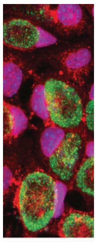

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RESEARCH ARTICLE

Bacteria that inhibit quorum sensing decrease biofilm formation and virulence in *Pseudomonas aeruginosa* PAO1

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This work nicely verifies that bacteria producing quorum sensing inhibiting compounds could be useful to attenuate virulence of bacteria like *P. aeruginosa* and possibly also other Gram-negative pathogens that use quorum sensing to regulate virulence.

Keywords
elastase; virulence; *Caenorhabditis elegans*; quorum quenching; biofilm; *Pseudomonas aeruginosa*.

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Abstract

In this study, we investigated the biotherapeutic potential of previously isolated quorum quenching (QQ) bacteria. Some of them produce and secrete small compounds that inhibit quorum sensing (QS), others quench QS by enzymatic degradation of *N*-acylhomoserine lactones (AHLs). The supernatant of cultures of these isolates was tested for inhibitory properties against *P. aeruginosa* PAO1 biofilms. Most isolates had a moderate effect on biofilm formation, as shown by viability staining and/or staining of the biofilm biomass. A substantial part of the isolates reduced *P. aeruginosa* elastase production in a concentration-dependent manner. Using *Caenorhabditis elegans* as an *in vivo* model system for virulence testing, we found that some of the isolates were able to increase survival of *P. aeruginosa* PAO1 and *Burkholderia cenocepacia* LGM16656-infected nematodes when co-administered with the pathogen. Altogether, these data indicate that some QQ bacteria, or the active compounds they produce, could be useful to attenuate virulence of *P. aeruginosa* PAO1 and possibly also other Gram-negative pathogens that use AHLs to regulate the production of virulence factors.

Introduction

Many bacteria use quorum sensing (QS) to monitor cell density and to coordinate their behavior (Williams *et al.*, 2007). Gram-negative bacteria contain a three-component network for QS, consisting of a signal synthase, the signal molecule itself, and a cytoplasmatic receptor (Fuqua *et al.*, 1994; Fuqua & Greenberg, 2002). As cell density increases, the concentration of diffusible signal molecules also increases, and upon reaching a certain threshold concentration, binding of the signal molecules to the receptor will result in a significant induction or repression of QS-regulated genes. The signal molecules most often used by Gram-negative bacteria are *N*-acylhomoserine lactones (AHLs), which contain a conserved lactone moiety, but may vary in length, as well as the degree of oxidation and saturation of the acyl side chain (Camilli & Bassler, 2006).

In various pathogenic Gram-negative bacteria, the production of virulence factors, and biofilm formation and maturation are (co-)regulated by QS (de Kievit & Iglewski, 2000; Winzer & Williams, 2001; Schuster *et al.*, 2003; Wagner & Iglewski, 2008). *Pseudomonas aeruginosa*, an opportunistic pathogen which commonly infects immunocompromised patients (Oberhardt *et al.*, 2008), uses two hierarchical QS regulatory systems (Las and Rhl) to regulate the expression of many of its virulence factors (Dekimpe & Deziel, 2009), including elastase, proteases, exotoxin A, pyocyanin, and siderophores (Schuster *et al.*, 2003; Schuster & Greenberg, 2007). Therefore, strategies interfering with this cell-to-cell signaling system are a promising novel approach to combat bacterial disease (Camara *et al.*, 2002). Indeed, it has already been shown *in vitro* and *in vivo* that inhibiting QS decreases the production of virulence factors of pathogenic bacteria (Hentzer & Givskov, 2003; Brackman

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et al., 2009, 2011). Furthermore, as QS systems are not directly involved in essential cellular processes, QS inhibition (QSI) is assumed not to impose harsh selective pressure on the population. Theoretically, ‘antipathogenic’ strategies such as QSI are less likely to result in the development of resistance (Rasmussen & Givskov, 2006).

Taking the architecture of the three-component network into account, QS systems in Gram-negative bacteria basically offer three points of attack: the signal synthase (LuxI homologue), the signal molecule (AHL) itself, and the signal receptor (LuxR homologue) (Kjelleberg *et al.*, 2008). Inhibition of the biosynthesis of the signaling molecule has been achieved by substrate analogues (Parsek & Greenberg, 1999), whereas some AHL analogues are reportedly able to interfere with signal reception (Schaefer *et al.*, 1996; Persson *et al.*, 2005; Brackman *et al.*, 2012). A third mechanism is degradation of the signaling molecules. AHLs are prone to enzymatic degradation, and two major classes of AHL inactivating hydrolases have been described thus far: AHL acylases and AHL lactonases. Members of the former family cleave the molecule into a free homoserine lactone and a fatty acid, while members of the latter family hydrolyze the lactone ring, yielding a homoserine. Degradation products of both enzymes are no longer active, thus shutting down the QS circuitry (Czajkowski & Jafra, 2009; Amara *et al.*, 2011).

We previously reported on the isolation and identification of bacteria with QSI activity from environmental samples. Some isolates were shown to secrete QSI compounds and therefore have extracellular QSI activity. Others did not show activity in their supernatant, but were able to degrade AHLs when whole cell cultures were incubated with synthetic signal molecules. Experiments in which the samples were treated with heat or proteinase K allowed us to further differentiate between isolates with enzymatic activity or isolates producing small molecule QS inhibitors (Christiaen *et al.*, 2011). In the present study, we have evaluated the biotherapeutic potential of these isolates *in vitro* and *in vivo*. To this end, the isolates were tested for their ability to inhibit biofilm formation and elastase production in *P. aeruginosa* PAO1. Additionally, the effect of co-administering QSI isolates with the pathogenic bacteria *P. aeruginosa* PAO1 or *Burkholderia cenocepacia* LMG16656 in a nematode model was also investigated.

Materials and methods

Organisms and culture conditions

A selection of isolates that had previously been shown to produce QSI compounds was made from a larger collection of QSI isolates (Christiaen *et al.*, 2011; Tang *et al.*, 2013). Selection criteria were the ability to decrease AHL levels with more than 50% and the presence of extracellular QSI activity. The isolate codes, their identity, the source of isolation, country of origin, and culture conditions are listed in Table 1. *Pseudomonas aeruginosa* PAO1 and *B. cenocepacia* LMG16656 were chosen as model pathogens as both of them use QS to regulate the production of virulence factors and biofilm formation. They were cultured aerobically at 37 °C in Mueller-Hinton Broth (MH). *Pseudomonas aeruginosa* QSIS2 was used as a QS biosensor strain and cultured as described previously (Rasmussen *et al.*, 2005). *Escherichia coli* OP50 was grown aerobically in tryptic soy broth (TSB) 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 (Stiernagle, 2006; Cooper *et al.*, 2009).

Biofilm formation

Biofilms were formed as described previously (Brackman *et al.*, 2009). In brief, *P. aeruginosa* PAO1 was grown overnight in MH, centrifuged, resuspended in double-concentrated MH (2 × MH), and diluted to an $OD_{590\ nm}$ = 0.1 in 2 × MH. Fifty microliters of the standardized cell suspension were transferred to the wells of a round-bottom 96-well microtiter plate (MTP). To the wells of the test conditions, 50 μL of the supernatant of the QS inhibitory isolates (QSI SN) was added. Negative controls contained 50 μL noninoculated medium. Bacteria were allowed to adhere and grow without agitation for 4 h at 37 °C. After 4 h, plates were emptied and washed with sterile physiological saline (PS; 0.9% NaCl). To test the influence of the QSI SN on the adhesion of *P. aeruginosa* PAO1, 100 μL MH was added to each well after this washing step. To test the influence on the entire process of biofilm formation (adhesion and maturation), the wells were filled as described above for the first 4 h of biofilm formation, washed with PS, and filled again with QSI SN (test conditions) or noninoculated medium (for negative controls). Plates were incubated for another 20 h of biofilm maturation at 37 °C. After 24 h, the medium was removed, the wells were rinsed with PS, and both cell viability and biofilm biomass were determined by resazurin (CellTiter-Blue, CTB) staining and crystal violet (CV) staining, respectively (Peeters *et al.*, 2008). Briefly, for CTB staining, 100 μL PS was added to the wells, followed by the addition of 20 μL CTB solution (Promega, Leiden, the Netherlands). After 60 min, fluorescence was measured (λ_{exc} = 535 nm, λ_{em} = 590 nm) using a MTP reader (Envision, Perkin Elmer, Waltham, MA). For CV staining, biofilms were fixed by adding 100 μL CV (Pro-lab Diagnostics, Richmond Hill, ON, Canada). After 20 min, CV was removed and wells were filled with 150 μL 33% glacial acetic acid (Sigma-Aldrich, Bornem, Belgium). The absorbance was measured at 590 nm using the Envision. Each SN was tested 12 times in each assay, and each assay was carried out twice (n = 24).

Elastin Congo Red (ECR) assay

Elastase production of *P. aeruginosa* PAO1 grown in the presence of increasing concentrations of QSI SN was determined with the ECR assay as described earlier, with slight modifications (Visca *et al.*, 1992; Luckett *et al.*, 2012). *Pseudomonas aeruginosa* PAO1 was cultured at 37 °C for 48 h in TSB containing 25%, 50%, or 75% QSI SN isolate supernatant. Next, 150 μL of sterile supernatant of these

Table 1 Isolates with QSI activity

QSI activity	Code	Name	Source	Country	Medium	Temperature (°C)
Extracellular	Li3-2	*Pseudomonas putida*	Rhizosphere (*Asteraceae*)	Belgium	TSB	37
	Li4-2	*Pseudomonas* sp.	Rhizosphere (*Caricaceae*)	Belgium	TSB	37
	MP2-1	*Pseudomonas putida*	Rhizosphere (*Gramineae*)	Belgium	TSB	37
	MP2-6	*Alcaligenes* sp.	Rhizosphere (*Gramineae*)	Belgium	TSB	37
	Le1-2	*Delftia* sp.	Rhizosphere (*Plumbaginaceae*)	Belgium	TSB	37
	Le2-1	*Arthrobacter* sp.	Rhizosphere (*Brassicaceae*)	Belgium	TSB	37
	Le2-4	*Delftia* sp.	Rhizosphere (*Brassicaceae*)	Belgium	TSB	37
	Le2-5	*Delftia* sp.	Rhizosphere (*Brassicaceae*)	Belgium	TSB	37
	Le3-4	*Pseudomonas* sp.	Rhizosphere (*Aristolochiaeceae*)	Belgium	TSB	37
	Le4-4	*Pseudomonas alcaligenes*	Rhizosphere (*Geraniaceae*)	Belgium	TSB	37
	Le4-5	*Pseudomonas putida*	Rhizosphere (*Geraniaceae*)	Belgium	TSB	37
	Lw1-1	*Diaphorobacter* sp.	Pond water	Belgium	TSB	37
	NFMI-T	*Pseudomonas fluorescens*	*Tetraselmis* culture	Belgium	TSB	37
	Th111	*Pseudoalteromonas paragorgicola*	Mucus of healthy flounders	China	MB	25
	Th120	*Muricauda olearia*	Mucus of healthy flounders	China	MB	25
	Th27	*Pseudoalteromonas paragorgicola*	Mucus of healthy flounders	China	MB	25
	T53	*Pseudoalteromonas marina*	Intestines of healthy flounders	China	MB	25
	T96	*Pseudoalteromonas marina*	Intestines of healthy flounders	China	MB	25
	T153	*Pseudoalteromonas marina*	Intestines of healthy flounders	China	MB	25
Intracellular	Le1-3	*Arthrobacter nicotinovorans*	Rhizosphere (*Plumbaginaceae*)	Belgium	TSB	37
	Le1-4	*Arthrobacter aurescens*	Rhizosphere (*Plumbaginaceae*)	Belgium	TSB	37
	Le3-3	*Arthrobacter* sp.	Rhizosphere (*Aristolochiaceae*)	Belgium	TSB	37
	Le3-5	*Pseudomonas* sp.	Rhizosphere (*Aristolochiaceae*)	Belgium	TSB	37
	Le4-2	*Achromobacter denitrificans*	Rhizosphere (*Geraniaceae*)	Belgium	TSB	37
	Le4-3	*Pseudomonas* sp.	Rhizosphere (*Geraniaceae*)	Belgium	TSB	37
	Le5-3	*Brevundimonas* sp.	Rhizosphere (*Fabaceae*)	Belgium	TSB	37
	Li1-1	*Arthrobacter* sp.	Rhizosphere (*Araceae*)	Belgium	TSB	37
	Li4-3	*Arthrobacter* sp.	Rhizosphere (*Caricaceae*)	Belgium	TSB	37
	MP2-4	*Delftia* sp.	Rhizosphere (*Gramineae*)	Belgium	TSB	37
	LCDR16	*Bacillus* sp.	Digestive tract of *Penaeus vannamei*	Belgium	TSB	37

Isolates codes, identity, source of isolation, and culture conditions are given (TSB, tryptic soy broth; MB, marine broth).

cultures was added to eppendorf tubes containing 20 mg of ECR (Sigma-Aldrich). Following addition of 850 μL of 100 mM Tris-Cl/1 mM $CaCl_2$ pH 7.5, overnight incubation at 37 °C with agitation, and removal of insoluble particles by centrifugation (13 000 ***g***, 1 min), the absorbance at 495 nm was measured.

Caenorhabditis elegans assay

Caenorhabditis elegans survival experiments were performed as described earlier, with minor modifications (Brackman *et al.*, 2011). Synchronized worms (L4 stage) were suspended in a medium containing 95% M9 buffer, 5% brain heart infusion broth, and 10 μg mL^{-1} cholesterol (Sigma-Aldrich), and 250 μL of this nematode suspension was transferred to the wells of a 24-well MTP. Stationary phase cultures of *P. aeruginosa* PAO1, *B. cenocepacia* LMG16656, and the QSI isolates were centrifuged, resuspended in the assay medium, standardized to $OD_{590\ nm} = 0.1$, and finally diluted 1 : 100. 250 μL aliquots of these standardized suspensions were added to each of the appropriate wells. In this way, three test conditions were obtained: one receiving only *P. aeruginosa* PAO1 or *B. cenocepacia* LMG16656, another receiving only the QSI isolate, and one condition in which both suspensions were added. Subsequently, assay medium was added to each well to obtain a final volume of 1 mL per well. Nematodes not being administered any bacteria were used as a control to correct for spontaneous mortality. Finally, the plates were incubated at 25 °C, and the fraction of dead nematodes was determined after 48 h by counting the number of dead worms and the total number of worms in each well, using a dissection microscope. Isolates of which the administration as such resulted in nematode killing were omitted for further data analysis. For the isolates that did not affect nematode survival, the increase in survival compared to as to when the pathogen is administered alone was calculated.

Size exclusion chromatography (SEC)

For the isolates with known extracellular QSI activity, the sterile SN of 24-h-old cultures was fractionated by size SEC. To this end, gravity flow columns with a cut-off value of 6000 Da (Econo-Pac 10DG) were used according to the minimal dilution protocol instructions supplied by the manufacturer (Bio-Rad Laboratories SA/NV, Eke, Belgium). In brief, 3 mL of sterile supernatant was applied onto the column, and a first fraction, containing the higher molecular weight components (> 6000 Da), was eluted with 4 mL

MilliQ water. Subsequently, lower molecular weight components were eluted with 8 mL of MilliQ water, yielding four subsequent fractions of 2 mL each. To determine which fraction contained the active compound, 900 μL of each fraction was mixed with a mixture of *N*-butyryl-DL-homoserine lactone and *N*-(3-oxododecanoyl)-L-homoserine lactone (final concentrations of 800 nM for each AHL in the reaction mixture). After 24-h incubation at 30 °C, 50 μL of the reaction mixtures was added to the wells of a MTP containing the *P. aeruginosa* QSIS2 biosensor, as described previously (Rasmussen *et al.*, 2005; Brackman *et al.*, 2009). Simultaneously, nonfractionated SN was also tested for QSI activity. Noninoculated medium with sterile MilliQ water or AHLs served as negative (0% activation of the QS system) and positive controls (100% activation of the QS system), respectively.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. The nonparametric Mann–Whitney U-test was used to compare the results.

Results and discussion

QSI isolates inhibit biofilm formation of *P. aeruginosa* PAO1

The purpose of these experiments was to assess whether the SN of isolates with known extracellular QSI activity could inhibit biofilm formation and maturation. Eighteen isolates previously shown to produce and secrete compounds that interfere with QS have been selected from a larger collection of QSI isolates. In Fig. 1a, the effect of adding QSI SN during the biofilm adhesion phase of *P. aeruginosa* PAO1 is shown, as quantified by CV staining and CTB staining. The relative biofilm formation in the presence of QSI SN during both biofilm adhesion and maturation is shown in Fig. 1b. Biofilms grown in the absence of QSI SN were used as a control (= 100%). From the data in Fig. 1, it is obvious that the influence of QSI SN on biofilm adhesion and/or maturation is strain dependent. Furthermore, isolates influencing total biofilm biomass do not necessarily affect viability of the *P. aeruginosa* PAO1 biofilm, or vice versa. Additionally, QSI isolates may primarily affect cell adhesion,

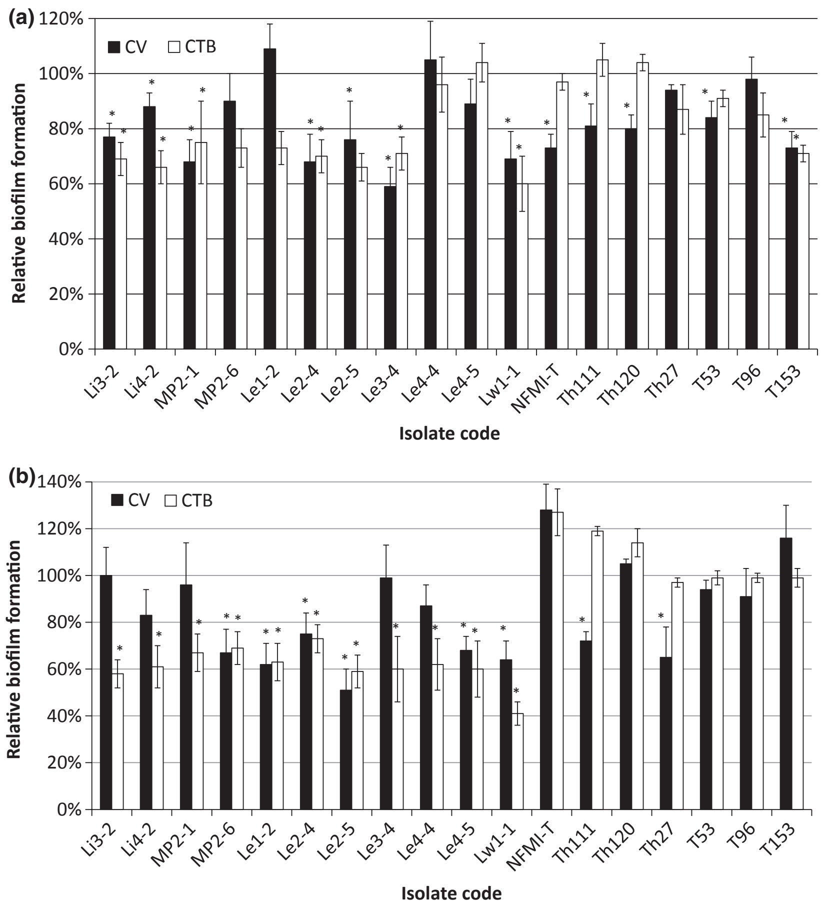

Fig. 1 Relative biofilm formation of *Pseudomonas aeruginosa* PAO1 grown in the presence of QSI SN, measured by CV staining (effect on biofilm biomass) and CTB staining (effect on cell viability), compared to a biofilm grown in the absence of SN (= 100%). The influence of QSI SN on biofilm adhesion (a) and biofilm maturation (b) is shown (n = 24; means ± SEM; *Significantly lower than control biofilm, $P \leq 0.05$).

primarily affect biofilm maturation, or affect both stages of biofilm formation. Isolates Lw1-1 (*Diaphorobacter* sp.) and Le2-5 (*Delftia* sp.) were the most active strains tested. These isolates significantly reduced both *P. aeruginosa* PAO1 cell adhesion and biofilm maturation. Furthermore, they decrease total biofilm biomass as well as cell viability of the *P. aeruginosa* PAO1 biofilm. However, in general, reductions in cell adhesion or biofilm maturation are moderate at best, with most isolates reducing biofilm adhesion or formation by *c.* 20–30%.

QSI isolates decrease elastase production by *P. aeruginosa* PAO1

These experiments were conducted to evaluate whether QSI SN could influence the production of the virulence factor elastase. The results of the ECR assay for the isolates that significantly ($P < 0.05$) decreased elastase production are shown in Fig. 2. Isolates for which no significant decrease in elastase production was observed are not included in the figure. Isolates Li3-2 (*P. putida*), Le2-5 (*Delftia* sp.), Lw1-1 (*Diaphorobacter* sp.), NFMI-T (*P. fluorescens*), Th111 and Th27 (*Pseudoalteromonas paragorgicola*), Th120 (*Muricauda olearia*), and T96 (*P. marina*) all decreased elastase production by *P. aeruginosa* PAO1 in a concentration-dependent manner. As the amount of QSI SN in the growth medium of *P. aeruginosa* PAO1 increased, elastase production decreased. As culturing *P. aeruginosa* PAO1 in the presence of QSI SN does not has a relevant impact on its growth (data not shown), we conclude that decreases in elastase production are to be attributed to interference with its QS system. *Delftia* sp. isolates have previously been reported to quench QS and thus reduce virulence of Gram-negative pathogens (Jafra *et al.*, 2006; Cirou *et al.*, 2007). *Pseudomonas fluorescens* strains have been reported to harbor the *pvdQ* gene for AHL-acylase (Koch *et al.*, 2010). Furthermore, Chernin *et al.* (2011) have recently shown that rhizobacterial volatiles of a *P. fluorescens* strain may also act as QS inhibitors.

Administration of QSI isolates increases survival of infected nematodes

Virulence of *P. aeruginosa* and *B. cenocepacia* toward *C. elegans* is regulated by QS (Kothe *et al.*, 2003; Papaioannou *et al.*, 2009). Therefore, this nematode was selected as an *in vivo* model organism to investigate the effect of simultaneous administration of isolates with QSI activity on survival of *P. aeruginosa* PAO1 and *B. cenocepacia* LMG16656-infected nematodes. For 14 isolates (Li3-2, Li4-2, MP2-1, Le1-2, Le2-4, Le2-5, Le3-4, Lw1-1, NFMI-T, Th111, Th120, Th27, T53, T96), a significant ($P < 0.05$) increase in survival was observed when the isolate was administered to the nematodes at the same time as *P. aeruginosa* PAO1, as compared to administration of the pathogen only (Fig. 3). To demonstrate that the principle of using biotherapeutic microorganisms for prevention or treatment of bacterial diseases can be extended toward Gram-negative pathogens other than *P. aeruginosa* PAO1, *B. cenocepacia* LMG16656 was also used to infect the nematodes. In this case, administration of 9 isolates (Li4-2, MP2-1, Le3-4, Lw1-1, NFMI-T, Th120, Th27, T53, T96) resulted a significant increase in survival when the isolate was administered at the same time as the pathogen (Fig. 3). The experiments with *P. aeruginosa* PAO1 and *B. cenocepacia* LMG16656 confirm the proof of concept that a broad variety of environmental bacteria have the potential for development as biotherapeutics.

To confirm that not only environmental QSI isolates with extracellular activity increase survival of infected

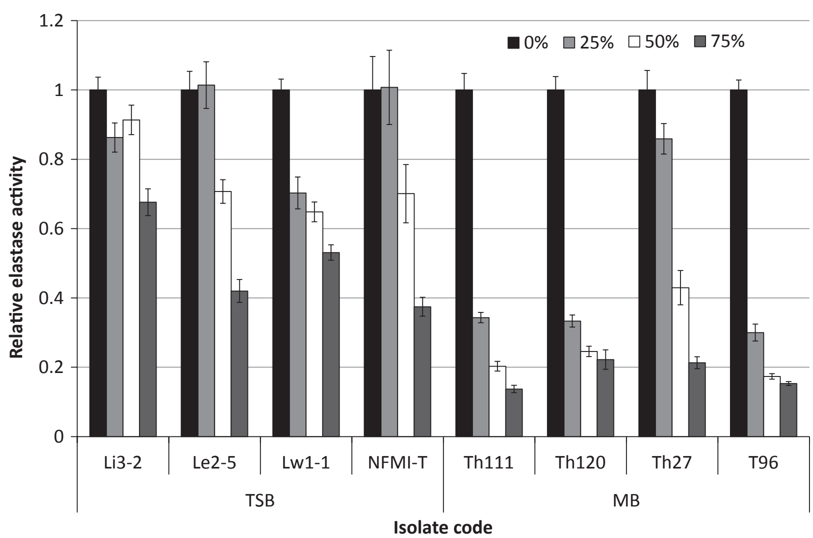

Fig. 2 Elastase production of *Pseudomonas aeruginosa* PAO1 (as measured by the absorbance of after the reaction of its supernatant with ECR) in the presence of isolate SN. The different bars represent the control (0%) and three test conditions, in which *P. aeruginosa* PAO1 was grown in the presence of 25%, 50%, and 75% QSI SN, respectively ($n = 3$; means ± SEM).

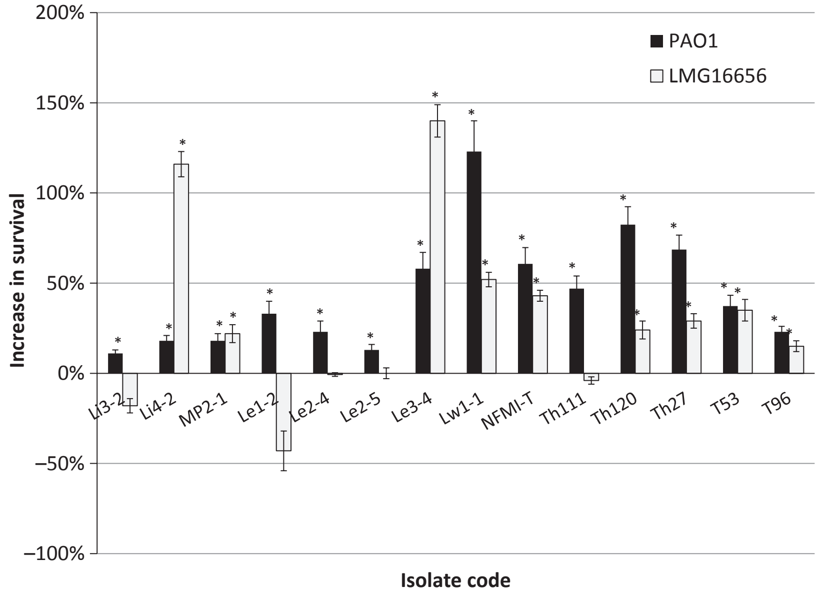

Fig. 3 Increase in survival of infected nematodes after co-administration of the isolate with extracellular QQ activity and the pathogen, relative to the survival in the absence of the isolate. Black bars represent *Pseudomonas aeruginosa* PAO1-infected nematodes, white bars represent *Burkholderia cenocepacia* LMG16656-infected nematodes. Only isolates of which administration to the nematodes resulted in a significant ($P < 0.05$) increase in survival for at least one of the two pathogens are displayed ($n = 3$; means ± SEM; *Significant increase in survival, $P \leq 0.05$).

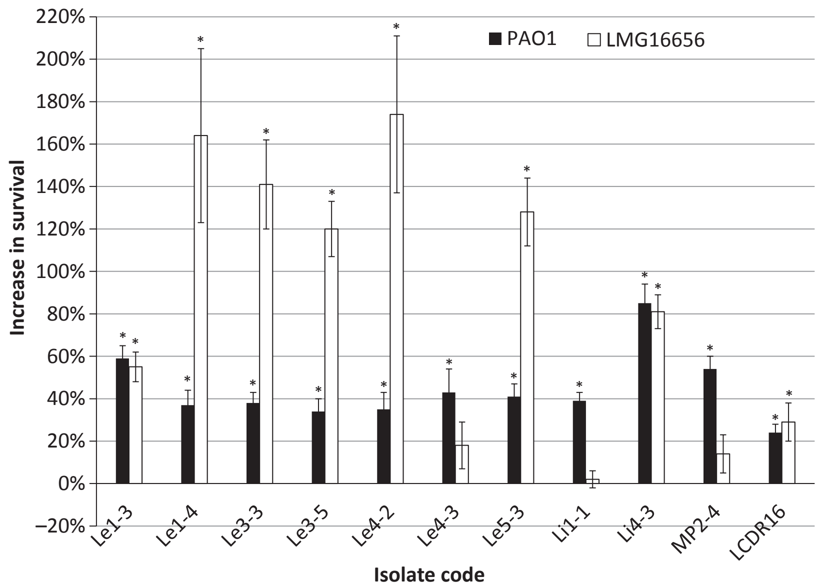

Fig. 4 Increase in survival of infected nematodes after co-administration of the isolate with intracellular QQ activity and the pathogen, relative to the survival in the absence of the isolate. Black bars represent *Pseudomonas aeruginosa* PAO1-infected nematodes, white bars represent *Burkholderia cenocepacia* LMG16656-infected nematodes. Only isolates of which administration to the nematodes resulted a significant ($P < 0.05$) increase in survival for at least one of the two pathogens are displayed ($n = 3$; means ± SEM; *Significant increase in survival, $P \leq 0.05$).

nematodes, isolates with previously shown intracellular QSI activity (Christiaen *et al.*, 2011) were also tested. In Fig. 4, the relative increase in survival of infected nematodes due to the administration of these isolates is shown. Again, diverse environmental isolates with QSI activity significantly increased survival of infected nematodes.

QSI SN activity in various fractions obtained with SEC

To determine whether the QS inhibitory activity in the QSI SN is caused by secreted enzymes or small molecules, SEC was used to separate the SN in a fraction containing the molecules > 6000 Da and fractions containing smaller

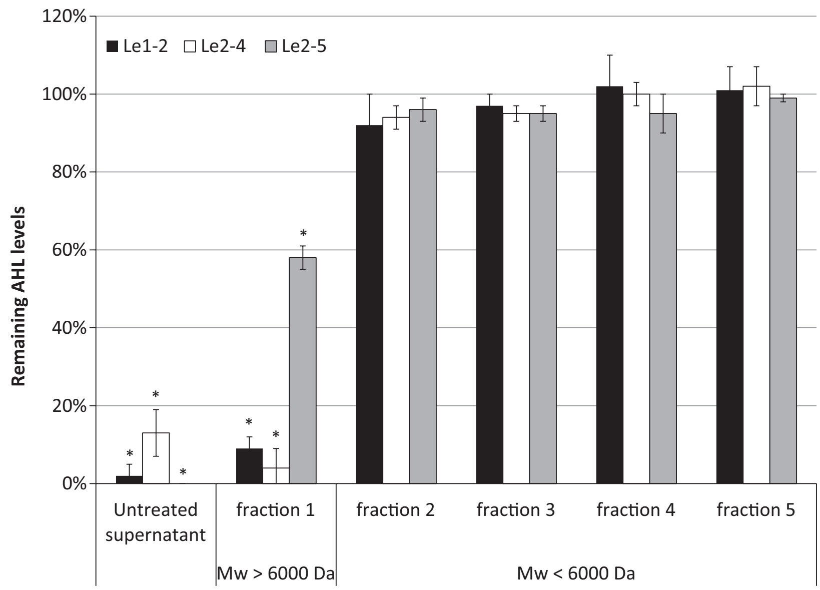

Fig. 5 Remaining AHL levels (as measured with the *Pseudomonas aeruginosa* QSIS2 biosensor) in the reaction mixture after incubating synthetic AHLs with the untreated supernatant of isolates belonging to the genus *Delftia* (Le1-2, Le2-4, Le2-5), and the different fractions obtained after SEC of the culture supernatant of Lw1-1 (mean ± SEM; *Significant decrease in AHL levels, $P \leq 0.05$).

molecules. For isolates Le1-2, Le2-4, and Le2-5, belonging to the genus *Delftia*, QSI activity was only present in the fraction containing molecules larger than 6000 Da, indicating that the QSI activity in this isolate is most likely due to enzymatic degradation of the signal molecules (Fig. 5). This is in agreement with previous studies (Jafra *et al.*, 2006; Christiaen *et al.*, 2011). The fact that for Le2-5 the level of remaining AHLs in the fractions is not as low as in the untreated sample is probably due to dilution of the sample. In contrast, for all other isolates, no detectable QSI SN activity was present in the fraction containing the large molecules (> 6000 Da) under the conditions tested. However, several fractions of the supernatant of these isolates containing lower molecular weight compounds did exhibit QSI activity, indicating the secretion of a low molecular weight inhibitory compound (Fig. 6). Although strains from the genus *Pseudomonas* and *Arthrobacter* have previously been shown to enzymatically degrade AHLs (with the enzymes PvdQ and AhlD, respectively), our data indicate that *Pseudomonas* spp. and *Arthrobacter* spp. also produce and secrete low molecular weight compounds with QSI activity.

Concluding remarks

To find a durable solution for the growing problem of bacterial resistance, the discovery of new, alternative therapeutic approaches is required (Cegelski *et al.*, 2008). Targeting bacterial virulence is such an approach, because it inhibits pathogenesis and its consequences rather than placing life-or-death pressure on the target bacterium. As it is generally recognized that a variety of pathogens use QS to control biofilm formation and the expression of their virulence factors, interfering with the bacterial communication system is considered as a promising new antipathogenic strategy (Hentzer *et al.*, 2003; Hentzer & Givskov, 2003). Much effort has already been put into finding chemical inhibitors of the QS circuitry (Galloway *et al.*, 2012; Bhardwaj *et al.*, 2013). Unfortunately, due to poor characterization and/or limited activity *in vivo*, the majority of these compounds are not very useful. Recently, however, various studies reported on the fact that probiotic bacteria produce compounds that interfere with QS (Valdez *et al.*, 2005; Medellin-Pena *et al.*, 2007). Together with the growing interest in the application of bacteria as biotherapeutic microorganisms (Reid *et al.*, 2003), this raised the question whether QSI plays a role in the biotherapeutic effects of some bacteria in diverse microbial environments. Furthermore, observations in aquaculture, where probiotics are already used as biological control agents (Verschuere *et al.*, 2000), suggested that QSI by bacteria is also relevant in important economic ecosystems, besides in human health care (Defoirdt *et al.*, 2012). The data in the present study confirm the biotherapeutic potential of bacteria, and they constitute a proof of concept that certain QSI bacteria could be used to prevent or treat QS-regulated infections. Still, future research is needed to determine whether these bacteria as such could be used as biotherapeutic microorganisms or whether the characterized active compounds produced could serve as lead molecules for drug development.

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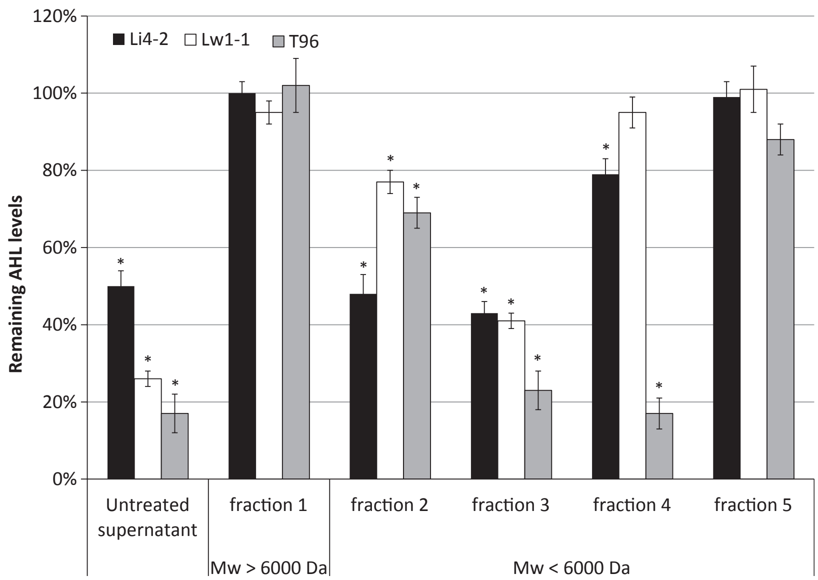

Fig. 6 Remaining AHL levels (as measured with the *Pseudomonas aeruginosa* QSIS2 biosensor) in the reaction mixture after incubating synthetic AHLs with the untreated supernatant of isolates Li4-2, Lw1-1 and T96 and the different fractions obtained after SEC of their culture supernatant (mean ± SEM; *Significant decrease in AHL levels, $P \leq 0.05$).

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