

# Quorum Sensing Inhibitors Increase the Susceptibility of Bacterial Biofilms to Antibiotics *In Vitro* and *In Vivo*<sup>∇†</sup>

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Although the exact role of quorum sensing (QS) in various stages of biofilm formation, maturation, and dispersal and in biofilm resistance is not entirely clear, the use of QS inhibitors (QSI) has been proposed as a potential antibiofilm strategy. We have investigated whether QSI enhance the susceptibility of bacterial biofilms to treatment with conventional antimicrobial agents. The QSI used in our study target the acyl-homoserine lactone-based QS system present in *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex organisms (baicalin hydrate, cinnamaldehyde) or the peptide-based system present in *Staphylococcus aureus* (hamamelitannin). The effect of tobramycin (*P. aeruginosa*, *B. cepacia* complex) and clindamycin or vancomycin (*S. aureus*), alone or in combination with QSI, was evaluated in various *in vitro* and *in vivo* biofilm model systems, including two invertebrate models and one mouse pulmonary infection model. *In vitro* the combined use of an antibiotic and a QSI generally resulted in increased killing compared to killing by an antibiotic alone, although reductions were strain and model dependent. A significantly higher fraction of infected *Galleria mellonella* larvae and *Caenorhabditis elegans* survived infection following combined treatment, compared to treatment with an antibiotic alone. Finally, the combined use of tobramycin and baicalin hydrate reduced the microbial load in the lungs of BALB/c mice infected with *Burkholderia cenocepacia* more than tobramycin treatment alone. Our data suggest that QSI may increase the success of antibiotic treatment by increasing the susceptibility of bacterial biofilms and/or by increasing host survival following infection.

Biofilm-associated infections are often very difficult to treat with conventional antibiotics (7, 17, 28, 38). Hence, novel targets are needed to combat biofilm infections. One of them could be the bacterial communication system (quorum sensing [QS]). Bacteria coordinate their cell-density-dependent gene expression by excreting small signaling molecules (26). When a certain extracellular threshold concentration is reached, these molecules bind to a receptor, thereby activating the QS system. The typical QS system in Gram-negative bacteria consists of three components: a LuxI synthase homolog, acyl-homoserine-lactone (AHL) signaling molecules, and a LuxR receptor homolog (10). Gram-positive bacteria generally use small peptide signaling molecules, which are transported out of the cell and bind to a membrane-associated two-component receptor (42). Binding to the receptor activates a signal transduction system leading to the transcription of QS-regulated genes. A third QS system using autoinducer 2 (AI-2) as signaling molecule is widespread in both Gram-positive and Gram-negative bacteria and is considered to be responsible for interspecies communication (43). QS has been shown to regulate biofilm formation in several bacterial species (15, 20). AHL QS mutants of *Burkholderia cenocepacia* and *Pseudomonas aeruginosa* form biofilms with a drastically altered structure and are impaired in their ability to maintain cells within the biofilm (18, 39, 44).

Similar results were observed when *Burkholderia multivorans*, *B. cenocepacia*, and *P. aeruginosa* biofilms were treated with QS inhibitors (QSI) such as baicalin hydrate (BH) or cinnamaldehyde (CA) (4, 44, 45). Biofilm formation in *Staphylococcus aureus* is enhanced by the *agr* QS system, while hamamelitannin (HAM; a nonpeptide analog of the RNAIII inhibiting peptide) decreases *S. aureus* attachment *in vitro* and *in vivo* (3, 21, 22). Due to the apparent role of QS in biofilm formation, QSI are proposed as promising antibiofilm agents (9). However, little is known about the relationship between the antibiofilm effect of QSI and the susceptibility of biofilms to antibiotics. In addition, biofilm model-dependent differences in susceptibility may affect the outcome. In the present study, we selected several QSI targeting the AHL-QS system or the QS system of Gram-positive bacteria and evaluated their effects on the susceptibility of medically important bacterial biofilms using *in vitro* and *in vivo* model systems.

## MATERIALS AND METHODS

**Strains and culture conditions.** *B. cenocepacia* LMG 16656, LMG 16659, and LMG 18828, *B. multivorans* LMG 13010, LMG 17588, and LMG 18822, *P. aeruginosa* PAO1, ATCC 9027, MH340, and MH710 (*ΔrhII ΔlasI*) (2), and *S. aureus* LMG 10147, Mu50 (methicillin-resistant *S. aureus* [MRSA]), and CS1 (a recent clinical MRSA isolate) were cultured in Mueller-Hinton broth (MH; Oxoid, Basingstoke, England). *Escherichia coli* OP50 and ATCC 25922 were grown in TSB (Oxoid) at 37°C.

*Caenorhabditis elegans* N2 (*glp-4; sek-1*) was propagated under standard conditions, synchronized by hypochlorite bleaching, and cultured on nematode growth medium using *E. coli* OP50 as a food source (5, 36). Adult *Galleria mellonella* larvae (Krekelonline, Turnhout, Belgium) were stored in wood chips at 15°C in darkness prior to use. Larvae weighing between 200 and 300 mg were used throughout.

**Antibiotics and QSI.** The following antibiotics were used: tobramycin (TOB; Sigma-Aldrich, Bornem, Belgium), vancomycin (VAN; Sigma-Aldrich), and clin-

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damycin (CLI; Certa, Braine-l'Alleud, Belgium). The QSI baicalin hydrate (BH), cinnamaldehyde (CA), and hamamelitannin (HAM) were purchased from Sigma-Aldrich.

**Determination of the MIC.** MICs of antibiotics were determined in triplicate according to the EUCAST broth microdilution protocol, using flat-bottom 96-well microtiter plates (TPP, Trasadingen, Switzerland) (31). The inoculum was standardized to approximately  $5 \times 10^5$  CFU/ml. The plates were incubated at 37°C for 20 h, and the optical density at 590 nm was determined by using a multilabel microtiter plate reader (Envision; Perkin-Elmer LAS, Waltham, MA). The lowest concentration of antibiotic for which a similar optical density was observed in the inoculated and the blank wells was recorded as the MIC. Test performance was monitored using *E. coli* ATCC 25922 and *S. aureus* LMG 10147 as control strains. For each antibiotic, the MIC was determined in the presence or absence of QSI. If the MIC determined in both conditions differed, checkerboard testing (34) was performed to determine whether the interaction is synergistic (fractional inhibitory concentration [FIC] index  $\leq 0.5$ ) or indifferent (FIC index  $> 0.5$ ).

**Biofilm formation on medical-grade silicone disks.** Biofilms were grown on medical grade silicone disks (Q7-4735; Dow Corning, Midland, MI) placed in the wells of a 24-well microtiter plate (TPP) as previously described (31), using MH medium, synthetic cystic fibrosis medium (29) supplemented with mucin (SCFMM), or Bolton broth with 50% plasma (Sigma-Aldrich) and 5% freeze-thaw laked horse blood (Lubbock chronic wound pathogenic biofilm medium [LCWPB]) (37). Inocula of  $10^8$  CFU/ml for MH medium and  $10^4$  CFU/ml for the other two media were used. Cell suspensions made in MH medium were incubated at 37°C for 4 h. After this adhesion step, wells were rinsed with physiological saline (PS), fresh sterile MH medium was added, and the biofilms were allowed to grow for an additional 20 h. Biofilms in the SCFMM and LCWPB media were incubated at 37°C for 24 h.

**Biofilm formation in a collagen matrix.** A collagen wound biofilm model (CWB) mimicking conditions in chronic wound and soft tissue infections was used (41). In brief, collagen matrices of polymerized rat-tail collagen type I (BD Biosciences, Erembodegem, Belgium) were prepared in 15-ml tubes. For 10 ml of collagen solution (2 mg/ml), 1 ml of  $10\times$  phosphate-buffered saline (PBS) was mixed with 0.125 ml of NaOH (1 M) and 3.475 ml of simulated wound fluid (SWF; 50% fetal calf serum and 50% PS in 0.1% peptone) and kept on ice. Cold collagen stock solution (3.68 mg/ml) was added and, after mixing, 350  $\mu$ l of the collagen solution was added to each tube. To achieve polymerization of the collagen, tubes were placed in an incubator at 37°C for 1 h. After polymerization, 350  $\mu$ l of a bacterial suspension ( $10^5$  CFU/ml in SWF) was added, and the biofilms were incubated for 24 h at 37°C.

**Biofilm formation on RHE.** Reconstituted human epithelium (RHE) tissue was obtained from SkinEthic Laboratories (Nice, France). For the biofilm assay, 0.5-cm<sup>2</sup> RHE surfaces were infected with a 50- $\mu$ l suspension containing  $2 \times 10^6$  CFU/ml and incubated in maintenance medium (SkinEthic Laboratories) at 37°C with 5% CO<sub>2</sub> at 100% humidity for 24 h.

**Treatment of 24-h-old biofilms and quantification of cells.** For all strains, the bactericidal effect of the antibiotics alone or in combination with QSI was determined using concentrations of 12  $\mu$ g/ml for CLI, 20  $\mu$ g/ml for VAN, and 8  $\mu$ g/ml for TOB. Against the three *B. cenocepacia* and three *B. multivorans* strains, TOB was used at four times the MIC. The QSI were added in sub-MICs, i.e., a concentration that did not affect growth (100  $\mu$ M for BH and 250  $\mu$ M for CA and HAM). Antibiotics and QSI were dissolved in sterile PS for the MH model system, in PBS for the RHE model, and in the respective biofilm growth media for the other model systems. After 24 h of biofilm formation, biofilms were rinsed once with PS and subsequently treated with an antibiotic alone, a QSI alone, a combination of an antibiotic and a QSI, or a blank control solution. After 24 h at 37°C, the biofilms were rinsed with PS. Sessile cells were removed from the disks or the RHE cells by three cycles of vortexing (30 s) and sonication (30 s; Branson 3510; Branson Ultrasonics Corp., Danbury, CT), and the number of CFU/biofilm was determined by plating the resulting suspensions. The bacteria present in the collagen matrices were quantified as previously described (41). In brief, 350  $\mu$ l of collagenase solution (500  $\mu$ g/ml) was added to the matrices, and the tubes were incubated for 30 min at 37°C, mixed, and incubated again for 60 min. Cells were washed twice by adding 700  $\mu$ l of PS and vortexing and were collected by centrifugation. The pellet was resuspended in 700  $\mu$ l of PS, and the number of CFU was determined by plating.

**C. elegans survival assay.** For the *C. elegans* survival assay, synchronized worms (L4 stage) were suspended in a medium containing 95% M9 buffer (3 g of KH<sub>2</sub>PO<sub>4</sub>, 6 g of Na<sub>2</sub>HPO<sub>4</sub>, 5 g of NaCl, and 1 ml of 1 M MgSO<sub>4</sub> · 7H<sub>2</sub>O in 1 liter of water), 5% brain heart infusion broth (Oxoid), and 10  $\mu$ g of cholesterol (Sigma-Aldrich)/ml. Then, 0.5 ml of this suspension of nematodes was transferred to the wells of a 24-well microtiter plate. An overnight bacterial culture

was centrifuged, resuspended in the assay medium, and standardized to  $10^8$  CFU/ml. Next, 250- $\mu$ l aliquots of this standardized suspension were added to each well, while 250  $\mu$ l of sterile medium was added to the positive control. QSI and/or antibiotics were added to the test wells in the same concentrations as were added to the ones used in the biofilm experiments. The assay plates were incubated at 25°C for up to 2 days. The fraction of dead worms was determined by counting the number of dead worms and the total number of worms in each well, using a dissecting microscope. Compounds were tested three times in each assay, and each assay was repeated at least six times ( $n \geq 18$ ).

**G. mellonella survival assay.** Prior to inoculation into *G. mellonella*, bacterial cells were washed with PBS and then diluted to an appropriate cell density ( $10^8$ ,  $10^4$ , and  $10^7$  CFU/ml for the *S. aureus*, *P. aeruginosa*, and *B. cenocepacia*/*B. multivorans* strains, respectively). A Hamilton syringe was used to inject 10- $\mu$ l aliquots in the *G. mellonella* last left proleg. The antibiotics, QSI, or both were administered by a 10- $\mu$ l injection into a different proleg within 1 h. The injected solution contained the compounds in the concentrations used in the biofilm experiments. Two control groups were used: the first group included larvae inoculated with PBS to monitor killing due to physical trauma; the second group included larvae receiving no injection. Experiments that had one or more dead larvae in either control group were discarded and repeated. For each treatment, at least 15 larvae were injected. Larvae were placed in the dark at 37°C and were scored as dead or alive every 24 h until 144 h postinfection. Larvae were considered dead when they displayed no movement in response to shaking or touch. For each treatment, data from at least four independent experiments were combined.

**Mouse lung infection model.** All experiments and procedures were approved by the ethical committee of the University of Antwerp. Female BALB/c mice were used. The mice were immunosuppressed by intraperitoneal injection of cyclophosphamide 24 h before infection (150 mg/kg; Endoxan; Baxter) and at 24 and 48 h postinfection (25 mg/kg). Before intranasal challenge with *B. cenocepacia* LMG 16656, the mice were anesthetized with ketamine hydrochloride (60 mg/kg subcutaneously; Anesketin; Eurovet). The challenge inoculum was prepared by diluting frozen stock cultures in PBS ( $5 \times 10^7$  CFU/ml). The mice were inoculated with a 10- $\mu$ l suspension in one nostril ( $5 \times 10^5$  CFU/10  $\mu$ l), followed by intranasal administration of 20  $\mu$ l of PBS or BH (2 mg/kg) at 24 and 48 h postinfection. The mice were sacrificed at 3 days postinfection by cervical dislocation, and the lungs were excised, weighed, and homogenized in 3 ml of PBS. A serial dilution of the lung homogenate was plated on *B. cepacia* selective agar (16) to enumerate the number of CFU.

**Statistics.** The normal distribution of the data was checked by using the Shapiro-Wilk test. Normally and non-normally distributed data were analyzed by using an independent sample *t* test and the Mann-Whitney U test, respectively. Statistics were determined using SPSS software, version 17.0. Survival curves were analyzed by using a log-rank (Mantel-Cox) test.

## RESULTS

**Effect of QSI on the susceptibility of planktonic cells.** All QSI (BH, CA, and HAM) were used in concentrations below the MICs (see Table S1 in the supplemental material) for all strains. MICs of TOB were determined for planktonic *B. cenocepacia* LMG 16656 (256  $\mu$ g/ml), LMG 16659 (512  $\mu$ g/ml), and LMG 18828 (256  $\mu$ g/ml), *B. multivorans* LMG 13010 (64  $\mu$ g/ml), LMG 17588 (64  $\mu$ g/ml), and LMG 18822 (128  $\mu$ g/ml), and *P. aeruginosa* PAO1 (2  $\mu$ g/ml) and ATCC 9027 (1  $\mu$ g/ml). The MICs of VAN and CLI were determined for planktonic *S. aureus* Mu50 (8 and 512  $\mu$ g/ml, respectively) and CS1 (4 and 0.256  $\mu$ g/ml, respectively). CA and BH did not alter the susceptibility of *B. cenocepacia*, *B. multivorans*, and *P. aeruginosa* strains toward TOB when grown planktonically. For VAN and CLI, minor differences in MIC toward *S. aureus* were observed depending on the presence or absence of QSI, but these differences were within the acceptable margin of error and were not considered relevant (see Table S1 in the supplemental material). The FIC indices were  $>0.5$  for all combinations, indicating that there was no synergistic activity and that the interactions observed are indifferent (data not shown).

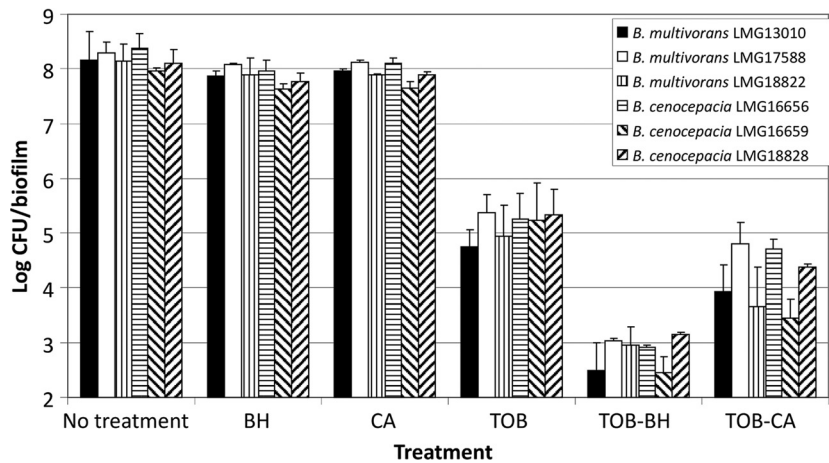


FIG. 1. Log CFU/biofilm (average ± the SD) in untreated 24-h-old *B. multivorans* and *B. cenocepacia* biofilms in MH medium and in biofilms treated with TOB or a QSI (BH or CA) alone or a combination of TOB and a QSI. Combined treatment was significantly more effective than treatment with TOB alone for both QSI and for all strains investigated ( $P < 0.05$ ).

**Effect of QSI on the susceptibility of *B. multivorans* and *B. cenocepacia* biofilms.** When used alone, BH or CA resulted in a minor reduction in the number of sessile *B. multivorans* and *B. cenocepacia* cells (Fig. 1). Treatment of the 24-h-old biofilms with TOB in combination with BH or CA resulted in significantly ( $P < 0.05$ ) more killing of *B. cenocepacia* and *B. multivorans* biofilm cells relative to treatment with TOB alone (Fig. 1). Similar results were obtained for *B. cenocepacia* and *B. multivorans* biofilms grown in SCFMM medium (see Table S2 in the supplemental material).

**Effect of QSI on the susceptibility of *P. aeruginosa* biofilms.** Use of BH and CA did not result in a significant decrease of the number of *P. aeruginosa* PAO1 and *P. aeruginosa* ATCC 9027 biofilm cells in any of the *in vitro* biofilm models (see Table S2 in the supplemental material). However, the susceptibility of the *P. aeruginosa* biofilms toward TOB was model and strain dependent (Table 1). TOB treatment decreased the number of living *P. aeruginosa* PAO1 cells by ca. 85% for biofilms grown in LCWPB medium and by ca. 40% when grown in the other three media (Table 1). For *P. aeruginosa* ATCC 9027, significant killing by TOB was only observed in the CWB medium (Table 1). Compared to TOB alone, treatment of 24-h-old biofilms with TOB in combination with BH or CA led to significantly ( $P < 0.05$ ) more killing of biofilm cells

of both *P. aeruginosa* strains in all *in vitro* biofilm model systems (Table 1). In addition, *P. aeruginosa* MH710 ( $\Delta rhII \Delta lasI$ ) was more susceptible to TOB compared to the corresponding WT strain MH340, even in the absence of QSI (see Table S3 in the supplemental material); BH or CA did not increase the susceptibility of the mutant strain toward TOB (see Table S3 in the supplemental material).

**Effect of QSI on the susceptibility of *S. aureus* biofilms.** When used alone, HAM resulted only in a minor reduction in the number of *S. aureus* sessile cells (see Table S2 in the supplemental material). The effect of VAN or CLI alone was model system and strain dependent (Table 2). Combined treatment with HAM and VAN resulted in significantly more killing of *S. aureus* Mu50 biofilm cells in all *in vitro* models tested (Table 2). Similar results were observed for the combination of HAM and VAN or CLI for *S. aureus* CS1 in all biofilm model systems tested (Table 2).

**Effect of the combination of QSI and antibiotics on survival of infected *C. elegans*.** The *in vivo* effect of a combined treatment with QSI and antibiotics was evaluated by using a *C. elegans* model system. None of the QSI alone affected the survival of uninfected *C. elegans* (data not shown). Infection of *C. elegans* with *B. cenocepacia* LMG 16656, *B. cenocepacia* LMG 18828, *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 9027, or

TABLE 1. Percent decrease in the number of CFU per biofilm when 24-h-old *P. aeruginosa* biofilms grown in different model systems were treated with TOB or a combination of TOB and a QSI (BH or CA)

<i>P. aeruginosa</i> strain	Treatment	Avg % decrease ± SD in CFU obtained in different model systems <sup>a</sup>			
		MH	SCFMM	LCWPB	CWB
PAO1	TOB	45.5 ± 14.2	39.9 ± 18.9	83.3 ± 7.8	35.2 ± 21.7
	TOB-BH	88.6 ± 7.2	80.9 ± 4.9	97.0 ± 2.4	80.6 ± 11.4
	TOB-CA	89.4 ± 1.9	86.9 ± 11.2	93.3 ± 3.7	77.0 ± 5.0
ATCC 9027	TOB	6.3 ± 10.3	5.8 ± 7.6	2.1 ± 17.8	34.6 ± 18.9
	TOB-BH	82.3 ± 10.3	72.9 ± 4.5	44.9 ± 7.6	89.5 ± 3.2
	TOB-CA	90.2 ± 1.5	76.3 ± 9.5	42.9 ± 13.7	80.2 ± 2.2

<sup>a</sup> All combined treatments were significantly more effective than treatment with TOB alone ( $P < 0.05$ ). MH, Mueller-Hinton; SCFMM, synthetic cystic fibrosis medium with mucin; LCWPB, Lubbock chronic wound pathogenic biofilm; CWB, collagen wound biofilm.

TABLE 2. Percent decrease in the number of CFU per biofilm when 24-h-old *S. aureus* biofilms grown in different model systems were treated with VAN or CLI or a combination of an antibiotic and HAM

<i>S. aureus</i> strain	Treatment	Avg % decrease $\pm$ SD in CFU obtained in different model systems <sup>a</sup>				
		MH	SCFMM	LCWPB	CWB	RHE
CS1	VAN	2.2 $\pm$ 11.2	78.9 $\pm$ 7.8	2.0 $\pm$ 12.9	94.2 $\pm$ 1.5	84.1 $\pm$ 4.4
	VAN-HAM	68.4 $\pm$ 10.1*	94.0 $\pm$ 3.0*	95.0 $\pm$ 1.0*	99.2 $\pm$ 0.2*	99.1 $\pm$ 1.2*
	CLI	13.5 $\pm$ 9.5	68.3 $\pm$ 18.4	4.6 $\pm$ 11.2	74.9 $\pm$ 18.0	75.2 $\pm$ 2.9
	CLI-HAM	69.0 $\pm$ 8.8*	97.8 $\pm$ 1.7*	63.0 $\pm$ 8.8*	96.8 $\pm$ 0.9*	98.8 $\pm$ 1.0*
Mu50	VAN	2.6 $\pm$ 11.7	65.9 $\pm$ 6.7	4.4 $\pm$ 13.4	66.9 $\pm$ 32.4	91.2 $\pm$ 2.3
	VAN-HAM	71.3 $\pm$ 6.9*	99.8 $\pm$ 0.1*	82.4 $\pm$ 7.7*	99.6 $\pm$ 1.7*	99.6 $\pm$ 0.1*
	CLI	8.3 $\pm$ 17.4	8.4 $\pm$ 10.2	5.3 $\pm$ 11.2	6.8 $\pm$ 9.2	ND
	CLI-HAM	6.2 $\pm$ 21.0	15.1 $\pm$ 12.2	12.1 $\pm$ 8.9	6.1 $\pm$ 8.5	ND

<sup>a</sup> \*, The results of combined treatment were significantly different from that of antibiotic treatment alone ( $P < 0.05$ ). MH, Mueller-Hinton; SCFMM, synthetic cystic fibrosis medium with mucin; LCWPB, Lubbock chronic wound pathogenic biofilm; CWB, collagen wound biofilm; RHE: reconstituted human epithelium. ND, not determined.

*S. aureus* Mu50 significantly ( $P < 0.01$ ) decreased the number of living nematodes after 48 h (Fig. 2). A significantly ( $P < 0.01$ ) higher fraction survived infection with *B. cenocepacia* LMG 16656, *B. cenocepacia* LMG 18828, *P. aeruginosa* PAO1, or *P. aeruginosa* ATCC 9027 after treatment with TOB compared to no treatment (Fig. 2). Increased survival was also observed after BH treatment of *C. elegans* nematodes infected with *B. cenocepacia* LMG 16656, *B. cenocepacia* LMG 18828, and *P. aeruginosa* ATCC 9027 (Fig. 2). Similar results were obtained for CA treatment (data not shown). In addition, a significantly ( $P < 0.01$ ) higher survival was observed for all BH/TOB combinations compared to treatment with TOB alone (Fig. 2). Similar results were obtained for *B. cenocepacia* LMG 16656, *B. cenocepacia* LMG 18828, and both *P. aeruginosa* strains treated with a TOB-CA combination (data not shown).

Both HAM and VAN alone significantly ( $P < 0.01$ ) in-

creased the survival of *C. elegans* infected with *S. aureus* Mu50 (Fig. 2). Further, the combination HAM-VAN significantly ( $P < 0.01$ ) enhanced survival compared to VAN alone (79%  $\pm$  13% versus 53%  $\pm$  14%).

**Effect of the combination of QSI and antibiotics on survival of infected *G. mellonella*.** None of the QSI or antibiotics was toxic for *G. mellonella* at the concentrations used (data not shown). All test strains significantly decreased *G. mellonella* survival within a 144-h postinfection time period (Fig. 3). A significantly higher percentage of *G. mellonella* larvae survived infection with *B. multivorans* LMG 18822 and *B. cenocepacia* LMG 16656 after treatment with TOB or BH compared to no treatment. Similar results were obtained for CA treatment (data not shown). A significantly ( $P < 0.05$ ) enhanced survival was observed for the combination BH-TOB compared to TOB treatment alone for infections with *B. cenocepacia* LMG 16656 and *B. multivorans* LMG 18822 (Fig. 3). Similar results were

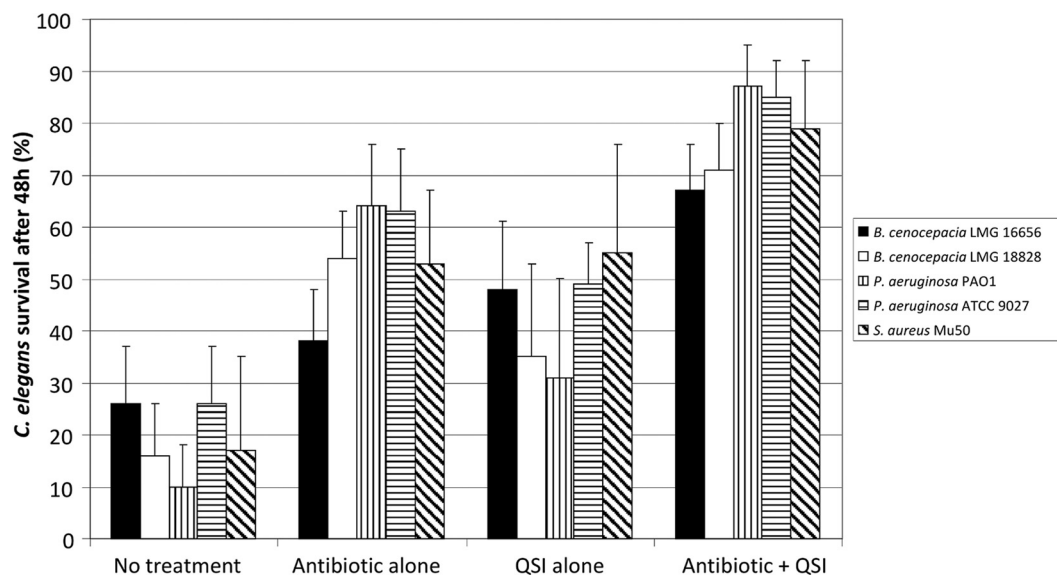


FIG. 2. Percent survival of infected *C. elegans* (average  $\pm$  the SD) after treatment with antibiotics (TOB for the *B. cenocepacia* and *P. aeruginosa* strains and VAN for *S. aureus* Mu50), QSI (BH for the *B. cenocepacia* and *P. aeruginosa* strains and HAM for *S. aureus* Mu50), or combinations of the respective compounds. The results are expressed as the percent survival after 48 h of infection and treatment. All combined treatments were significantly more effective than treatment with antibiotic alone ( $P < 0.01$ ).

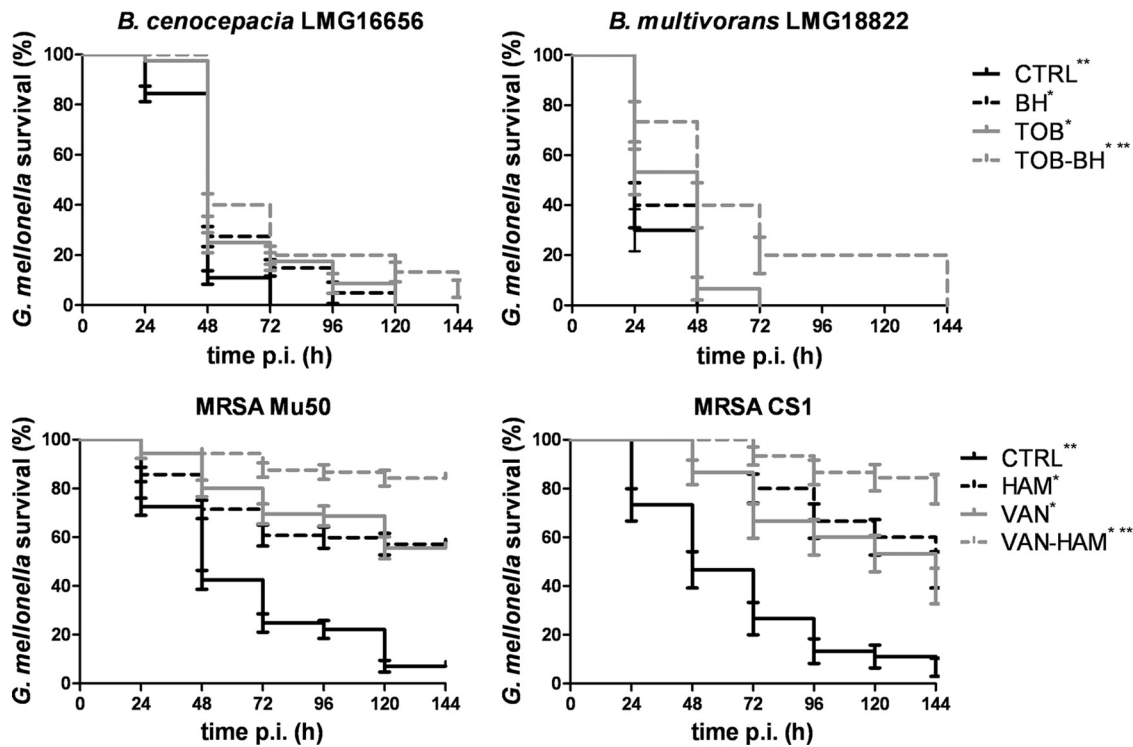


FIG. 3. Kaplan-Meier survival-curve of infected *G. mellonella* receiving no treatment (CTRL) or a treatment with an antibiotic (TOB or VAN), a QSI (BH or HAM), or a combination of both. \*, Significantly different compared to no treatment ( $P < 0.05$ ); \*\*, significantly increased survival compared to antibiotic treatment alone ( $P < 0.05$ ).

obtained for CA-TOB (data not shown). Both HAM and VAN significantly ( $P < 0.05$ ) enhanced *G. mellonella* survival after infection with *S. aureus* Mu50 or CS1. In addition, the combination VAN-HAM significantly ( $P < 0.05$ ) increased survival compared to either of the compounds used alone (Fig. 3).

**Effect of BH in combination with TOB for the treatment of mice infected with *B. cenocepacia* LMG 16656.** The results of the *in vitro* and *in vivo* studies presented above, warranted further exploration of the therapeutic effect of BH in combination with TOB in a murine *B. cenocepacia* lung infection model. Significantly fewer *B. cenocepacia* LMG 16656 cells were recovered from TOB-treated mice than from the control mice (decreases of 47.5, 88.8, and 91.9% for the 10-, 20-, and 30-mg/kg TOB doses, respectively) (Table 3). At lower concentrations of TOB (10 or 20 mg/kg), the addition of BH to the treatment had no effect. However, the combination of a higher dose of TOB (30 mg/kg) with BH (2 mg/kg) resulted in a significantly ( $P < 0.05$ ) reduced pulmonary bacterial load compared to the TOB treatment alone (Table 3).

**DISCUSSION**

The biofilm mode of living of bacteria is associated with an increased resistance toward antibacterials, making successful treatment of biofilm-related infections difficult (17). Although much remains to be learned about the involvement of QS in biofilm formation, maintenance, and dispersal, QSI have been proposed as promising antibiofilm agents (24). In the present study we evaluated the potential of combining antibiotics with QSI. We selected various bacteria known to form clinically

relevant biofilms and evaluated them under conditions mimicking the *in vivo* situation. *B. multivorans*, *B. cenocepacia*, *P. aeruginosa*, and *S. aureus* often infect cystic fibrosis patients and were consequently evaluated in SCFMM medium mimicking the conditions found in the cystic fibrosis lung (13, 29, 35). Both *P. aeruginosa* and *S. aureus* cause soft tissue infections (23, 25), and for this reason the biofilm susceptibility of these species was evaluated in two *in vitro* wound models and on RHE (36, 40). QSI targeting different QS systems and different classes of antibiotics were included, the latter in clinically relevant concentrations (8, 12). Although QSI and antibiotics did not act synergistically against planktonic cells, a different picture was observed for biofilm cells. Although there were con-

TABLE 3. Number of *B. cenocepacia* LMG 16656 CFU in the lungs of mice after treatment with TOB alone or in combination with BH<sup>a</sup>

Treatment (mg/kg)		No. of mice	CFU/g of lung (avg ± SEM)	Reduction in bacterial load (%) compared to untreated control mice
TOB	BH			
0	0	18	56,752 ± 13,003	
10	0	14	29,233 ± 3,753	47.5
10	2	11	26,793 ± 3,561	52.1
20	0	7	6,267 ± 1,813	88.8
20	2	4	8,128 ± 4,609	85.9
30	0	10	4,560 ± 1,714	91.9
30	2	7	559 ± 119*	99.0*

<sup>a</sup>\*, Significantly different compared to treatment with antibiotic alone ( $P < 0.05$ ).

siderable differences in results obtained in various model systems, overall, the use of QSI increased the effect of the antibiotic. QSI, such as BH, CA, and HAM, do not kill or inhibit the growth of bacterial cells but may influence biofilm formation and/or matrix production, which may lead to the altered susceptibility of these biofilms. To rule out the possibility that other processes besides QS are involved in this, we used a *P. aeruginosa* mutant incapable of QS (due to a disruption of both *rhlI* and *lasI* this strain cannot produce AHL signaling molecules) (2). Compared to the wild type, this mutant was much more susceptible toward TOB, which confirms the role of a functional QS system in resistance. In addition, while BH or CA resulted in an increased susceptibility of the wild-type strain toward TOB, this increase was not observed in the QS mutant strain, indicating that a functional QS system is required for the QSI to exert their effect. Although we cannot exclude that the QSI have other effects, our data strongly suggest that these are minor at best and that the increased antibiofilm effect is due to QS inhibition. However, since effects obtained in *in vitro* model systems cannot always be reproduced under *in vivo* conditions (1, 9, 14, 30), it was unclear whether combination therapy would also improve survival *in vivo*. For this reason, we investigated the effect of combined treatments on the survival of infected *C. elegans* and *G. mellonella*. These invertebrates have been shown to be good models for studying the pathogenicity of *B. cenocepacia*, *P. aeruginosa*, and *S. aureus* (5, 6, 11, 27, 32, 33, 40). Biofilm formation is also an important factor in staphylococcal virulence against *C. elegans* (1). A significantly higher fraction of *G. mellonella* larvae and *C. elegans* survived bacterial infection after treatment with a combination of QSI and antibiotics compared to treatment with an antibiotic alone. However, QSI alone also resulted in an increased survival, indicating that these compounds probably interfere with other cellular processes besides biofilm formation. QS is known to be involved in the production of virulence factors. For example, the production of the nematocidal protein AidA in *B. cenocepacia* is regulated by QS, and QS inhibition interferes with the swarming motility of bacteria and possibly affecting their dissemination in the host during infection (18, 19). RIP and its nonpeptide analog hamamelitannin were previously shown to affect *S. aureus* virulence factor production (21). In addition, bacterial QS mutants were observed to be significantly less virulent in several *in vivo* models (22, 40). For this reason, the increased survival in the presence of QSI is probably not only the result of an increased susceptibility of the bacteria to the antibiotic. Despite this, the effect observed in several *in vitro* and *in vivo* model systems encouraged us to further explore the potential effect of BH-TOB in a BALB/c lung infection model. Significantly fewer *B. cenocepacia* LMG 16656 cells were recovered from the lungs of mice treated with a combination of TOB-BH (30 and 2 mg/kg) compared to TOB (30 mg/kg) alone. Although the results from the present study seem promising, it will be important to determine whether similar results can be obtained for other organisms in other *in vivo* infection models. Altogether, our data suggest that combining antibiotics with QSI may increase the success of a treatment by increasing the efficacy of the former against biofilm-related infections and/or by increasing host survival after infection.

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