Pitfalls associated with evaluating enzymatic quorum quenching activity: the case of MomL and its effect on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilms

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Background. The enzymatic degradation of quorum sensing (QS) molecules (called quorum quenching, QQ) has been considered as a promising anti-virulence therapy to treat biofilm-related infections and combat antibiotic resistance. The recently-discovered QQ enzyme MomL has been reported to efficiently degrade different *N*-acyl homoserine lactones (AHLs) of various Gram-negative pathogens. Here we investigated the effect of MomL on biofilms formed by two important nosocomial pathogens, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. **Methods.** MomL was expressed in *E.coli* BL21 and purified. The activity of MomL on AHLs with hydroxyl substituent was tested. Biofilms of *P. aeruginosa* PAO1 and *Acinetobacter* strains were formed in 96-well microtiter plates. Biofilm formation was evaluated by crystal violet staining, plating and fluorescence microscopy. The effect of MomL on biofilm susceptibility to antibiotics was also tested. We further evaluate MomL in dual-species biofilms formed by *P. aeruginosa* and *A. baumanii*, and in biofilms formed in a wound model. The effect of MomL on virulence of *A. baumanii* was also tested in the *Caenorhabditis elegans* model. **Results.** MomL reduced biofilm formation and biofilm susceptibility to different antibiotics in biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in microtiter plates *in vitro*. However, no significant differences were detected in the dual-species biofilm and in wound model biofilms. In addition, MomL did not affect virulence of *A. baumannii* in the *C. elegans* model. Finally, the effect of MomL on biofilm of *Acinetobacter* strains seems to be strain-dependent. **Discussion.** Our results indicate that although MomL showed a promising anti-biofilm effect against *P. aeruginosa* and *A. baumanii* biofilms formed in microtiter plates, the effect on biofilm formation under conditions more likely to mimic the real-life situation was much less pronounced or even absent. Our data indicate that in order to obtain a better picture of potential applicability of QQ enzymes for the treatment of biofilm-related infections, more elaborate model systems need to be used.

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Abstract

Background. The enzymatic degradation of quorum sensing (QS) molecules (called quorum quenching, QQ) has been considered as a promising anti-virulence therapy to treat biofilm-related infections and combat antibiotic resistance. The recently-discovered QQ enzyme MomL has been reported to efficiently degrade different *N*-acyl homoserine lactones (AHLs) of various Gram-negative pathogens. Here we investigated the effect of MomL on biofilms formed by two important nosocomial pathogens, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Methods. MomL was expressed in *E.coli* BL21 and purified. The activity of MomL on AHLs with hydroxyl substituent was tested. Biofilms of *P. aeruginosa* PAO1 and *Acinetobacter* strains were formed in 96-well microtiter plates. Biofilm formation was evaluated by crystal violet staining, plating and fluorescence microscopy. The effect of MomL on biofilm susceptibility to antibiotics was also tested. We further evaluate MomL in dual-species biofilms formed by *P. aeruginosa* and *A. baumanii*, and in biofilms formed in a wound model. The effect of MomL on virulence of *A. baumanii* was also tested in the *Caenorhabditis elegans* model.

Results. MomL reduced biofilm formation and biofilm susceptibility to different antibiotics in biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in microtiter plates *in vitro*. However, no significant differences were detected in the dual-species biofilm and in wound model biofilms. In addition, MomL did not affect virulence of *A. baumannii* in the *C. elegans* model. Finally, the effect of MomL on biofilm of *Acinetobacter* strains seems to be strain-dependent.

Discussion. Our results indicate that although MomL showed a promising anti-biofilm effect against *P. aeruginosa* and *A. baumanii* biofilms formed in microtiter plates, the effect on biofilm formation under conditions more likely to mimic the real-life situation was much less pronounced or even absent. Our data indicate that in order to obtain a better picture of potential applicability of QQ enzymes for the treatment of biofilm-related infections, more elaborate model systems need to be used.

Introduction

Quorum sensing (QS) is a widespread communication process that allows bacteria to coordinate their group behavior based on the production, detection and response to extracellular signal molecules (Bassler & Losick 2006; Williams et al. 2007). QS regulates gene expression related to biofilm formation, motility and production of virulence factors in many Gram-negative and Gram-positive pathogens, and interfering with QS has been intensively studied as a promising anti-virulence therapy to combat bacterial infections and antibiotic resistance (Hentzer & Givskov 2003; LaSarre & Federle 2013; Rutherford & Bassler 2012). Many natural and synthetic compounds have been found to inhibit QS, and several quorum quenching (QQ) enzymes mainly targeting *N*-acyl homoserine lactone (AHL) based QS in Gram-negative bacteria have been described as well (Brackman & Coenye 2015; Fetzner 2015; Rasmussen & Givskov 2006; Tang & Zhang 2014). Some of these QS inhibitors (QSIs) and QQ enzymes have shown promising anti-virulence effects both *in vitro* and *in vivo*. For instance, furanones have been reported to reduce biofilm formation and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice (Hentzer et al. 2002; Wu et al. 2004). Baicalin hydrate and cinnamaldehyde (QSIs targeting AHL-based QS in *P. aeruginosa* and *Burkholderia cepacia* complex) as well as hamamelitannin (a QSI targeting the peptide-based system present in *Staphylococcus aureus*) increase biofilm susceptibility to antibiotics and survival of infected *Galleria mellonella* larvae and *Caenorhabditis elegans*, as well as decrease the microbial load in a mouse pulmonary infection model (Brackman et al. 2011). As for QQ enzymes, an AiiM-producing *P. aeruginosa* mutant showed reduced lung injury and increased survival in an *in vivo* study on mice with pneumonia (Migiyama et al. 2013), and an inhaled lactonase *Sso*Pox-I was also reported to reduce virulence of *P. aeruginosa* and mortality in rat pneumonia (Hraiech et al. 2014).

Previously MomL, a novel AHL lactonase belonging to the metallo-β-lactamase superfamily was identified and characterized (Tang et al. 2015). It has high degrading activities towards short- and long-chain AHLs with or without an oxo-group at the C-3 position (Tang et al. 2015). MomL can reduce pyocyanin and protease production by *P. aeruginosa* and attenuated the virulence of *P. aeruginosa* in a *C. elegans* infection model (Tang et al. 2015), but its effect on biofilm formation of *P. aeruginosa* and other Gram-negative pathogens was not tested yet.

Besides *P. aeruginosa*, *Acinetobacter baumannii* has also been recognized as an increasingly prevalent Gram-negative opportunistic pathogen responsible for severe nosocomial infections (Gonzalez-Villoria & Valverde-Garduno 2016; Peleg et al. 2008). Resistance of *P. aeruginosa* and *A. baumannii* strains to multiple antibiotic classes complicates the treatment for these

infections and poses considerable therapeutic challenges worldwide (Potron et al. 2015). One of the main factors contributing to their reduced antibiotic susceptibility and to treatment failure is biofilm formation both on tissues and abiotic surfaces (Donlan & Costerton 2002; Hall-Stoodley et al. 2004; Longo et al. 2014). Biofilms of both *P. aeruginosa* and *A. baumannii* are known to be regulated by AHL-based QS. In *P. aeruginosa*, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl-L-homoserine lactone (C_4-HSL) are used by the Las and Rhl QS system, respectively (Pesci et al. 1997). One AHL synthase belonging to LuxI family, AbaI, has been reported to catalyze the synthesis of *N*-(3-hydroxydodecanoyl)-L-homoserine lactone (3-OH-C_{12}-HSL) in *Acinetobacter nosocomialis* M2 (Niu et al. 2008), but other AHLs with varied chain lengths and substituents are also found in *Acinetobacter* strains (Bhargava et al. 2010; González et al. 2009). The biofilm-forming ability of an *abaI* mutant was reduced by around 40 % compared to the corresponding wildtype strain (Niu et al. 2008). Compared to the extensive literature on inhibiting QS pathways and virulence in *P. aeruginosa* (Aybey & Demirkan 2016; Furiga et al. 2016; Hentzer et al. 2003; O'Loughlin et al. 2013; Yin et al. 2015), there are fewer reports on inhibiting QS and biofilm formation in *A. baumannii* (Bhargava et al. 2015; Chow et al. 2014; Saroj & Rather 2013).

In the present study, we tested the anti-biofilm activity of MomL against *P. aeruginosa* and *A. baumannii*, and further evaluated the effect of MomL under more complex conditions such as in dual-species biofilm and in a wound model system with the aim to obtain a better knowledge base regarding the possible development of QQ enzymes as anti-virulence therapy.

Material & Methods

Bacterial strains, culture conditions and chemicals

P. aeruginosa PAO1, *A. calcoaceticus* LMG 10517, *A. nosocomialis* M2 and *A. baumannii* LMG 10520, LMG 10531 and AB5075 were cultured on tryptic soy agar (TSA) or in Mueller-Hinton broth (MH) at 37°C. *Escherichia coli* BL21(DE3) harboring MomL expression plasmid pET24a(+)-momL-(-SP) (Tang et al. 2015) was cultured on Luria-Bertani (LB) agar supplemented with kanamycin (50 μg/mL) at 37°C. The AHL biosensor *Agrobacterium tumefaciens* A136 (pCF218) (pCF372) (Zhu et al. 1998) was maintained on LB agar supplemented with spectinomycin (50 μg/mL) and tetracycline (4.5 μg/mL), and grown in AT

minimal medium (Tempé et al. 1977) containing 0.5% (wt/vol) glucose for detecting AHLs in the liquid X-Gal (5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside) assay. 3-OH-C_{12}-HSL was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) as stock solution (100 mM). *C. elegans* N2 (Δ*glp-4*; Δ*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 1999).

MomL expression and purification

MomL was expressed and purified according to Tang et al., 2015. In brief, protein expression was induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when *E. coli* cells in LB reached an optical density at 600 nm (OD600) of 0.5 to 0.7. The induction was carried out at 16°C with moderate shaking (150 rpm) for 12h. Cells were harvested and sonicated, and the obtained supernatant was loaded on NTA-Ni (Qiagen) columns for purification according to the manufacturer's instruction. Desalting of the protein solution was accomplished by Amicon Ultra-15 centrifugal filter devices, and the purified MomL was stored at -20°C in Tris-HCl buffer (50mM, pH 6.5) with 25% glycerol.

Detection for degradation of 3-OH-C_{12}-HSL

The amount of 3-OH-C_{12}-HSL was quantified using *A. tumefaciens* A136 liquid X-gal assay and expressed as the normalized *β*-galactosidase activity as previously described (Tang et al. 2013). The correction factor a and b were obtained and calculated for our experimental conditions, and the final formula to calculate the normalized *β*-galactosidase activity is

$$\frac{0.716 \times OD492 - OD620}{0.205 \times OD620 - OD492}$$

. To test the degradation of 3-OH-C_{12}-HSL by MomL, 3-OH-C_{12}-HSL (10 μM) was mixed with MomL in different concentrations (0.05-5 μg/mL) and incubated at 37°C for 1h. Afterwards the residual 3-OH-C_{12}-HSL was quantified by adding 10 μL solution to the A136 biosensor, as described previously (Tang et al. 2013).

120 **Biofilm formation assays**

121 Overnight cultures of *P. aeruginosa* and *Acinetobacter* strains in MH broth were diluted to
122 contain approximately 5×10^7 CFU/mL. Ninety µL of this diluted bacterial suspension was
123 transferred to the wells of a round-bottomed 96-well microtiter plate. To test the effect of MomL
124 on biofilm formation, 10 µL purified enzyme (in different concentrations) was added to the wells,
125 while 10 µL Tris-HCl buffer (50mM, pH 6.5) with 25% glycerol was added to the control. The
126 plate was incubated at 37°C for 4 h before the supernatant was removed. The wells were rinsed
127 once with sterile physiological saline (PS) and re-filled with fresh media (90 µL) and MomL (10
128 µL). The plate was incubated at 37°C for an additional 20 hours. The biofilm biomass was
129 quantified by crystal violet (CV) staining as described previously (Peeters et al. 2008). After
130 rinsing the wells with sterile PS, the biofilm was fixed with 100 µL 99% methanol for 15 min and
131 stained with 100 µL 0.1% CV for 20 min. The excess CV was removed by washing the plates
132 under running tap water and bound CV was released by adding 150 µl of 33% acetic acid. The
133 absorbance was measured at 590 nm.

134 **Biofilm susceptibility assays**

135 After a 24h-biofilm of *P. aeruginosa* or *A. baumannii* strains was formed as described above
136 either in presence of MomL or not, the plate was emptied and biofilm cells were rinsed with
137 sterile PS. Antibiotics were dissolved in PS and 90 µL of these solutions were added to treat the
138 biofilm for another 24h, either with or without 10 µl MomL. Tobramycin (TOB; 4 µg/mL as final
139 concentration), ciprofloxacin (CIP; 0.5 µg/mL), meropenem (MEM; 16 µg/mL) and colistin
140 (CST; 16 µg/mL) were used to treat the biofilm of *P. aeruginosa* PAO1; TOB (6 µg/mL), CIP (4
141 µg/mL), MEM (8 µg/mL) and CST (16 µg/mL) were used to treat the biofilm of the *A.*
142 *baumannii* strains. The supernatant was removed and the wells were washed once with sterile
143 PS. To release bacterial cells from biofilm, two cycles of vortex (5 mins) and sonication (5 mins)
144 were performed, and the number of CFU/biofilm was determined by plating the resulting
145 suspensions on TSA.

146 **Fluorescence microscopy**

147 Biofilms of *P. aeruginosa* PAO1 or *A. baumannii* strains were formed in the absence or presence
148 of MomL and treated with antibiotics as described above using a flat-bottomed 96-well microtiter
149 plates. 3 µL SYTO9 and 3 µL propidium iodide were diluted to 1mL in sterile PS, and 100 µL of
150 this staining solution was transferred to each well. The plate was incubated for 15min at room
151 temperature and fluorescence microscopy was performed with EVOS FL Auto Imaging System
152 (Life Technologies).

153 **Dual-species biofilm formation**

154 Overnight cultures of *P. aeruginosa* and *A. baumannii* strains in MH broth were diluted to contain
155 approximately 5×10^5 CFU/mL and 5×10^7 CFU/mL, respectively, and equal volume of
156 suspensions of *P. aeruginosa* and *A. baumannii* were mixed. MomL (200 µg/mL) and tobramycin
157 (6 µg/mL) were added as described above. To quantify CFU in the dual-species biofilm,
158 *Pseudomonas* Isolation Agar (Difco) and TSA supplemented with 5 µg/mL cefsulodin were used
159 as selective media for *P. aeruginosa* and *A. baumannii* respectively.

160 **Biofilm formation in wound model**

161 Artificial dermis composed of hyaluronic acid and collagen was used in our wound model, as
162 described before (Brackman et al. 2016). Each disk of artificial dermis was placed in 24-well
163 microtiter plate. One mL media containing Bolton Broth, heparinized bovine plasma and freeze-
164 thaw laked horse blood cells was added on and around the dermis. Suspensions (10 µL) of *P.*
165 *aeruginosa* or *A. baumannii* containing 10^4 bacterial cells were added on the top of dermis. Final
166 concentrations of MomL added were 200 µg/mL and 10 µg/mL for *P. aeruginosa* and *A.*
167 *baumannii*, respectively. Tobramycin (10 µg/mL) was added after 8 h incubation at 37°C. After
168 24h, the infected dermis was washed with 1 mL PS and was transferred into 10 ml PS. Biofilm
169 cells on the dermis were loosen and collected by three cycles of vortex (30 s) and sonication (30
170 s). The number of CFU/dermis was quantified by standard plating techniques.

171 ***C. elegans* survival assay**

The *C. elegans* survival assay was carried out as described before with minor modification (Brackman et al. 2011). Synchronized worms (L4 stage) were suspended in medium containing 95% M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, and 1 ml of 1 M $MgSO_4 \cdot 7H_2O$ in 1 liter of water) and 5% brain heart infusion broth (Oxoid), and 25 µL of this nematode suspension was transferred to the wells of a 96-well microtiter plate. Overnight culture of *A. baumannii* was suspended in the assay media and added in a final concentration of $2.5x10^7$ CFU/ml. MomL was added in a final concentration of 10 µg/mL. The plates were incubated at 25°C for 24 h. The fraction of dead worms was determined by counting the number of dead worms and the total number of worms in each well.

Statistics

Data were expressed as means ± standard deviations (SD), and there were at least six replicates per treatment in biofilm assays. Student's *t* test was used to determine the significance.

Results

Degradation of 3-OH-C_{12}-HSL by purified MomL

MomL was produced in *E. coli* and subsequently successfully purified (Fig. 1). Although MomL had been shown to degrade various AHLs (Tang et al. 2015), its activity on AHLs with hydroxyl substituent at the C3 position was not tested yet. We could demonstrate that MomL, in a concentration of 1 µg/mL or higher, can degrade almost all 3-OH-C_{12}-HSLs (10 µM) under the experimental conditions used in the present study (Fig. 2).

Effect of MomL on biofilm formation by *P. aeruginosa* and *A. baumannii*

Following biofilm formation in 96-well microtiter plates and quantification by crystal violet staining, a significant difference was observed between *P. aeruginosa* PAO1 control biofilms and biofilms grown in the presence of MomL (concentration > 50 µg/mL) (Fig. 3A). When grown with 150 µg/mL MomL, an average decrease of approximately 35% was observed. MomL inhibited *A. baumannii* LMG 10531 biofilm formation at concentrations as low as 0.1 µg/mL, and

197 the biofilm biomass was reduced by approximately 42% when exposed to 5 μg/mL MomL (Fig.
198 3B). No further decrease was observed when *A. baumannii* LMG 10531 biofilms were grown in
199 the presence of higher concentrations of MomL.

200 **Effect of MomL on biofilm susceptibility to antibiotics**

201 Application of MomL alone (200 μg/mL for *P. aeruginosa* PAO1 and 10 μg/mL for *A. baumannii*
202 LMG 10531) reduced the number of cultivable biofilm cells by approximately 50% in both *P.*
203 *aeruginosa* PAO1 and *A. baumannii* LMG 10531. For *P. aeruginosa* PAO1, combining CIP or
204 MEM with MomL led to >70% more reduction compared to treatment with CIP or MEM alone
205 (Fig. 4A). For *A. baumannii* LMG 10531, MomL also increased killing of biofilm cells when
206 antibiotics were used together with MomL (Fig. 4B). In case of TOB, cell number was reduced
207 by 80% when used in combination with MomL compared to TOB alone. Consistent with results
208 obtained by plating, fewer living cells were observed in fluorescence microscope images of
209 biofilms treated with MomL, TOB, or a combination of both, compared to control biofilms (Fig.
210 5).

211 **Effect of MomL on dual-species biofilm formed by *P. aeruginosa* and *A. baumannii***

212 We also evaluated the effect of MomL on dual-species biofilm formed by *P. aeruginosa* PAO1
213 and *A. baumannii* LMG 10531. We found that *P. aeruginosa* PAO1 inhibited growth of *A.*
214 *baumannii* LMG 10531 in dual-species biofilm, and most *A. baumannii* LMG 10531 cells were
215 killed by *P. aeruginosa* PAO1 after 48h (Fig. 6). When MomL was added, there was a reduction
216 in *A. baumannii* LMG 10531 cell numbers; however no difference was observed in either total
217 cell numbers or number of surviving *P. aeruginosa* PAO1 cells (Fig. 6A). MomL in combination
218 of TOB was also tested, but no change in susceptibility to TOB was observed in the dual-species
219 biofilm (Fig. 6B).

220 **Effect of MomL on other *Acinetobacter* strains**

We also tested MomL on four other *Acinetobacter* strains. However, only *A. baumannii* LMG 10520 showed reduction in biofilm biomass when treated with MomL at 50 μg/mL (Fig. 7). No significant difference was observed for *A. calcoaceticus* LMG 10517, *A. nosocomialis* M2 and *A. baumannii* AB5075. The effect of MomL on susceptibility of *A. baumannii* LMG 10520 and *A. calcoaceticus* LMG 10517 biofilms was also tested. For *A. baumannii* LMG 10520, significant differences were detected when MomL was added alone or in combination with antibiotics (Fig. 8). For *A. calcoaceticus* LMG 10517, no difference was observed between biofilms receiving MomL treatment and biofilms receiving the control treatment, either by plating or fluorescence microscope.

Effect of MomL in a biofilm wound model system and in the *C. elegans* model

An *in vitro* wound model was used to mimic the conditions in an infected wound. For both *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531, MomL had no effect on biofilm formation in this wound model (Fig. 9).

The *C. elegans* model was used to further evaluate whether MomL can increase survival of nematodes infected with *A. baumannii*. However, no significant increase of *C. elegans* survival was found after treating nematodes infected with *A. baumannii* LMG 10520 or *A. baumannii* LMG 10531 with MomL (Fig. 10).

Discussion

QS disruption has been considered as a promising anti-infectious strategy to substitute or at least supplement treatment with antibiotics, and could inhibit production of virulence factors and the formation of biofilms (Brackman et al., 2011). Compared to QS inhibitors, QQ enzymes can degrade AHLs from different pathogens and might be more effective in treating multispecies infections. In addition, QQ enzymes do not need to enter the cells as they can act extracellularly, making it less likely resistance will develop (Bzdrenga et al. 2016). The recently-discovered QQ enzyme, MomL has strong degrading activity towards AHLs with different acyl-chain length and substituents (oxo or hydroxyl) (Tang et al. 2015), and this could be an advantage when targeting bacteria like *Acinetobacter* strains that produce various AHLs. In the present study we

investigated the possible use of MomL for treating biofilm infections, and evaluate its effect on two important Gram-negative nosocomial pathogens, *P. aeruginosa* and *A. baumannii* in different models.

First we tested the effect of MomL on single-species biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in microtiter plates; a reduction of biofilm biomass was observed for both strains. The maximum decrease in biofilm of *A. baumannii* LMG 10531 was achieved at a concentration of 5 μg/mL and no further decrease was observed with higher concentrations of MomL, which indicated that other mechanism beside QS might also be involved in *A. baumannii* biofilm regulation. When used in combination with antibiotics fewer biofilm cells survived compared to antibiotic treatment alone, both for *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531. All these *in vitro* results seem promising and suggest possible use of MomL to treat biofilm infections of *P. aeruginosa* and *A. baumannii*.

We subsequently investigated the effect of MomL in a dual-species biofilm formed by *P. aeruginosa* and *A. baumannii* and in a wound biofilm model. Surprisingly, MomL had no effect on the overall cell number in the mixed species biofilm and the same disappointing results were obtained in biofilms formed in wound model system. In this wound model system, media containing plasma, serum, horse blood and heparin was used to reflect nutritional condition in wounds. An artificial dermis was used to mimic a wound like surface and an inoculum of 10^4 cells was used to reflect the microbial load of a wound prior to infection. Additionally, in contrast to what we observed for the mono-species biofilms formed in 96-well microtiter plates, MomL did not potentiate the activity of TOB in this model system.

To our knowledge, this is the first study to evaluate the anti-biofilm activity of a QQ enzyme in more advanced biofilm models (including multispecies biofilms and a wound biofilm model). Our data strongly suggest that the effect of MomL (and potentially also other QQ enzymes) on *in vivo* grown bacterial biofilms may be much less pronounced than the effect observed with biofilms formed under simple *in vitro* conditions. Factors affecting the anti-biofilm activity in more complex systems could include stability of the enzyme, penetration of the enzyme through the biofilm matrix, and the composition of the environment.

Different outcomes were also observed when we evaluated the effect of MomL on different *Acinetobacter* strains, and no effects of MomL on biofilm formation was detected for three out of five *Acinetobacter* strains tested. In addition, for *A. baumanii* LMG 10520, a considerably higher

concentration of MomL was required to obtain a pronounced inhibitory effect than for *A. baumannii* LMG 10531. These results confirm that the anti-biofilm activity of QQ enzymes is strain-dependent, which is likely to reduce their clinical efficacy.

Conclusion

The results of the present study highlight that there are considerable hurdles to be cleared before QQ enzymes could potentially be used to combat infections. Our data indicate that demonstrating AHL degrading activity *in vitro* and/or anti-biofilm activity in simple *in vitro* biofilm model systems is not sufficient to predict an anti-biofilm effect in more complex systems.

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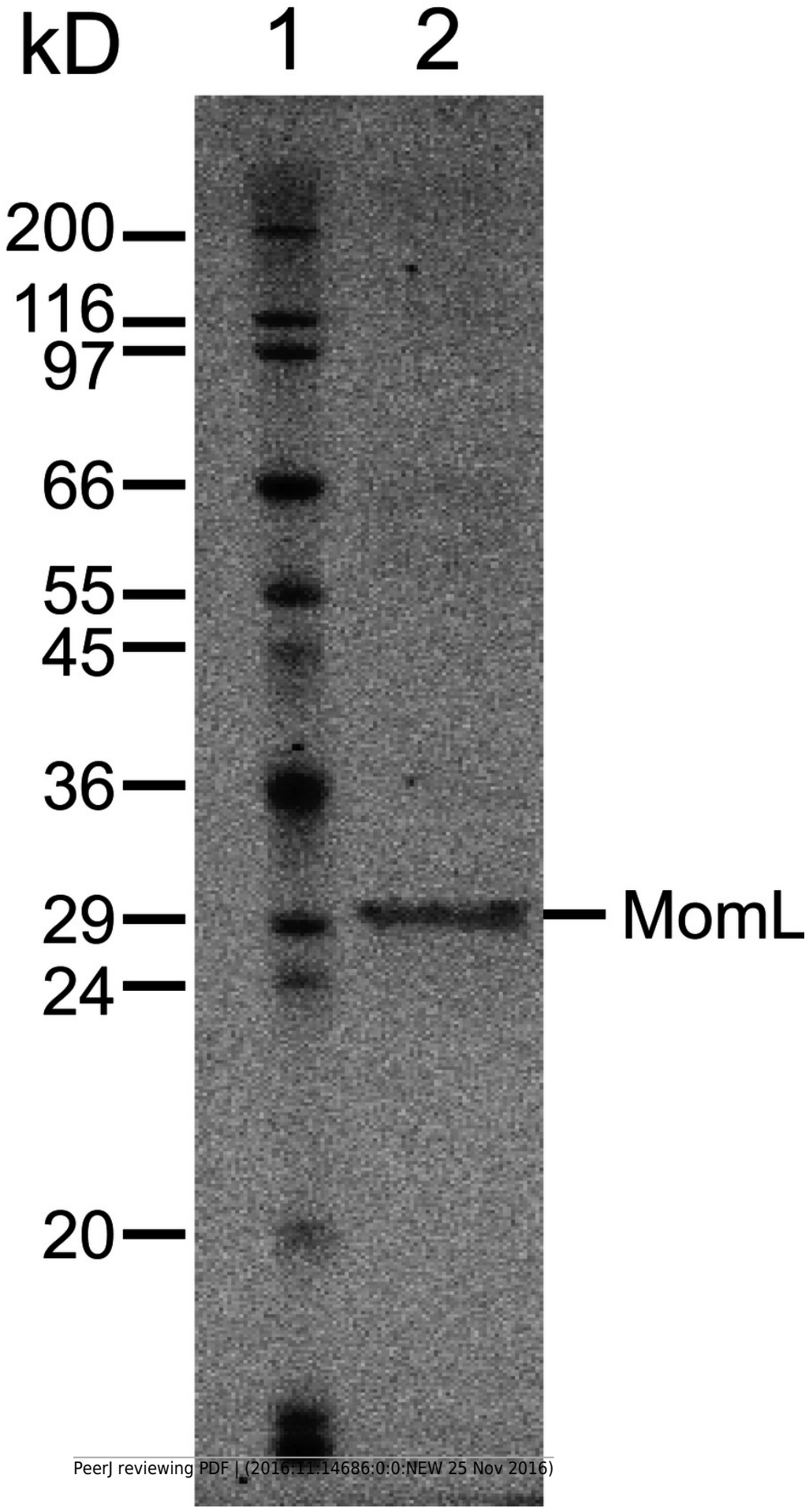
Figure 1

SDS-PAGE of purified MomL

Figure 1. SDS-PAGE of purified MomL. Lane1, molecular mass markers; Lane 2, purified recombinant MomL with molecular mass of nearly 31 kD.


kD
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MomL

Figure 2

Degradation of 3-OH-C_{12}-HSL by MomL

Figure 2. Degradation of 3-OH-C_{12}-HSL by MomL. The amount of residual 3-OH-C_{12}-HSL was expressed as the normalized β-galactosidase activity. Data shown are average (n = 3), error bars represent standard deviation. *, P<0.05 in T-test when compared with control.

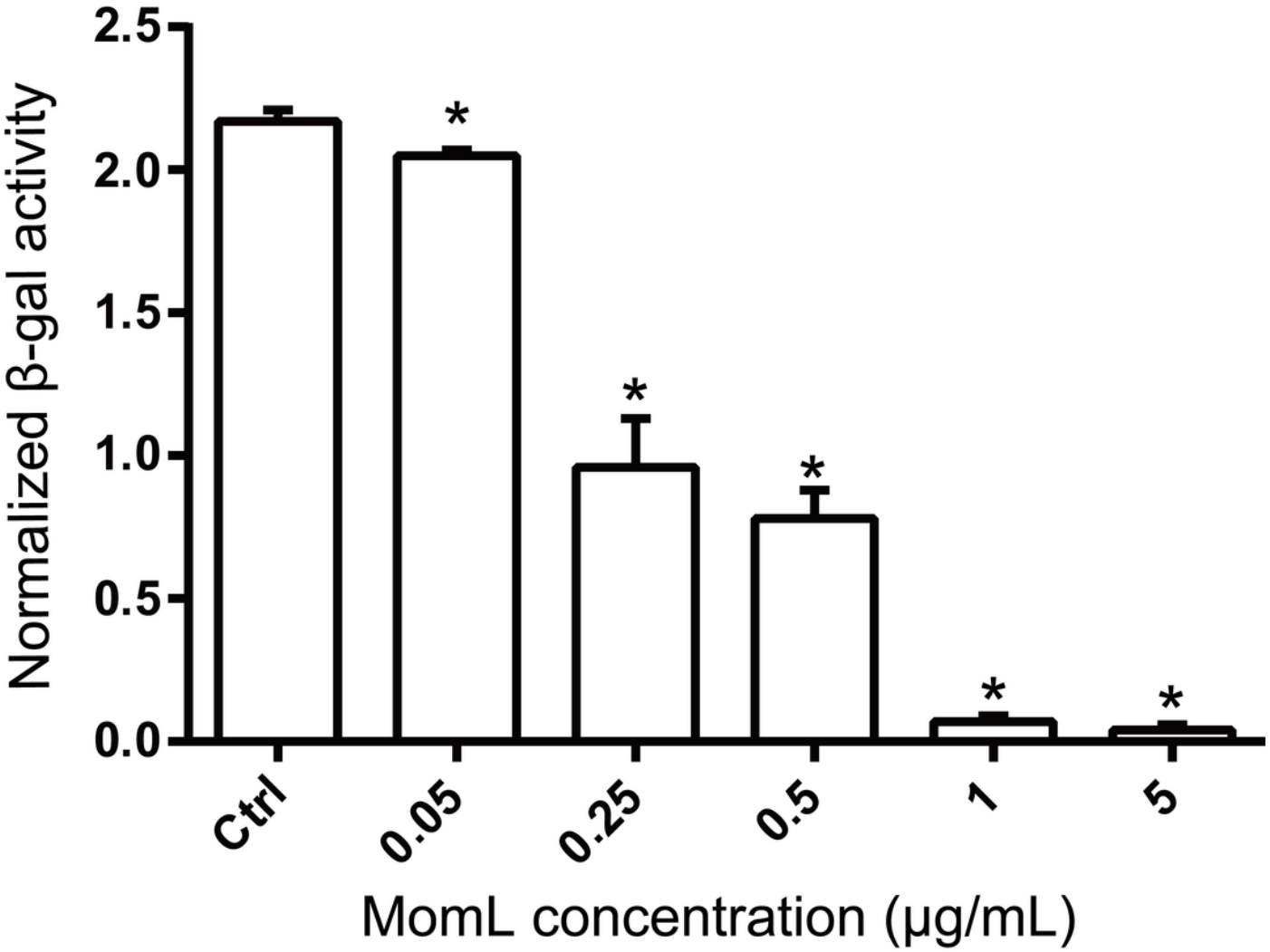


Figure 3

Effect of MomL on biofilm formation by *P. aeruginosa* PAO1 (A) and *A. baumannii* LMG 10531 (B) .

Figure 3. Effect of MomL on biofilm formation by *P. aeruginosa* PAO1 (A) and *A. baumannii* LMG 10531 (B) . Biofilms were quantified by CV staining and amount of biofilm left is expressed as percentage of OD 590 compared to control. Data shown are average (n ≥ 27), error bars represent standard deviation. *, 0.005<P<0.05; **, P<0.005 in T-test when compared with control.

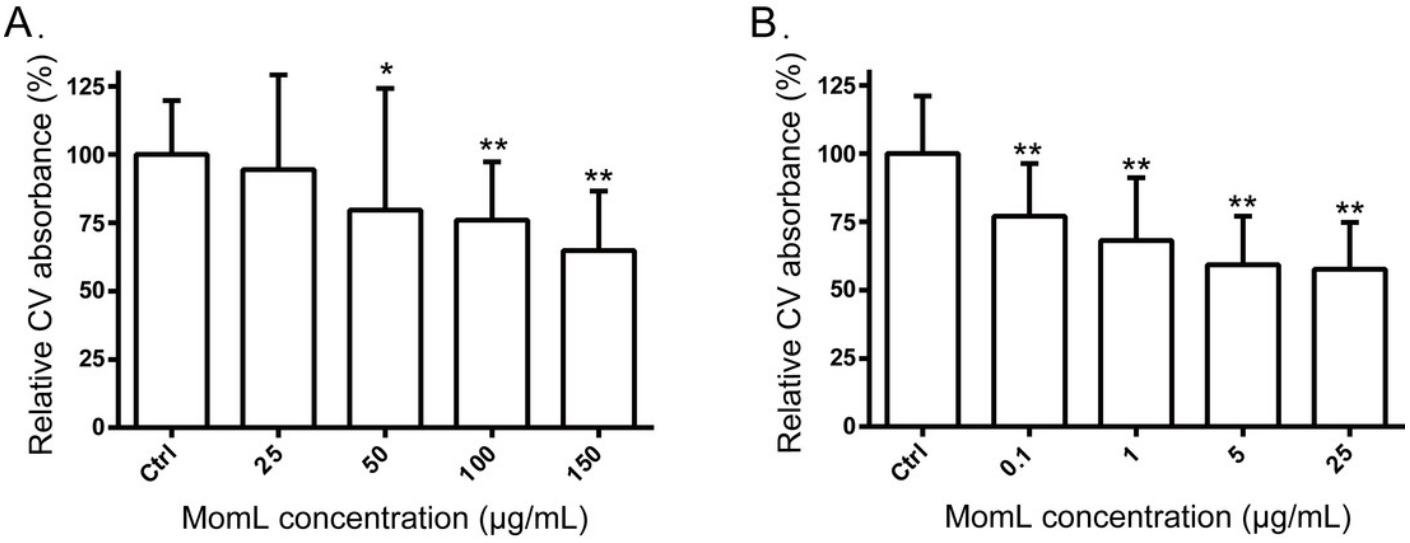


Figure 4

Effect of MomL on susceptibility of *P. aeruginosa* PAO1 (A) and *A. baumannii* LMG 10531 (B) biofilms to different antibiotics.

Figure 4. Effect of MomL on susceptibility of *P. aeruginosa* PAO1 (A) and *A. baumannii* LMG 10531 (B) biofilms to different antibiotics. The percentage of CFU/biofilm compared to untreated control is shown. MomL was added in a final concentration of 200 µg/mL for *P. aeruginosa* PAO1 and 10 µg/mL for *A. baumannii* LMG 10531. Data shown are average (n = 9), error bars represent standard deviation. T-tests were performed to compare control and MomL or antibiotic treatment alone and in combination with MomL (*, 0.005<P<0.05; **, P<0.005)

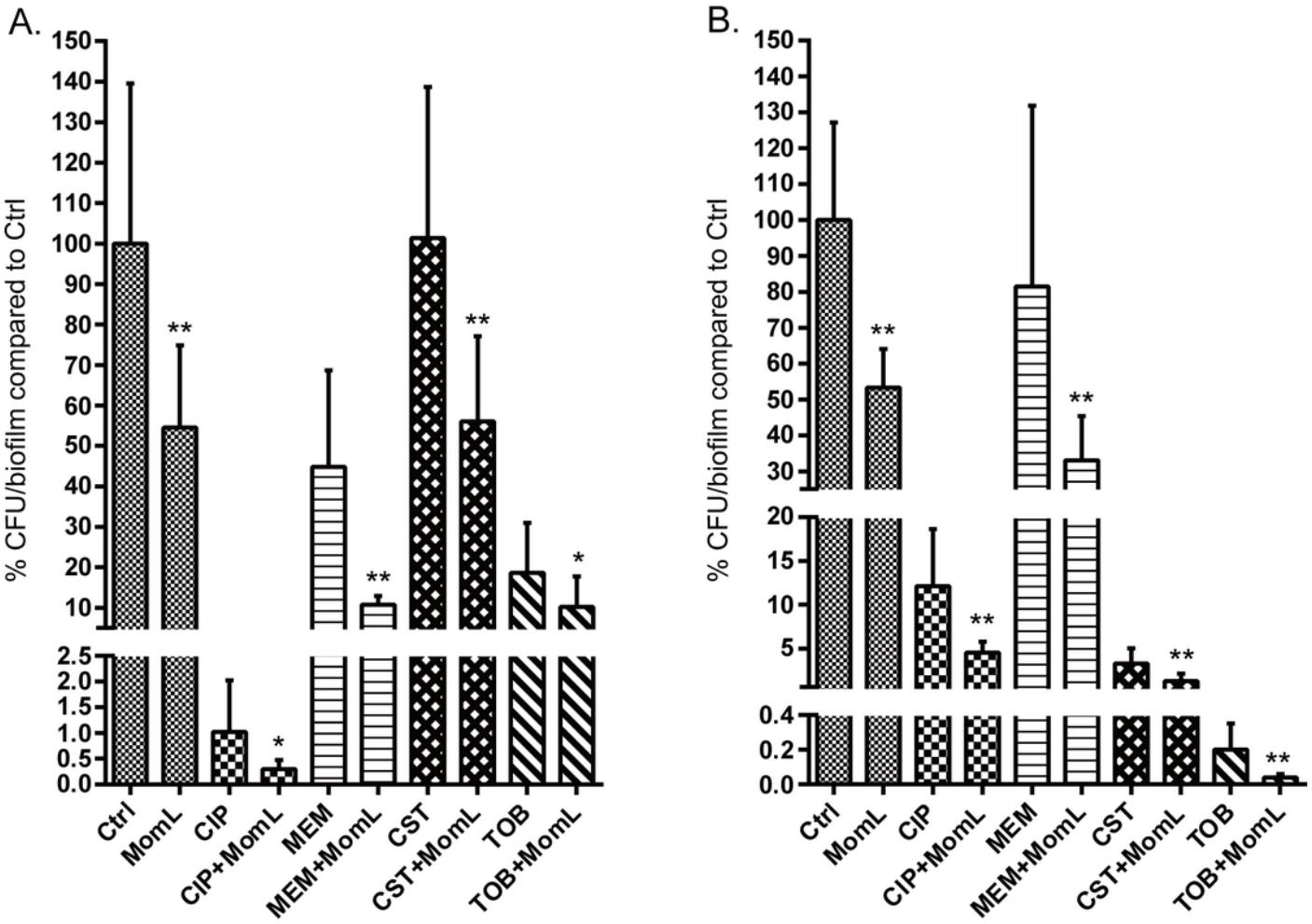


Figure 5

Representative fluorescence images of biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531.

Figure 5. Representative fluorescence images of biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531. Biofilms were treated with MomL alone, TOB alone or a combination of both and stained with Syto9 and propidium iodide. The scale bar represents 100 µm.

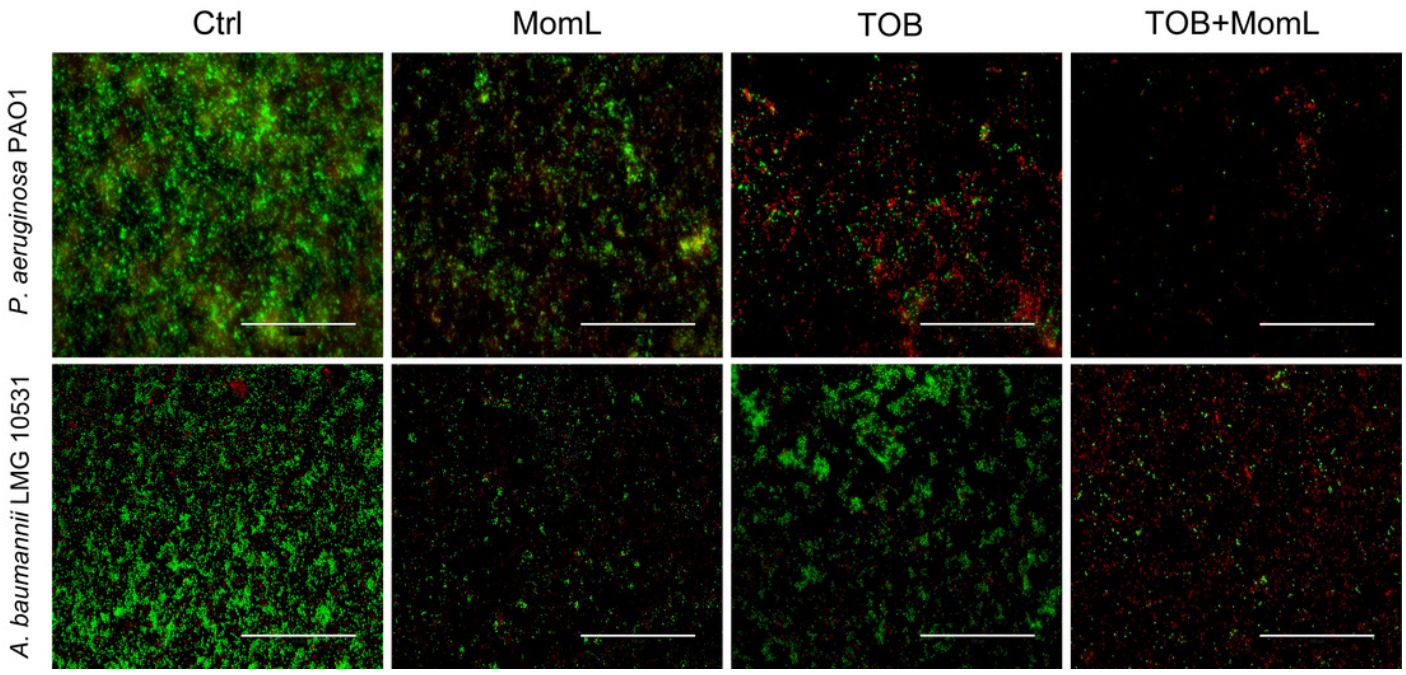


Figure 6

Effect of MomL on dual-species biofilms.

Figure 6. Effect of MomL on dual-species biofilms. Total number of CFU/biofilm, number of *P. aeruginosa* PAO1 CFU/biofilm and number of *A. baumannii* LMG 10531 CFU/biofilm in each dual-species biofilm were determined by plating. (A). 24h-biofilm treated with MomL alone; (B). 48h-biofilm treated with MomL alone, TOB alone or a combination of both. Data shown are average (n = 9 for A and n = 6 for B), error bars represent standard deviation. T-tests were performed to compare total, *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 cell numbers respectively between untreated or MomL-treated dual-species biofilm (**, P<0.005).

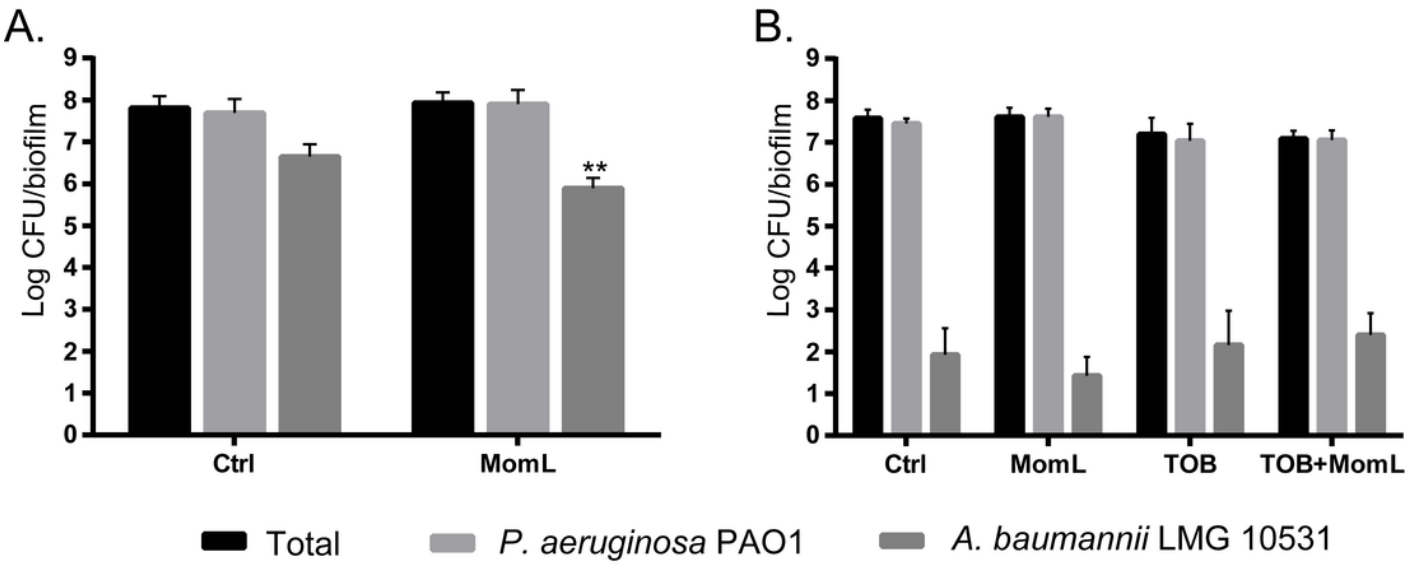


Figure 7

Effect of MomL on biofilms formed by other *Acinetobacter* strains.

Figure 7. Effect of MomL on biofilms formed by other *Acinetobacter* strains. Biofilms of *A. calcoaceticus* LMG 10517, *A. nosocomialis* M2, *A. baumannii* LMG 10520 and *A. baumannii* AB5075 were treated with different concentration of MomL and quantified by CV staining. Data shown are average (n ≥ 27), error bars represent standard deviation. T-tests were performed to compare MomL treatment and untreated control for each strain (**, P<0.005).

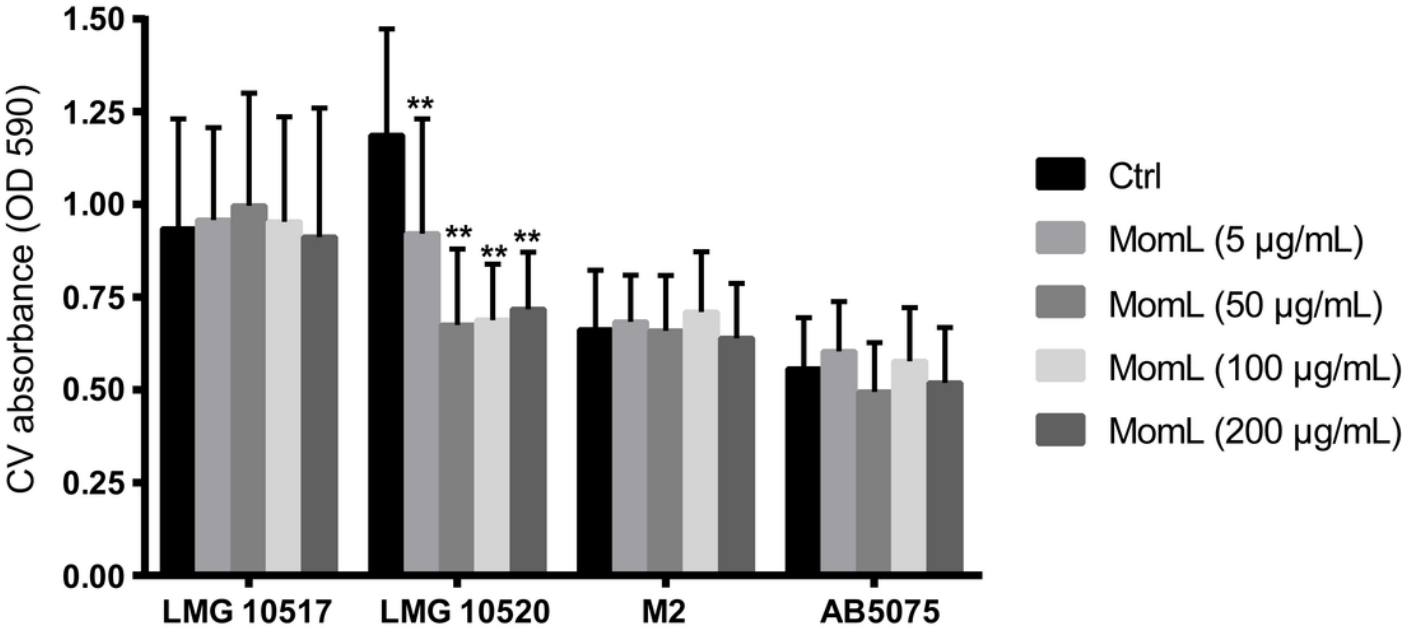
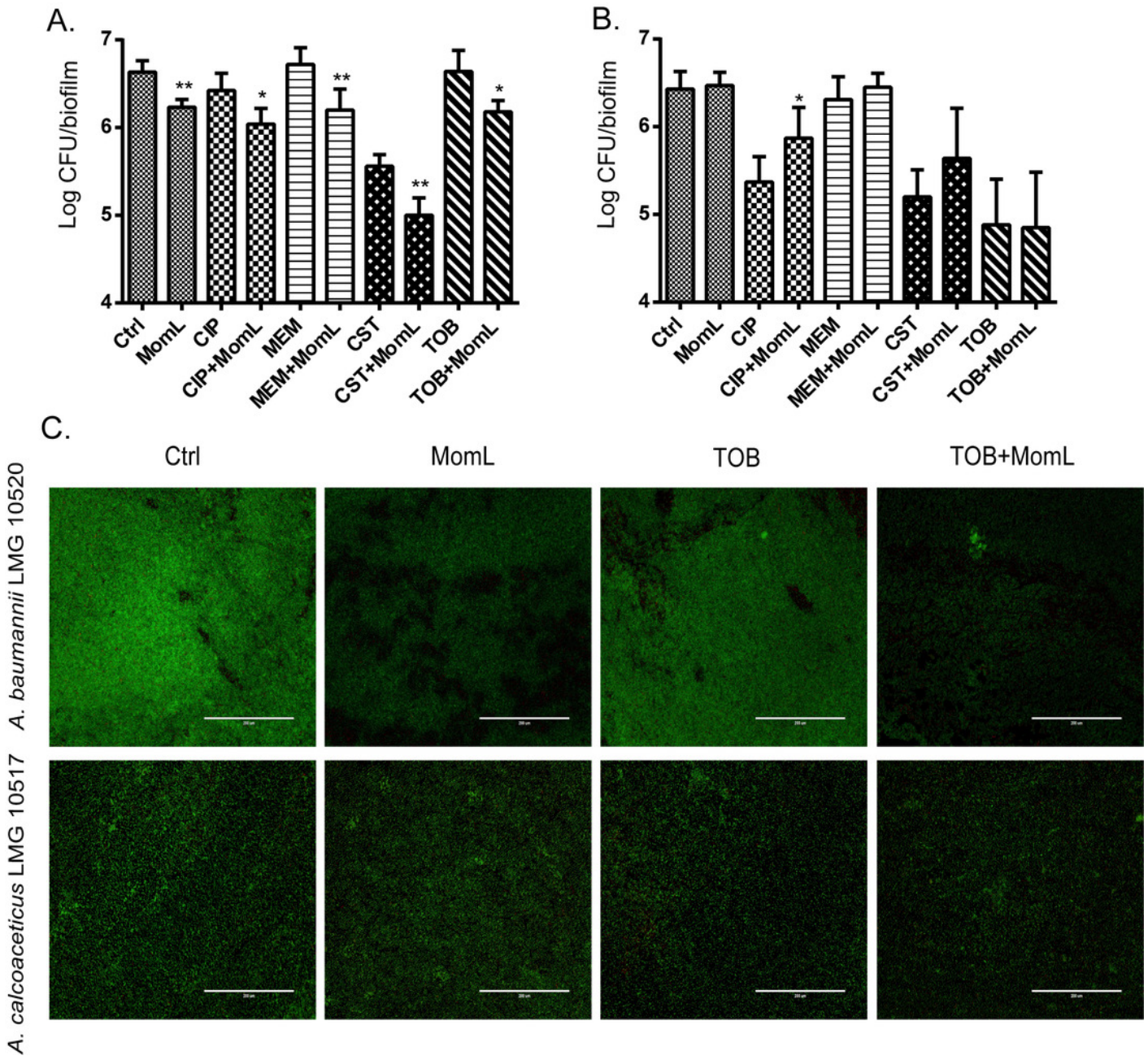


Figure 8

Effect of MomL on biofilm susceptibility of *A. baumannii* LMG 10520 and *A. calcoaceticus* LMG 10517.

Figure 8. Effect of MomL on biofilm susceptibility of *A. baumannii* LMG 10520 and *A. calcoaceticus* LMG 10517. (A). Plating results for biofilms of *A. baumannii* LMG 10520 exposed to CIP, MEM, CST, TOB alone or in combination with MomL (50 µg/mL) ; (B), Plating results for biofilms of *A. calcoaceticus* LMG 10517 exposed to CIP, MEM, CST, TOB alone or in combination with MomL (200 µg/mL). Data shown are average (n = 6), error bars represent standard deviation. T-tests were performed to compare control and MomL or antibiotic treatment alone and in combination with MomL (*, $0.005<P<0.05$; **, $P<0.005$). (C). Representative fluorescence images of *A. baumannii* LMG 10520 and *A. calcoaceticus* LMG 10517. Biofilms were treated with MomL alone or in combination with tobramycin and stained with Syto9 and propidium iodide. The scale bar represents 200 µm.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C.
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MomL
TOB
TOB+MomL
A. baumannii LMG 10520
A. calcoaceticus LMG 10517

Figure 9

Effect of MomL on biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in wound model.

Figure 9. Effect of MomL on biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in wound model. Data shown are average (n = 6), error bars represent standard deviation. T-tests were performed to compare control and MomL treatment, or TOB and TOB in combination with MomL.

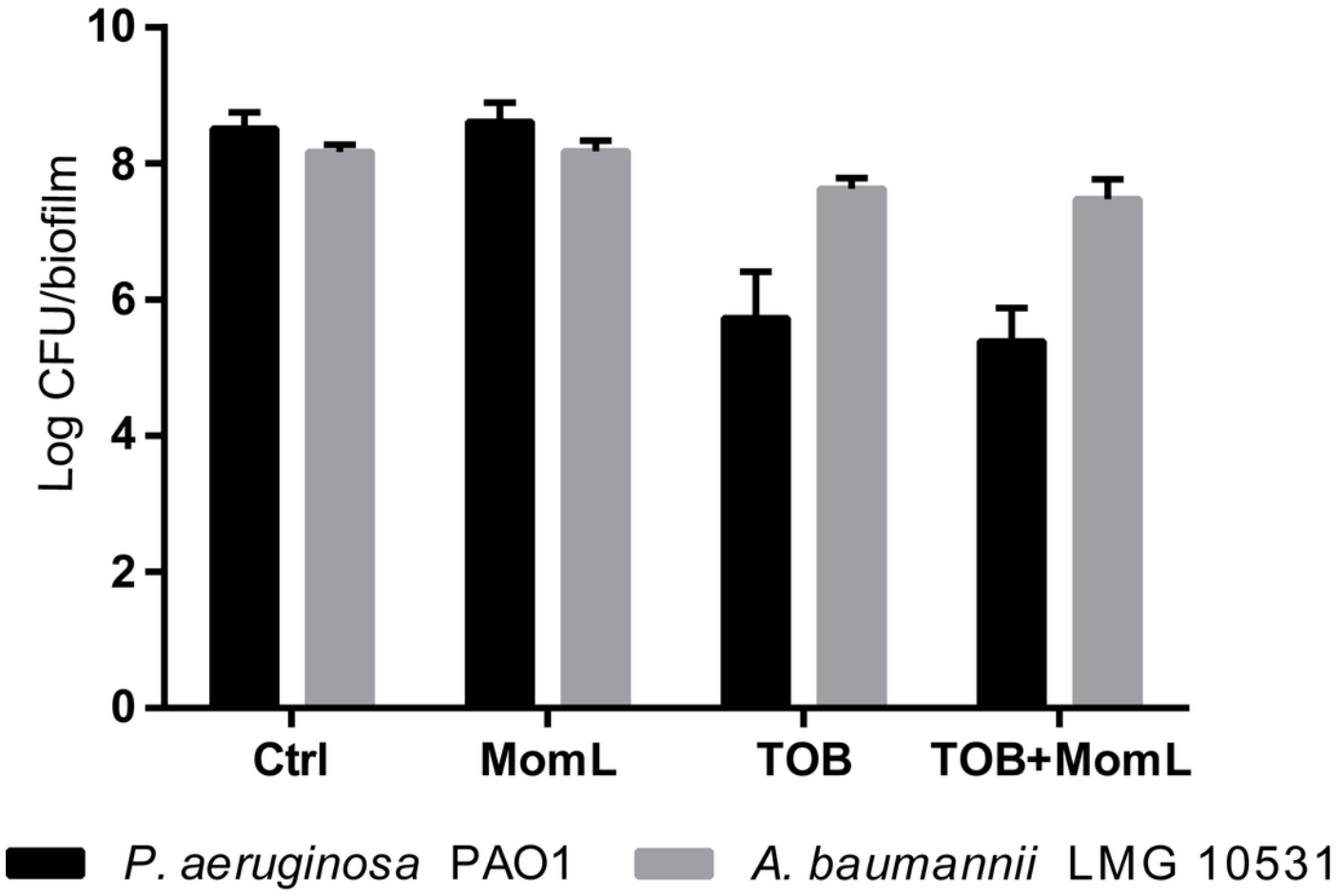

Figure 10

Effect of MomL on the virulence of *A. baumanii* strains in *C. elegans* model.

Figure 10. Effect of MomL on the virulence of *A. baumanii* strains in *C. elegans* model. Percent survival of *C. elegans* infected by *A. baumannii* LMG 10520 and LMG 10531. Data shown are average (n = 9), error bars represent standard deviation.