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Original article

Fighting *Acinetobacter baumannii* infections with the acylase PvdQ

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ABSTRACT

Acinetobacter baumannii is an opportunistic Gram-negative bacterial pathogen that poses a threat for frail patients worldwide. The high ability to withstand environmental stresses as well as its resistance towards a broad range of antibiotics make *A. baumannii* an effective hard-to-eradicate pathogen. One of the key mechanisms mediating tolerance against antibiotic treatment is the formation of biofilms, a process that is controlled by a multitude of different regulatory mechanisms. A key factor with major impact on biofilm formation is cell-to-cell communication by quorum-sensing, which in *A. baumannii* is mediated by acyl homoserine lactone signaling molecules. Here we show that the Ntn-Hydrolase PvdQ from *Pseudomonas aeruginosa* can reduce biofilm formation by the *A. baumannii* ATCC 17978 type strain and several clinical isolates on abiotic surfaces. Further, our study shows that a combination treatment of PvdQ-mediated quorum-quenching with the antibiotic gentamicin has a synergistic effect on the clearance of *A. baumannii* biofilms and possible biofilm dispersal. Moreover, we demonstrate in a *Galleria mellonella* larval infection model that PvdQ administration significantly prolongs survival of the larvae. Altogether, we conclude that the acylase-mediated irreversible cleavage of quorum-sensing signaling molecules as exemplified with PvdQ can set a profound limit to the progression of *A. baumannii* infections.

Acinetobacter baumannii is a Gram-negative, non-flagellated coccobacillus, which is strictly aerobic, non-fermentative and does not form spores [1]. The bacterium caught worldwide attention during the second Golf-War, as it was commonly isolated from wounded soldiers [1]. *A. baumannii* also emerged to be a pathogen frequently found in hospital environments in the U.S.A., as well as in Europe, causing hospital-acquired respiratory tract infections, urinary tract infections, skin and soft tissue infections, and nosocomial meningitis [2]. Remarkably, *Acinetobacter* species are omnipresent in the environment, and are even part of the natural skin microbiota. However, *A. baumannii* is almost exclusively encountered in nosocomial environments and reports about community-acquired *A. baumannii* infections are scarce [1]. *A. baumannii* is a member of a group of highly virulent pathogenic bacteria collectively referred to with the acronym ESKAPE, which stands for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. [3]. *A. baumannii* emerged as a priority pathogen, being notorious for its persistence on medical surfaces and showing remarkable resistance towards several classes of antibiotics. Noteworthy are the multiple efflux pumps that can push potent antibiotics, such as aminoglycosides, cephalosporins, penicillins, meropenem and imipenem out of the cell [4]. Nowadays many multi-drug-resistant (MDR) strains have emerged in hospitals posing a serious threat to hospitalized patients worldwide [5,6].

So far it remains unknown what precisely enables *A. baumannii* to be such an effective pathogen, as systematic studies could not identify a particular virulence factor responsible for its clinical presentation [2]. However, it is notable that *A. baumannii* can withstand and survive harsh conditions. For example, it shows a remarkable desiccation resistance, which allows it to stay viable on dry surfaces for at least 100 days [7]. It has thus been postulated that the high mortality rates are a result of Acinetobacter's high persistence and resistance. In a recent study reviewing cases of carbapenem resistant *A. baumannii* infections, the mortality rate amongst patients receiving an appropriate antibiotic treatment was

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33.7%, which underscores the high morbidity caused by this pathogen [8].

A. baumannii is able to organize itself in biofilms, where the bacteria are embedded in a thick matrix of polysaccharides, DNA and proteins. Such biofilms form an impermeable barrier for various antibiotics, resulting in higher antibiotic tolerance and eventually resistance [9]. The ability of *A. baumannii* to form biofilms is largely dependent on pili, which mediate attachment to abiotic as well as biotic surfaces. This attachment represents the crucial first step in biofilm formation. The responsible genes for pilus formation are clustered in the *csu* operon of *A. baumannii* [10].

Quorum-sensing is a form of bacterial communication. Bacteria are known to excrete various small molecules and, upon reaching a threshold concentration, the bacteria will respond to them. In Gram-negative bacteria these signaling molecules, the so-called autoinducers are produced by a LuxI type synthase and recognized by a LuxR type response regulator [11]. The synthase typically utilizes S-adenosyl methionine and an acyl chain bound to an acyl-carrier protein to synthesize acyl homoserine lactones (AHLs) as autoinducers. These signal molecules modulate the activity of a transcriptional regulator, which will accordingly up- or down-regulate the target genes under its control [12].

In *A. baumannii* the identified quorum-sensing circuit consists of the autoinducer synthase AbaI and the cognate response regulator AbaR [13], which is a transcription factor binding to a consensus DNA sequence called the "*lux*-box". The presence of this sequence in front of the gene for the autoinducer synthase *abaI* suggests a positive feedback loop promoting its own expression [14]. In *A. baumannii* autoinducers have acyl side chains varying in size from 10 to 16 carbon atoms. The most predominant signaling molecule is the 3-hydroxy C12 homoserine lactone (HSL) [13], which has a role in controlling motility and biofilm formation [15].

Quorum-quenching is the term for the inhibition of quorum-sensing in general. There are many possible targets when aiming at interference of bacterial communication. One mechanism involves the synthesis of small molecules, which are able to block quorum-sensing receptors. The second mechanism involves inactivation of the LuxI type synthase [16,17]. The third mechanism involves degradation of the autoinducers outside of the cell [18,19]. The PvdQ enzyme of *P. aeruginosa* is a heterodimeric acylase with an N-terminal serine in the active center [20]. Its ability to cleave long chain AHLs makes the enzyme a powerful tool for degrading the signal molecules and ultimately interfering with quorum-sensing [20,21]. In our group quorum-quenching was shown to be a promising approach to fight infectious diseases. In a recent study, we demonstrated that the acylase PvdQ has beneficial effects on the course of a pulmonary infection caused by *P. aeruginosa* in a mouse model [22].

In view of the clinicals challenges imposed by *A. baumannii* infections, the aim of the present study was to investigate whether the quorum-sensing system of *Acinetobacter* could be an appropriate target for antimicrobial therapy. Here we show for the first time that the Ntn-hydrolase PvdQ is effective in counteracting biofilm formation by *A. baumannii*. Moreover, we demonstrate the impact of PvdQ in a *Galleria mellonella* infection model, and that targeting the 3-OH-C12 HSL with PvdQ results in the same phenotype as a Δ*abaI* mutation that eliminates AHL synthesis.

1. Materials and methods

1.1. Biosensor assay for autoinducers

The enzymatic degradation of long chain AHLs was measured by employing the biosensor strain *E. coli* JM109 carrying the reporter plasmid pSB1075 [23]. This vector contains a *lux* operon under control of the *lasI* promotor. The corresponding transcription factor is the quorum-sensing response regulator LasR. Upon the binding of long chain AHLs with carbon chains ranging from C10 to C14, the activated response regulator LasR upregulates transcription of the *lux* operon, which results in light emission. The amount of light emitted by the biosensor was measured every 5 min over a 15 h time course using a multifunctional microplate reader (FLUOstar Omega, BMG Labtech). Data obtained immediately prior maximum light emission were used for comparisons.

1.2. PvdQ purification

PvdQ was produced and purified as reported previously [20]. Briefly, *E. coli* DH10B carrying the plasmid pMCT-PvdQ was grown in 2xTY medium containing 16 g/l Tryptone, 10 g/l Yeast extract, supplemented with chloramphenicol (50 μg/ml) for 30 h at 30 °C, 200 rpm. The cells were harvested and sonicated in a three times volume of lysis buffer (50 mM Tris−HCl pH 8.8; 2 mM EDTA). The cell lysate was centrifuged at 17.000 rpm for 1 h and the cleared lysate supernatant fraction was collected. All subsequent purification steps were performed with an Äkta Pure system from GE Healthcare Life Sciences. The cleared lysate was first applied to an anion exchange HiTrap Q-sepharose column, where PvdQ elutes in the flow-through. This step is required in order to remove contaminant proteins as a first purification step. Next, the collected flow-through fraction was diluted in buffer with 2.8 M ammonium sulfate to a final concentration of 750 mM and applied to a hydrophobic interaction phenyl sepharose column. PvdQ was eluted from the column using an ammonium sulfate gradient, ranging from 0 M to 1 M. Here PvdQ elutes at the end of the gradient. Lastly the PvdQ-containing fraction was applied to a gel filtration superdex75 16/60 column with phosphate-buffered saline (PBS) as running buffer. The collected PvdQ was stored in PBS buffer at −80 °C until further use. All protein chromatography columns were obtained from GE Healthcare Life Sciences.

1.3. Biofilm assay

The ability of *A. baumannii* strains to attach to surfaces as an initial step in biofilm formation was measured by using crystal violet staining following the protocol of O'Tool et al. with minor modifications [24]. Briefly, bacterial strains were grown overnight at 37 °C and, subsequently, diluted in Lysogeny Broth (LB) to an optical density at 600 nm (OD_{600}) of 0.8. Aliquots of 100 μl were added to wells of a 96-well plate and incubated for 24 h at 37 °C under static conditions. The resulting biofilms were fixed for 60 min at 60 °C and stained for 15 min using 150 μl 0.1% (v/v) crystal violet solution. The wells were washed with distilled water, and the residual water was removed. The plate was then air-dried and the dye was eluted with 110 μl 33% acetic acid. The eluted crystal violet signal was measured using a FLUOstar Omega plate reader (BMG Labtech) at a wavelength of 585 nm. Measurements were performed in three biological replicates, each involving four technical replicates. The reduction of cell mass was assessed by comparing treated and untreated samples. As a negative technical control to assess the amount of background dye, an additional staining was performed with wells only containing medium without bacterial cells.

1.4. Galleria mellonella *killing assay*

G. mellonella larvae in the final developmental stage were acquired at Frits Kuipers, Groningen. The larvae were stored at 18 °C in the dark and used within 3 days after purchase. For every experimental group, ten randomly chosen larvae between 250 mg

and 350 mg in weight were used. *Acinetobacter* cells were cultured in LB medium until an OD_{600} nm of 0.1 and concentrated by centrifugation to achieve 10^7 colony-forming units (CFUs). Prior to inoculation, the bacterial cells were washed with sterile PBS. 10 μl of the bacterial suspension (10^5 CFU) were injected into the hemocoel of each larva via the last left proleg. An insulin pen (HumaPen Luxura; Lilly Nederland) was used for injection. Bacterial colony counts on LB agar were used to confirm all inocula. Experiments comparing two or more bacterial strains in which the inoculum of one of the strains was more than 0.5 log CFU/larva different from its comparator were discarded and the experiment was repeated. After injection, the larvae were incubated at 37 °C. The number of dead larvae was scored daily for 5 days. The larvae were considered dead when they displayed no movement in response to touch.

1.5. Cloning

A marker-less *abaI* gene deletion in *A. baumannii* ATCC 17978 was achieved as previously published [25]. In short, the flanking upstream and downstream regions of the *abaI* gene were amplified from the genomic DNA *of A. baumannii* ATCC 17978 using the Q5 Polymerase (New England Biolabs). The fragments were inserted in the pMol130-TelR vector by restriction digestion and ligation. The resulting plasmid was then used to transform the *E. coli* S17-1 conjugational strain. Conjugational transfer of pMol130-TelR was achieved by co-inoculation of the donor strain *E. coli* S17-1 and the recipient *A. baumannii* strain ATCC 17978. The counter selection of the conjugational helper strain *E. coli* S17-1 was performed with 20 μg/ml chloramphenicol overnight at 37 °C. Subsequently the excision of the plasmid backbone from the *A. baumannii* chromosome was promoted by culturing the transformants in LB medium containing 20% glucose. The successful deletion of the *abaI* gene was confirmed by PCR.

1.6. Biofilm elimination assay

The biofilm elimination assay was performed as described by Zhang et al. [26] with minor modifications. In short: after the initial biofilm formation by *A. baumannii* for 24 h in the absence or presence of 500 μg/ml PvdQ, planktonic cells were removed and the wells were washed with sterile PBS. This concentration of PvdQ was based on calibration experiments (Data not shown). Gentamicin was added to sterile LB medium to a final concentration of 5 μg/ml. The medium containing the antibiotic (100 μl) was added to the washed wells containing the biofilm and incubated for another 24 h. In the case of the untreated samples LB medium without antibiotics was used. After 24 h of incubation the supernatant was removed and the wells were washed once with sterile PBS. The cells were detached by sonicating twice for 5 min, and the number of CFU/biofilm was determined by plating the resulting suspensions on LB agar plates.

2. Results

2.1. PvdQ degrades the A. baumannii autoinducer 3-OH-C12 HSL

In a previous study it was shown that the main autoinducer of the *P. aeruginosa* LasIR dependent quorum-sensing system, 3-oxo-C12 HSL, is a substrate of PvdQ [27]. Since the quorum-sensing system of *A. baumannii* depends on 3-OH-C12 HSL, we tested the capability of PvdQ to cleave 3-OH-C12 HSL in the same fashion. Fig. 1 shows the results of a biosensor setup using the strain *E. coli* DH10B carrying the reporter plasmid pSB1075 [23]. In this strain, the expression of the plasmid-borne *lux* genes and, hence, the emission of light is directly correlated with the presence of the cognate AHLs. Indeed, the presence of either 3-OH-C12 HSL or the 3oxo-C12 HSL control resulted in a detectable light signal, indicating the presence of autoinducer. Importantly, when the assay was performed in the presence of PvdQ, the emission of light was in both cases strongly diminished, showing that PvdQ is also able to accept 3-OH-C12 HSL as a substrate (Fig. 1) and, thus, to degrade the main quorum-sensing autoinducer of *A. baumannii* ATCC 17978.

2.2. PvdQ treatment reduces biofilm formation in A. baumannii

Since biofilm formation is partially controlled by quorum-sensing, we assessed whether PvdQ would also have a quorum-quenching effect on *A. baumannii* biofilm formation. To this end, *A. baumannii* biofilms were grown with or without the addition of PvdQ for 3 h or 24 h. As shown in Fig. 2, the addition of PvdQ led to a significant reduction in biofilm formation, which was clearly detectable, both after 3 and 24 h incubation. Already 3 h incubation with PvdQ reduced the surface-attached cell mass by approximately 60% and after 24 h the biofilm reduction still amounted approximately 40%.

2.3. Clinical A. baumannii isolates are susceptible to PvdQ quorum-quenching

Biofilms of *A. baumannii* are notoriously difficult to treat in clinical settings [7]. Yet, it is known that clinical isolates may differ in their ability to form biofilms. Therefore, we explored possible quorum-quenching effects of PvdQ on clinical isolates collected from patients between 2010 and 2016 (Table S1). Three of the 10 clinical isolates formed strong biofilms, comparable to the *A. baumannii* ATCC 17978 control strain, whereas four strains were relatively poor biofilm formers and three strains formed no biofilms under the tested conditions (Fig. 3, Table S1). From the seven isolates that formed biofilms, five isolates showed a significant biofilm reduction in the presence of PvdQ. This reduction was particularly

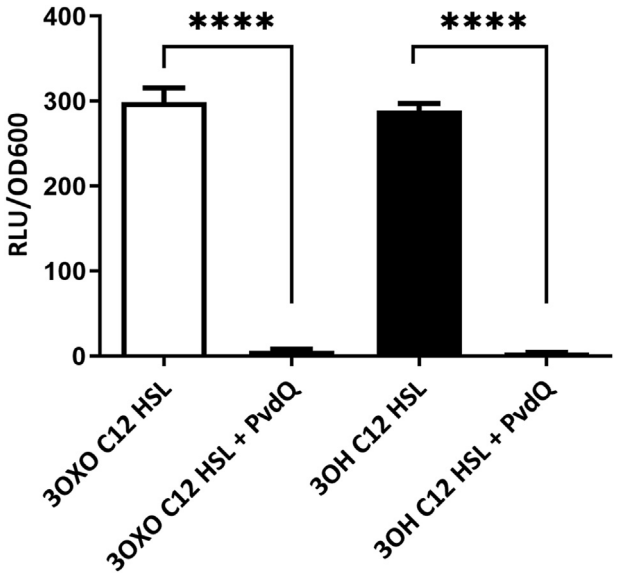

Fig. 1. Comparison of the PvdQ-dependent degradation of autoinducers 3oxoC12 HSL and 3OH C12 HSL. A biosensor assay based on the *E. coli* JM109 strain carrying the reporter plasmid pSB1142 was applied to detect the presence of 3-oxo C12 HSL and 3-OH C12 HSL and the respective degradation of these autoinducers through the PvdQ enzyme of *P. aeruginosa* (100 μg/ml). Both autoinducers were added to a final concentration of 0.1 mM. Statistical significance: $p < 0.0001$ (****); RLU/OD describes the normalized luminescence emitted by the *E. coli* biosensor strain.

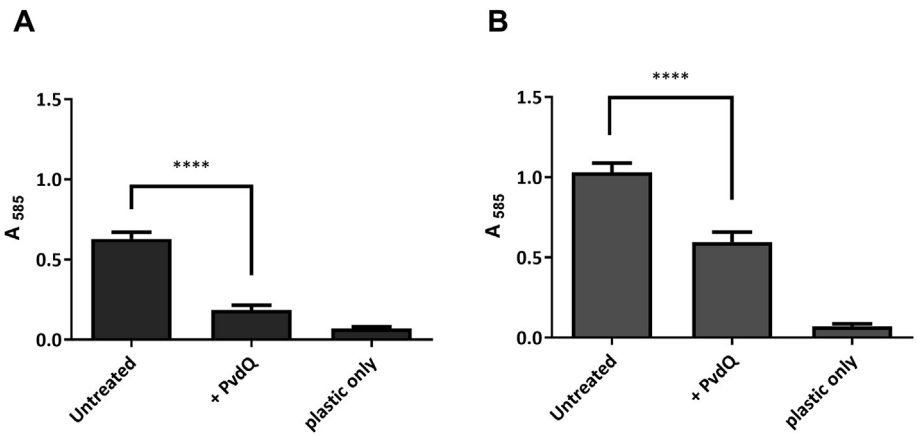

Fig. 2. PvdQ significantly reduces *A. baumannii* biofilm formation. Bacteria were incubated in the absence (untreated) or presence of PvdQ (500 μg/ml), and biofilm formation was assessed after 3 h (A) and 24 h (B) based on crystal violet binding and re-extraction. Extracted crystal violet was assayed by measuring absorption at 585 nm (A_{585}) Statistical significance: $p < 0.0001$ (****).

evident for the clinical isolates 2 and 6, and for the model strain ATCC 17978.

2.4. AHL synthase-deficient A. baumannii shows reduced attachment in biofilm formation

To elucidate the potency of enzymatic quorum-quenching with the acylase PvdQ, we constructed the *A. baumannii* mutant ATCC 17978 Δ*abaI* (Δ*abaI*). The deleted gene encodes the AHL−synthase AbaI and, therefore, the Δ*abaI* mutant is unable to produce the autoinducer 3OH−C12-HSL. To ensure the loss of AbaI activity, we created a marker-less *abaI* gene deletion as described in the publication of Amin et al. [25], and the absence of long chain AHL production by the Δ*abaI* mutant was verified using the biosensor setup based on *E. coli* DH10B carrying the pSB1075 reporter plasmid (Fig. S1). As expected, the quorum-sensing deficient Δ*abaI* strain showed a significantly reduced biofilm formation (Fig. 4A). The same reduction was also observed for the wild-type strain when treated with PvdQ for 24 h. This reduction is reflected also in the CFU counting, as the biofilms of the PvdQ-treated bacteria showed fewer viable bacteria (Fig. 4B) similar as the Δ*abaI* mutant. Furthermore, we detected an even further biofilm reduction upon addition of PvdQ to the mutant. These observations show that biofilm formation by *A. baumannii* ATCC 17978 is AHL-dependent and can be abrogated by quorum-quenching using PvdQ.

2.5. Gentamicin in combination with PvdQ has a positive effect on biofilm clearance

The intention of quorum-quenching based treatment is to limit the production of virulence factors as well as hindering the formation of a protective biofilm. However, this approach does not actively kill bacteria. To explore further the possible clinical applicability of the acylase PvdQ, a combination treatment approach with the antibiotic gentamicin was explored. To this end, we evaluated the impact of a gentamicin treatment on biofilms grown

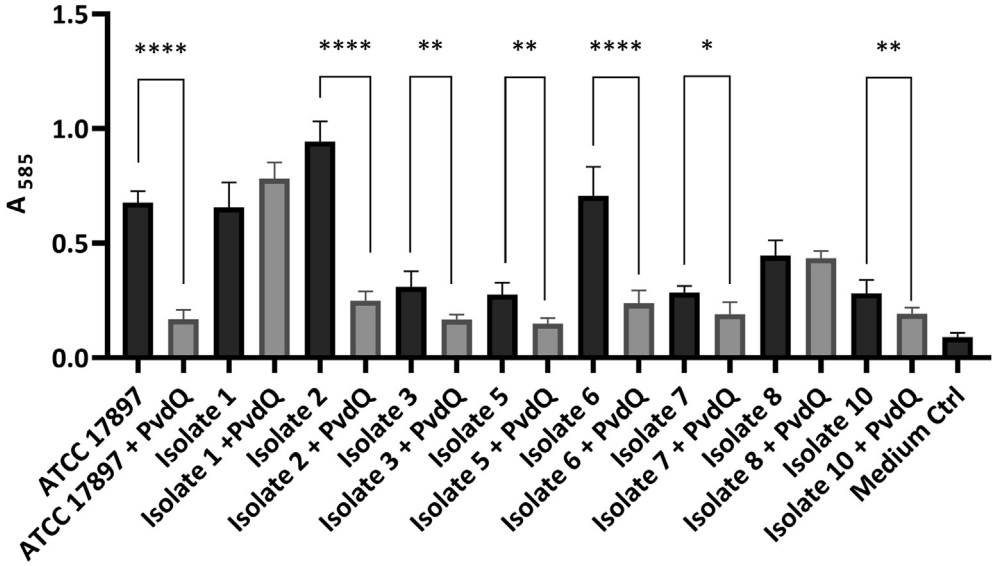

Fig. 3. Reduced biofilm formation by clinical *A. baumannii* isolates in the presence of PvdQ. Assessment of biofilm formation by clinical isolates of *A. baumannii* and the ATCC 17897 type strain, as well as the quorum-quenching potential of PvdQ was performed by crystal violet staining and re-extraction. Crystal violet binding was quantified by A_{585} readings. The measurements highlight the differences of individual clinical isolates with respect to the formation of biofilms, and their sensitivity to PvdQ treatment. All strains were grown at 37 °C for 24 h under static conditions. Statistical significance: $p > 0.05$ (ns), $p \leq 0.0332$ (*), $p \leq 0{,}0021$ (**), $p < 0.0001$ (****).

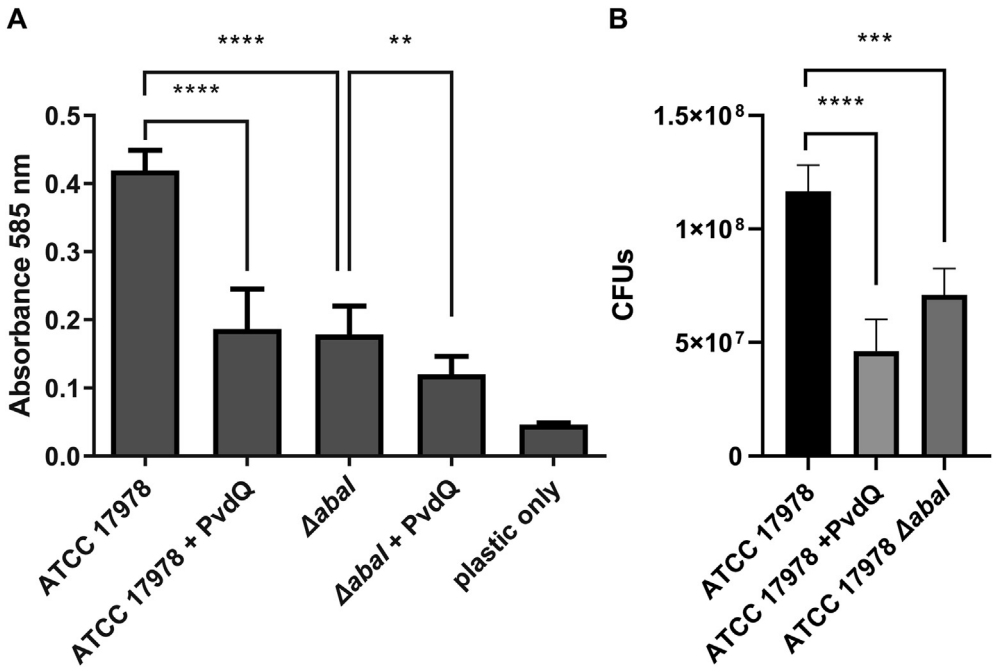

Fig. 4. Comparison of biofilm formation by *A. baumannii* ATCC17978 and the ATCC17978 Δ*abaI* mutant. Biofilm formation measured by crystal violet binding and re-extraction (A), and the number of colony-forming units (B), were determined after 24 h incubation with or without PvdQ (500 μg/ml). A quorum-quenching deficient mutant (Δ*abaI*) was included in the analysis. Statistical significance: $p \leq 0.0002$ (***), $p < 0.0001$ (****).

under static conditions for 24 h. The *A. baumannii* bacterial cells were grown with or without PvdQ pretreatment for the first 24 h of the experiment. Subsequently, the biofilm growth was allowed to continue for another 24 h, after which the biofilm-associated CFUs were counted. As shown in Fig. 5, treatment with either PvdQ or gentamicin alone reduced the biofilm significantly. Importantly, the combined application of PvdQ and gentamicin has a synergistic effect, significantly reducing the viable cells in the biofilm by another 50% compared to gentamicin alone (Fig. 5).

2.6. *PvdQ treatment prolongs* G. mellonella *survival in an infection model*

As a final step in the evaluation whether PvdQ could be a useful addition in the treatment of *A. baumannii* infections, the *in vivo G. mellonella* larval infection model was applied. Larvae of this greater wax moth have an innate immune system, which partially resembles the immune systems of higher organisms [28]. Larvae were challenged with a bacterial load of 10^5 CFUs of *A. baumannii* ATCC 17978 to induce infection with a survival rate of ~25% after 5 days. One group of larvae received an injection with PvdQ while the control group was injected with sterile PBS. Survival was monitored every 24 h. PBS injection was used as a negative control. Indeed, the PBS-treated larvae showed a survival rate of 97% after 5 days (Fig. 6). The larvae challenged with *A. baumannii* showed a mortality of 77%. In contrast, the PvdQ-treated larvae showed a mortality of only 20% over the 5 days of the experiment. A similar effect was observed when the larvae were infected with the ATCC 17978 Δ*abaI* strain. It can thus be concluded that the larvae can be protected against the lethal effect of *A. baumannii* infection by the administration of PvdQ.

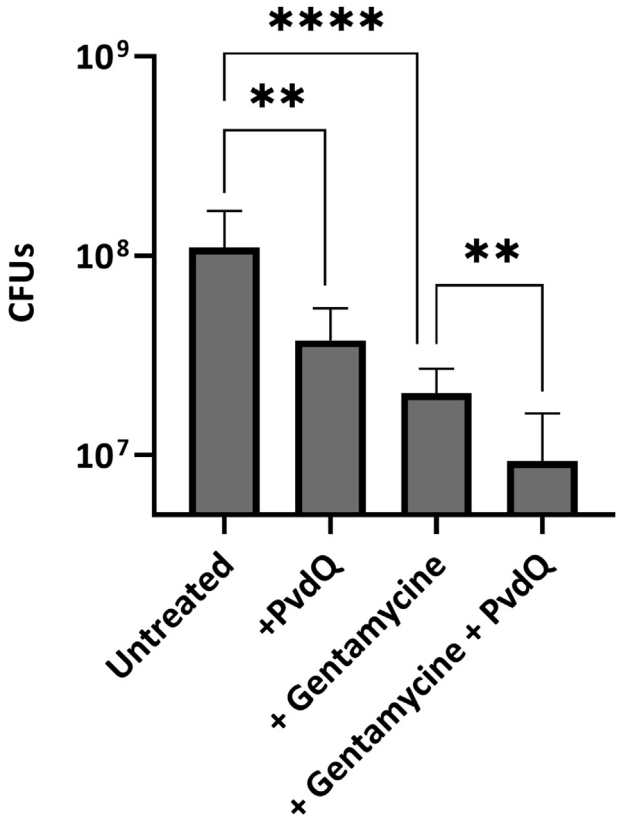

Fig. 5. Synergistic effect of PvdQ and gentamicin in the clearance of *A. baumannii* biofilm. *A. baumannii* cells were treated with PvdQ (500 μg/ml), gentamicin (5 μg/μl) and a combination of both. The results were evaluated based on the CFU counts after 48 h of incubation. Treatment with PvdQ was started upon culture inoculation (day 0), whereas the gentamicin was added after 24 h. Statistical significance: $p \leq 0.0021$ (**), $p < 0.0001$ (****).

3. Discussion

A. baumannii infections haunt IC units all around the globe, because the bacteria can infest nosocomial environments spreading

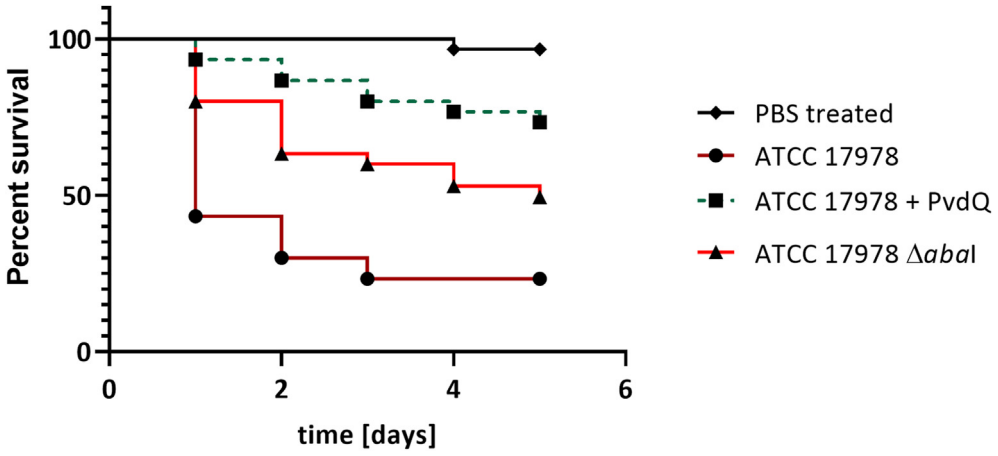

Fig. 6. PvdQ-enhanced survival of *Galleria mellonella* larvae upon infection with *A. baumannii*. Groups of ten *G. mellonella* larvae were challenged by injection with 10^5 CFUs of *A. baumannii* ATCC 17978. Larva were injected with 10 μl PvdQ solution in PBS (500 μg/ml). The *A. baumannii* ATCC 17878 Δ*abaI* strain was used as a control. For negative control, larvae were injected only with PBS. Data was collected with n = 30 larvae per group. The comparison of the survival curves gives (Logrank) a statistical significance of p = 0.0026.

from colonized patients via different transmission routes especially to patients with a reduced immunity [29]. It has been proposed that the success of this pathogen is based on a "resist and persist strategy". In addition, the ability of *A. baumannii* to attach to abiotic surfaces may explain, at least in part, the success of this pathogen in hospital environments [1]. The subsequent formation of biofilms is an evolutionary conserved wide-spread adaptation that allows bacteria to increase their tolerance against predation, escape from killing by antimicrobial substances in their environments, and to survive environmental insults. Accordingly, managing the formation of biofilms can offer a strategy to control bacterial populations in certain environments, most significantly in hospital settings. Especially with the rise of multi-drug resistant pathogens, it is now more important than ever to be careful with the usage and dosage of antimicrobials [30].

The quorum-sensing systems of *P. aeruginosa* and *A. baumannii* share a strong similarity, which was suggested to go deeper than only the regulation of the biofilm formation. In particular, *A. baumannii* exhibits a similar quorum-sensing circuit as *Aliivibrio fischeri* (formerly known as *Vibrio fischerii*) with homologues LuxR type response regulators and a LuxI type AHL-synthase, respectively named AbaR and AbaI. Analogous to other pathogens, like the well characterized *P. aeruginosa*, the *A. baumannii* quorum-sensing system regulates biofilm formation as well as motility [13]. Accordingly, *abaI*-deficient strains show impaired biofilm formation by ~40% compared to the respective wild-type strains in previous studies [13]. To our knowledge no further quorum-sensing circuits or AHL synthases are characterized in *A. baumannii*. However, we observed that treatment of the Δ*abaI* mutant with PvdQ resulted in a further reduction of biofilm formation compared to the same mutant without PvdQ treatment. This might suggest the presence of additional long chained AHLs and, accordingly, more layers of quorum-sensing regulation that we are not yet aware of.

All in all, our present observations suggest that enzymatic quorum-quenching mediated by the *P. aeruginosa*-derived hydrolase PvdQ can prolong the time *A. baumannii* needs to form a differentiated biofilm. Since the biofilm formation by various bacteria is associated with an increased tolerance towards antibiotics and decreased accessibility for the host immune system, inhibiting the formation of this structure seems to be a promising approach to fight bacterial infections [31]. In *P. aeruginosa* several studies attest for the attenuation of virulence by quorum-quenching [21]. In both species quorum-sensing mechanisms promote the production of antioxidant enzymes, such as catalase and superoxide dismutase. A quorum-quenching intervention could thus lower the oxidative stress tolerance in both strains [32,33]. In this context, it should be noted that biofilms serve as a reservoir for bacterial infections and it is therefore important to assess not only the presence of a biofilm, but also the number of biofilm-resident viable bacterial cells. The present study shows that, despite the fact that quorum-quenching approaches do not kill bacterial cells, the number of biofilm-associated bacterial cells was reduced upon treatment with PvdQ. This means that lowering the numbers of dispersing cells, which potentially could cause metastatic infections can be reduced by quorum-quenching approaches. Thus, a prophylactic administration of PvdQ could be beneficial for reducing bacterial infection, and may as well allow the protection of abiotic surfaces against microbial attachment.

The attenuation of virulence by quorum-quenching was previously demonstrated for *P. aeruginosa* PA01 infections in a mouse model [22]. Based on the impact of quorum-quenching mediated by PvdQ in the *A. baumannii* biofilm model we also investigated this approach in a *G. mellonella* infection model. Indeed, it was demonstrated that PvdQ protects infected *G. mellonella* larvae against *A. baumannii* infection. This suggests that PvdQ supports the larval innate immune system to clear the infection by quorum-quenching. How this exactly takes place is presently unclear as the precise mechanism of *A. baumannii* virulence is not very well understood [7]. Wand et al. showed that there is a complex correlation between *Acinetobacter* strains and their ability to form biofilms with respect to virulence in a *G. mellonella* infection model. In particular, this group reported that bacterial cells dispersed from a biofilm exhibited higher virulence than planktonic cells [34]. Based on our present observations, we propose that by reducing the ability to form a biofilm, we can interfere with the persistence of this pathogen in its fight with the innate immune defenses and potentially reduce the number of biofilm-dispersed *A. baumannii* cells. Furthermore, the repression of the 3-OH-C12 HSL QS system can lead to a mis-regulation of virulence cascades under its control. In combination, this would explain how the administration of PvdQ allows infected larvae to clear an *A. baumannii* infection. Importantly, our present study shows that a combination treatment of PvdQ with the antibiotic gentamicin has a synergistic effect with respect to biofilm clearance *in vitro*, reducing the load of surface-attached bacterial cells. This implies that the combined

administration of PvdQ and antibiotics may help to successfully fight *A. baumannii* infections.

It is interesting to note that the ability to form biofilms varies significantly between the individual clinical *A. baumannii* isolates (Table S1). For example, isolate 2 from the sputum of a hospitalized patient was observed to be a strong biofilm former, whereas isolate 4 from a tissue sample was a very poor biofilm former. Further, we demonstrated that the biofilms of five biofilm-forming clinical isolates could be significantly reduced by incubation in the presence of PvdQ, whereas two isolates were resistant to PvdQ treatment. Since the complete quorum-sensing system of *A. baumannii* is not yet fully understood, at this point we cannot predict to what extent our quorum-quenching strategy will inactivate the quorum-sensing systems of clinical *A. baumannii* isolates, or whether PvdQ will target also alternative autoinducers, which would add to the beneficial effects we presented in our present study.

The location of an infection seems to be an important factor in the PvdQ quorum-quenching treatment scenario, taking into account that PvdQ has to retain its catalytic activity when applied. If we consider PvdQ treatment as a strategy to fight, for example, sputum-resident *Acinetobacter* species, the most beneficial way of administration would be a topical application, or in the case of pneumonia administration with an inhalation device. Quorum-quenching strategies for medical application have been investigated since many years, and researchers have been assessing various different strategies. These range from the use of small molecules that inhibit the synthesis of autoinducers, as well as the inhibition of the response regulator to the enzymatic degradation of autoinducers [35]. Most of the published work in the field of enzymatic quorum-quenching was performed with lactonases. These enzymes open the homoserine-lactone ring at the ester bond, rendering the molecule no longer recognizable for the cognate response regulator. However, this approach has the downside that the opened homoserine lactone ring can close again and that the targeted compound can act again as an active signaling molecule. With the acylase PvdQ this is not the case, because the autoinducer molecule is cleaved into two parts, which leads to an irreversible inactivation of all downstream signaling. PvdQ is therefore a highly potent acylase with high quorum-quenching potential. Especially in the face of MDR bacteria colonizing intensive care units around the globe, alternatives to antibiotics to control bacterial diseases in general and *A. baumannii* infections in particular will be increasingly important in the years to come.

Declaration of competing interest

There are no conflicts of interest for any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2022.104951.

References

[1] Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: emergence of a successful pathogen. Clin Microbiol Rev 2008;21:538–82. https://doi.org/10.1128/CMR.00058-07.

[2] Moubareck CA, Halat DH. Insights into *Acinetobacter baumannii*: a review of microbiological, virulence, and resistance traits in a threatening nosocomial pathogen. Antibiotics 2020;9. https://doi.org/10.3390/antibiotics9030119.

[3] Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, No drugs: No ESKAPE! An update from the infectious diseases society of America. Clin Infect Dis 2009;48:1–12. https://doi.org/10.1086/595011.

[4] Amin M, Navidifar T, Saleh Shooshtari F, Rashno M, Savari M, Jahangirmehr F, et al. Association between biofilm formation, structure, and the expression levels of genes related to biofilm formation and biofilm-specific resistance of Acinetobacter baumannii strains isolated from burn infection in Ahvaz, Iran. Infect Drug Resist 2019;12:3867–81. https://doi.org/10.2147/IDR.S228981.

[5] Mezzatesta ML, D'Andrea MM, Migliavacca R, Giani T, Gona F, Nucleo E, et al. Epidemiological characterization and distribution of carbapenem-resistant *Acinetobacter baumannii* clinical isolates in Italy. Clin Microbiol Infect 2012;18:160–6. https://doi.org/10.1111/j.1469-0691.2011.03527.x.

[6] Huang G, Yin S, Xiang L, Gong Y, Sun K, Luo X, et al. Epidemiological characterization of *Acinetobacter baumannii* bloodstream isolates from a Chinese Burn Institute: a three-year study. Burns 2016;42:1542–7. https://doi.org/10.1016/j.burns.2016.02.021.

[7] Harding CM, Hennon SW, Feldman MF. Uncovering the mechanisms of *Acinetobacter baumannii* virulence. Nat Rev Microbiol 2017;16:91–102. https://doi.org/10.1038/nrmicro.2017.148.

[8] Du X, Xu X, Yao J, Deng K, Chen S, Shen Z, et al. Predictors of mortality in patients infected with carbapenem-resistant *Acinetobacter baumannii*: a systematic review and meta-analysis. Am J Infect Control 2019;47:1140–5. https://doi.org/10.1016/j.ajic.2019.03.003.

[9] Rao R, Karthika R, Singh S, Shashikala P, Kanungo R, Jayachandran S, et al. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *Acinetobacter baumannii*. Indian J Med Microbiol 2008;26:333–7. https://doi.org/10.4103/0255-0857.43566.

[10] Tomaras AP, Flagler MJ, Dorsey CW, Gaddy JA, Actis LA. Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. Microbiology 2008;154:3398–409. https://doi.org/10.1099/mic.0.2008/019471-0.

[11] Fuqua WC, Winans SC, Greenberg EP. Quorum sensing in bacteria: the LuxR-LuxI family of cell density- responsive transcriptional regulators. J Bacteriol 1994;176:269–75. https://doi.org/10.1111/j.1462-5822.2006.00734.x.

[12] Saipriya K, Swathi CH, Ratnakar KS, Sritharan V. Quorum-sensing system in *Acinetobacter baumannii*: a potential target for new drug development. J Appl Microbiol 2020;128:15–27. https://doi.org/10.1111/jam.14330.

[13] Niu C, Clemmer KM, Bonomo RA, Rather PN. Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii*. J Bacteriol 2008;190:3386–92. https://doi.org/10.1128/JB.01929-07.

[14] Bhargava N, Sharma P, Capalash N. Quorum sensing in *Acinetobacter* : an emerging pathogen. Crit Rev Microbiol 2010;36:349–60. https://doi.org/10.3109/1040841X.2010.512269.

[15] Keller L, Surette MG. Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol 2006;4:249–58. https://doi.org/10.1038/nrmicro1383.

[16] Kalia VC. Quorum sensing inhibitors: an overview. Biotechnol Adv 2013;31:224–45. https://doi.org/10.1016/J.BIOTECHADV.2012.10.004.

[17] Grandclément C, Tannières M, Moréra S, Dessaux Y, Denis D. Faure. Quorum quenching: role in nature and applied developments. FEMS Microbiol Rev 2015;40:86–116. https://doi.org/10.1093/femsre/fuv038.

[18] Koch G, Nadal-Jimenez P, Reis CR, Muntendam R, Bokhove M, Melillo E, et al. Reducing virulence of the human pathogen *Burkholderia* by altering the substrate specificity of the quorum-quenching acylase PvdQ. Proc Natl Acad Sci Unit States Am 2014;111:1568–73. https://doi.org/10.1073/pnas.1311263111.

[19] Bzdrenga J, Daudé D, Rémy B, Jacquet P, Plener L, Elias M, et al. Biotechnological applications of quorum quenching enzymes. Chem Biol Interact 2017;267:104–15. https://doi.org/10.1016/J.CBI.2016.05.028.

[20] Bokhove M, Nadal Jimenez P, Quax WJ, Dijkstra BW. The quorum-quenching N-acyl homoserine lactone acylase PvdQ is an Ntn-hydrolase with an unusual substrate-binding pocket. Proc Natl Acad Sci U S A 2010;107:686–91. https://doi.org/10.1073/pnas.0911839107.

[21] Papaioannou E, Wahjudi M, Nadal-Jimenez P, Koch G, Setroikromo R, Quax WJ. Quorum-quenching acylase reduces the virulence of Pseudomonas aeruginosa in a Caenorhabditis elegans infection model. Antimicrob Agents Chemother 2009;53:4891–7. https://doi.org/10.1128/AAC.00380-09.

[22] Utari PD, Setroikromo R, Melgert BN, Quax WJ. PvdQ quorum quenching acylase attenuates *Pseudomonas aeruginosa* virulence in a mouse model of pulmonary infection. Front Cell Infect Microbiol 2018;8:119. https://doi.org/10.3389/fcimb.2018.00119.

[23] Winson MK, Swift S, Fish L, Throup JP, Jørgensen F, Chhabra SR, et al. Construction and analysis of luxCDABE -based plasmid sensors for investigating N -acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol Lett 1998;163:185–92. https://doi.org/10.1111/j.1574-6968.1998.tb13044.x.

[24] O'Toole GA. Microtiter dish Biofilm formation assay. JoVE 2011. https://doi.org/10.3791/2437.
[25] Amin I, Richmond GE, Sen P, Koh T, Piddock LJ, Chua K. A Method for generating marker-less gene deletions in multidrug-resistant Acinetobacter baumannii. BMC Microbiol 2013;13:158. https://doi.org/10.1186/1471-2180-13-158.
[26] Zhang Y, Brackman G, Coenye T. Pitfalls associated with evaluating enzymatic quorum quenching activity: the case of MomL and its effect on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilms. PeerJ 2017;5:e3251. https://doi.org/10.7717/peerj.3251.
[27] Sio CF, Otten LG, Cool RH, Diggle SP, Braun PG, Bos R, et al. Quorum quenching by an N-acyl-homoserine lactone acylase from Pseudomonas aeruginosa PAO1. Infect Immun 2006;74:1673–82. https://doi.org/10.1128/IAI.74.3.1673.
[28] Ramarao N, Nielsen-Leroux C, Lereclus D. The insect Galleria mellonella as a powerful infection model to investigate bacterial pathogenesis. JoVE 2012: e4392. https://doi.org/10.3791/4392.
[29] Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. Nat Rev Microbiol 2007;5:939–51. https://doi.org/10.1038/nrmicro1789.
[30] Fodor A, Abate BA, Deák P, Fodor L, Gyenge E, Klein MG, et al. Multidrug resistance (MDR) and collateral sensitivity in bacteria, with special attention to genetic and evolutionary aspects and to the perspectives of antimicrobial peptides—a review. Pathogens 2020;9:1–55. https://doi.org/10.3390/pathogens9070522.
[31] Römling U, Balsalobre C. Biofilm infections, their resilience to therapy and innovative treatment strategies. J Intern Med 2012;272:541–61. https://doi.org/10.1111/joim.12004.
[32] Bhargava N, Sharma P, Capalash N. Pyocyanin stimulates quorum sensing-mediated tolerance to oxidative stress and increases persister cell populations in Acinetobacter baumannii. Infect Immun 2014;82:3417–25. https://doi.org/10.1128/IAI.01600-14.
[33] Davenport PW, Griffin JL, Welch M. Quorum sensing is accompanied by global metabolic changes in the opportunistic human pathogen Pseudomonas aeruginosa. J Bacteriol 2015;197:2072–82. https://doi.org/10.1128/JB.02557-14.
[34] Wand ME, Bock LJ, Turton JF, Nugent PG, Mark Sutton J, Sutton JM. *Acinetobacter baumannii* virulence is enhanced in Galleria mellonella following biofilm adaptation. J Med Microbiol 2012;61:470–7. https://doi.org/10.1099/jmm.0.037523-0.
[35] Fetzner S. Quorum quenching enzymes. J Biotechnol 2014;201:2–14. https://doi.org/10.1016/j.jbiotec.2014.09.001.