mCTL were assayed at 100 μM for endpoint assays and at concentrations ranging from 200 nM to 200 μM for titrations following 17 h of aerobic growth with shaking at 37 °C. Cells were separated from culture fluids via centrifugation at 16.3 × 1000 *g* for 15 min. Culture fluids were passed through 0.22- μm syringe-driven filters (Millipore). Cell-free culture fluids were analyzed for pyocyanin on a Beckman Coulter DU-800 spectrophotometer from 200 to 800 nm; 695 nm was chosen for graphical representation. Titration data were fit with a variable-slope sigmoidal dose–response curve using GraphPad Prism to determine the IC_{50} values.

LasR and RhlR GFP Assays. The LasR–GFP assays were performed in *E. coli* strain BL21 DE3 Gold (Agilent) carrying pET23b (Novagen) containing *lasR* (maintained with 100 μg/mL ampicillin) and carrying plasmid pEVS141 (30) containing the *rsaL* promoter-driving expression of *gfp* (maintained with 50 μg/mL of kanamycin.) The RhlR–GFP assays were performed in *E. coli* strain BL21 DE3 Gold (Agilent) carrying pET23b (Novagen) containing *rhlR* (maintained with 100 μg/mL ampicillin) and carrying plasmid pEVS141 (30) containing the *rhlA* promoter-driving expression of *gfp* (maintained with 50 μg/mL of kanamycin.) These *E. coli* strains were grown overnight and subcultured 1:100 into fresh medium with appropriate antibiotics and grown shaking for 8 h at 37 °C for the LasR–GFP strain and 12 h for the RhlR–GFP construct. 3OC12–HSL (50 nM) or C4–HSL (20 μM) was added to the LasR–GFP and RhlR–GFP preparations, respectively. Compounds were tested at 1 mM for antagonism and at 100 nM or 20 μM for agonism. These concentrations were chosen for agonism studies to match the concentrations of autoinducers used in our experiments. For antagonism studies, we used the EC_{95} concentration for each receptor. GFP was measured on an Envision plate reader.

LasR and RhlR Overexpression. Overexpression of LasR and RhlR were performed as previously reported for CviR (21). See *SI Appendix* for complete method.

RNA Extraction and Microarray Analysis. Overnight, *P. aeruginosa* PA14 cultures were back-diluted 1:1,000 into 5 mL of fresh LB and 100 μM mBTL, or an equivalent amount of DMSO, was added to cultures which were grown aerobically with shaking at 37 °C for 17 h. Nine ODs of cells were harvested for each treatment. Lysozyme (1 mg/mL in Tris-EDTA buffer) (Sigma) was added for 10 min at RT. Total RNA was prepared using the RNeasy Midi Kit (Qiagen). cDNA was synthesized and normalized as previously reported (35). cDNA was hybridized using the Gene Expression Hybridization Kit (Agilent) to a custom microarray (Agilent design ID 43307), which was designed using the Agilent eArray tool with two probes for most genes. Samples were hybridized for 22 h at 65 °C with continuous rotation at 10 rpm using a rotisserie hybridization oven (Agilent model #G2545A). Microarrays were scanned using an Agilent G2505C scanner and analyzed using Agilent Feature Extract software version 9.5. Resulting microarray intensity data have been deposited in the Princeton University MicroArray database (http://puma.princeton.edu) for archiving and analyzed using Matlab R2013a (MathWorks).

***C. elegans* Life Span Assays (8, 25).** *C. elegans* fast killing assays were conducted with 90 WT N2 worms for each condition (30 worms per replicate, three replicates performed). *C. elegans* were propagated on NGM plates before eggs being harvested from gravid adults using a standard bleaching protocol (30 mL 5% bleach, 15 mL 5-M KOH, 55 mL dH_2O). Harvested eggs were placed on lawns of fresh *E. coli* HB101 and allowed to grow for 48 h (to reach the L4 stage) at 20 °C before being moved to lawns of *P. aeruginosa* and placed at 25 °C on sorbitol, glucose, and cholesterol plates. Nematodes were scored for survival every hour for 5 h and again at 24 h. The percent living worms was calculated in triplicate for each time point and 50 μM mBTL or an equivalent volume of DMSO was added to plates and to the bacterial cultures during growth.

A549 Human Lung Cell Infections. Human A549 cells were grown in CellStar tissue culture flasks. Before infection, the A549 cells were treated with trypsin–EDTA (CellGro), split, counted, and aliquotted into 96-well plates at 20,000 mammalian cells per well [cell counts were estimated using Trypan Blue (CellGro) exclusion]. Cells were grown for 20 h at 37 °C in DMEM (Invitrogen). Cells were washed 3× with warm PBS (Gibco) before 100 μL of "master mix" was added to each well for each condition. Master mix contained 1 mL prewarmed PBS, 5 μL of 2 mg/mL propidium iodide (Bioprobes), 1 μL of a 100-mM inhibitor stock or DMSO, and 10 μL of OD_{600} = 2 *P. aeruginosa* PA14 grown in the presence of mBTL or DMSO. Infections were monitored using a Perkin Elmer Wallac EnVision plate reader every 2 h with the Texas Red FP filter available from Perkin Elmer.

Biofilm-Induced Clogging of Microfluidic Flow Cells. Clogging experiments were performed as previously described (26) in the presence or absence of mBTL. See *SI Appendix* for the detailed method.

Biofilm Growth in Static Culture. Overnight, *P. aeruginosa* cultures were back-diluted 1:1,000 into tryptone broth with 100 μM mBTL, or an equivalent concentration of DMSO, and grown to midlogarithmic phase (OD_{600} = 0.5). A 96-well plate with glass bottom (Thermo Fisher), which was filled with 200 μL of tryptone broth containing 100 μM mBTL or DMSO, was then inoculated

with 2 μL of the midlogarithmic culture. The 96-well plates were incubated for 24 h before adding 5 μM SYTO 9 nucleic acid stain (Invitrogen). Biofilm thickness was measured using confocal microscopy (Nikon).

Chemistry Materials and Methods. The following compounds were synthesized as previously described: CL, CTL, mBTL, mCTL (21), itc-13 (20), and PD-12 (36). See *SI Appendix* for complete synthesis and evaluation methods.

ACKNOWLEDGMENTS. We thank Christina Kraml and Neal Byrne (Lotus Separations, LLC) for chiral separation of mBTL; Donna Storton, Jessica Buckles, and John Matese for assistance with microarray experiments; Anisa Ismail and Kristen Werner for assistance with tissue culture and *C. elegans* maintenance, respectively; Lee Swem and Jessica Schaffer for plasmids; and Frederick Hughson for thoughtful advice and discussion. PUMAdb is funded in part by National Institutes of Health (NIH) Grant P50 GM071508. This work was supported by the Howard Hughes Medical Institute, NIH Grant 5R01GM065859 and National Science Foundation Grant MCB-0343821 (to B.L.B.) A.S. is supported by Grant F32AI095002 (a Ruth L. Kirschstein National Research Service Award fellowship from NIH National Institute of Allergy and Infectious Diseases). K.D. is supported by the Human Frontier Science Program.

1. Ng W-L, Bassler BL (2009) Bacterial quorum-sensing network architectures. *Annu Rev Genet* 43(1):197–222.
2. Rutherford ST, Bassler BL (2012) Bacterial quorum sensing: Its role in virulence and possibilities for its control. *Cold Spring Harbor Perspect Med* 2(11):a012427.
3. Zhu J, Winans SC (1999) Autoinducer binding by the quorum-sensing regulator TraR increases affinity for target promoters in vitro and decreases TraR turnover rates in whole cells. *Proc Natl Acad Sci USA* 96(9):4832–4837.
4. Zhu J, Winans SC (2001) The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. *Proc Natl Acad Sci USA* 98(4):1507–1512.
5. Sadikot RT, Blackwell TS, Christman JW, Prince AS (2005) Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med* 171(11):1209–1223.
6. Pesci EC, Pearson JP, Seed PC, Iglewski BH (1997) Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 179(10):3127–3132.
7. Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: A transcriptome analysis. *J Bacteriol* 185(7):2066–2079.
8. Tan M-W, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM (1999) Pseudomonas aeruginosa killing of Caenorhabditis elegans used to identify P. aeruginosa virulence factors. *Proc Natl Acad Sci USA* 96(5):2408–2413.
9. Gambello MJ, Iglewski BH (1991) Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. *J Bacteriol* 173(9):3000–3009.
10. Pearson JP, et al. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* 91(1):197–201.
11. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *J Bacteriol* 185(7):2080–2095.
12. Gilbert KB, Kim TH, Gupta R, Greenberg EP, Schuster M (2009) Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR. *Mol Microbiol* 73(6):1072–1085.
13. Pearson JP, Passador L, Iglewski BH, Greenberg EP (1995) A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 92(5): 1490–1494.
14. Ochsner UA, Fiechter A, Reiser J (1994) Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa* rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. *J Biol Chem* 269(31): 19787–19795.
15. Latifi A, et al. (1995) Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* 17(2):333–343.
16. Brint JM, Ohman DE (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(24): 7155–7163.
17. Parsek MR, Greenberg E (2000) Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: A signaling mechanism involved in associations with higher organisms. *Proc Natl Acad Sci USA* 97(16):8789–8793.
18. Müh U, et al. (2006) Novel *Pseudomonas aeruginosa* quorum-sensing inhibitors identified in an ultra-high-throughput screen. *Antimicrob Agents Chemother* 50(11): 3674–3679.
19. Geske GD, O'Neill JC, Miller DM, Mattmann ME, Blackwell HE (2007) Modulation of bacterial quorum sensing with synthetic ligands: Systematic evaluation of N-acylated homoserine lactones in multiple species and new insights into their mechanisms of action. *J Am Chem Soc* 129(44):13613–13625.
20. Amara N, et al. (2009) Covalent inhibition of bacterial quorum sensing. *J Am Chem Soc* 131(30):10610–10619.
21. Swem LR, et al. (2009) A quorum-sensing antagonist targets both membrane-bound and cytoplasmic receptors and controls bacterial pathogenicity. *Mol Cell* 35(2):143–153.
22. Lau GW, Hassett DJ, Ran H, Kong F (2004) The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol Med* 10(12):599–606.
23. Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM (1999) Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. *Cell* 96(1):47–56.
24. Dietrich LEP, Price-Whelan A, Petersen A, Whiteley M, Newman DK (2006) The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol* 61(5):1308–1321.
25. Cezairliyan BVN, et al. (2013) Identification of *Pseudomonas aeruginosa* phenazines that kill *Caenorhabditis elegans*. *PLoS Pathog* 9(1):e1003101.
26. Drescher K, Shen Y, Bassler BL, Stone HA (2013) Biofilm streamers cause catastrophic disruption of flow with consequences for environmental and medical systems. *Proc Natl Acad Sci USA* 110(11):4345–4350.
27. Kalia VC (2013) Quorum sensing inhibitors: An overview. *Biotechnol Adv* 31(2): 224–245.
28. Limmer S, et al. (2011) *Pseudomonas aeruginosa* RhlR is required to neutralize the cellular immune response in a *Drosophila melanogaster* oral infection model. *Proc Natl Acad Sci USA* 108(42):17378–17383.
29. Lazenby JJGP, Griffin PE, Kyd J, Whitchurch CB, Cooley MA (2013) A quadruple knockout of lasIR and rhlIR of *Pseudomonas aeruginosa* PAO1 that retains wild-type twitching motility has equivalent infectivity and persistence to PAO1 in a mouse model of lung infection. *PLoS ONE* 8(4):e60973.
30. Dunn AK, Millikan DS, Adin DM, Bose JL, Stabb EV (2006) New rfp- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and lux expression in situ. *Appl Environ Microbiol* 72(1):802–810.
31. Liberati NT, et al. (2006) An ordered, nonredundant library of Pseudomonas aeruginosa strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* 103(8): 2833–2838.
32. Lesic B, Rahme LG (2008) Use of the lambda Red recombinase system to rapidly generate mutants in *Pseudomonas aeruginosa*. *BMC Mol Biol* 9(1):20.
33. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP (1998) A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: Application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212(1):77–86.
34. Shen Y, Siryaporn A, Lecuyer S, Gitai Z, Stone HA (2012) Flow directs surface-attached bacteria to twitch upstream. *Biophys J* 103(1):146–151.
35. Rutherford ST, van Kessel JC, Shao Y, Bassler BL (2011) AphA and LuxR/HapR reciprocally control quorum sensing in vibrios. *Genes Dev* 25(4):397–408.
36. Morkunas B, et al. (2012) Inhibition of the production of the *Pseudomonas aeruginosa* virulence factor pyocyanin in wild-type cells by quorum sensing autoinducer-mimics. *Org Biomol Chem* 10(42):8452–8464.