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Antibody catalyzed hydrolysis of a quorum sensing signal found in Gram-negative bacteria

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Abstract—*N*-(3-Oxo-acyl) homoserine lactones are used by Gram-negative bacteria to signal the establishment of specific population densities and coordinate population-wide gene expression. Herein we report the antibody-catalyzed hydrolysis of *N*-(3-oxo-acyl) homoserine lactone (AHL) using a reactive immunization strategy with a squaric monoester monoamide hapten. Kinetic analysis of the most efficient antibody revealed a modest k_{cat}, with AHL hydrolysis competitively inhibited by original squaric monoester monoamide hapten. These studies suggest that antibody catalysis could provide a new avenue for blocking quorum sensing in bacteria.

Bacteria can communicate with members of their own species and others to coordinate their behavior upon reaching a given cell density. This phenomenon, known as quorum sensing, ensures that a sufficient number of bacteria are present to organize a synchronized action that enables the species to function as a multicellular organism.[1–3] The mechanism is based on the production and sensing of one or more secreted signaling molecules. In *Pseudomonas aeruginosa*, an opportunistic pathogen causing a wide variety of nosocomial infections, the transcription of several virulence genes is controlled by regulatory mechanisms depending on quorum sensing. Two QS systems, *las* and *rhl*, were identified in these Gram-negative bacteria and each system is defined by a pair of proteins: a regulation protein and an autoinducer enzyme. Importantly, the autoinducer enzyme contributes to the synthesis of small molecules, a family of *N*-acyl homoserine lactones (AHLs) with a diversity of acylation patterns providing specificity to Gram-negative bacteria. AHLs can diffuse freely across the bacterial cell membranes and upon reaching a critical threshold concentration, they bind to their cognate receptor proteins, triggering the expression of target genes.[4–7]

A number of different approaches are being actively pursued for the development of potential therapeutics for the treatment or prevention of *Pseudomonas aeruginosa* infections.[8–14] Traditional routes include the development of new antibiotic drugs.[15–17] Nevertheless, the remarkable ability of bacteria to develop resistance to specific antibiotics has led researchers to generate new methods that do not cause cell death directly, but target extracellular signaling molecules in order to control the virulence of strains and reduce biofilm formation during *Pseudomonas aeruginosa* infections. The inactivation of these diffusible signal molecules, autoinducers, can in essence reduce quorum sensing.[18–20] Exciting non-conventional strategies employed to inhibit the activity of AHLs include the development of AHL inactivating enzymes that catalyze the degradation of AHLs such as lactonases[21,22] and acylases[23], and the sequestering of AHLs by antibodies.[24,25] Other recent reports describe the synthesis of AHL mimics that compete with natural AHLs for receptor binding on the bacteria, but that do not trigger QS related responses such as biofilm formation and virulence factor production.[26–31] The first use of antibodies to inhibit QS was recently reported by our group.[32] In our communication, we discovered several specific antibodies to AHLs that possess the ability

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to inhibit 3-oxo-C_{12}-AHL-based QS signaling. In order to further our immunotherapeutic program utilizing antibodies to inhibit AHL-mediated quorum sensing in *Pseudomonas aeruginosa*, we have investigated catalytic antibodies as a method to block the signaling cascade of AHLs. In this vein, we have tested a panel of antibodies that were previously obtained through immunization of mice with a reactive hapten based on a squaric monoester monoamide motif **1** (Fig. 1),[33] in this study, we uncovered antibodies that catalyzed the hydrolysis of paraoxon. In our current manuscript, we investigated hapten **1** for the discovery of catalytic antibodies to AHLs. We note that **1**, while not specifically designed for the generation of catalytic antibodies to AHLs, does present a reactive head group, (squaric monoester) which we believed would provide insight as to whether antibody catalysis to such structures was feasible. Herein, we detail our findings of examining antibodies procured to **1** for the hydrolysis of the autoinducer **2** (Scheme 1).

We commenced our studies by screening the panel of antibodies obtained against **1** for their ability to catalyze the hydrolysis of homoserine lactone 3-oxo-C_{12}-AHL **2** to its ring-opened hydrolysis product **3** (Scheme 1). The respective concentrations of these two compounds can be detected accurately by LC-MS (see materials and methods). From the 17 monoclonal antibodies that were examined, XYD-**11G2** was found to catalyze the hydrolysis of 3-oxo-C_{12}-AHL over the background reaction.

The reaction catalyzed by mAb XYD-**11G2** obeys classical Michaelis–Menten kinetics. Therefore, we were able to calculate K_m and k_{cat} constants, at low values of substrate, the velocity rises linearly (Fig. 2). We performed a non-linear regression analysis with GraFit software to calculate a K_m of 33.10 μM, with a maximum velocity of the reaction (V_{max}) of 6.84×10^{-3} μM min^{-1}. The catalytic efficiency, E, as defined by k_{cat}/K_m was determined to be 207 min^{-1} M^{-1}. Therefore, mAb XYD-**11G2** catalyzes the hydrolysis of 3-oxo-C_{12}-AHL with moderate catalytic efficiency.

In order to probe the catalytic nature of the reaction, the ability of hapten **1** to inhibit the mAb XYD-**11G2** mediated hydrolysis of 3-oxo-C_{12}-AHL was investigated. Instead of studying the effect of inhibitor concentration on enzyme activity under conditions of varying substrate concentrations, a titration under fixed substrate concentration was performed,[34,35] as initial studies showed tight binding of hapten **1** to mAb XYD-**11G2.** The dose–response plot constructed from these data furnishes an IC_{50} value for the corresponding inhibitor of 23.6 μM (Fig. 3). This confirms catalysis is occurring, at least in part, in the antibody combining site since the reaction was readily inhibited by hapten **1**.

Inhibition of 3-oxo-C_{12}-AHL-mediated QS in P. aeruginosa by mAb XYD-**11G2**. Next, we set out to examine the potential disruption of QS-based production of

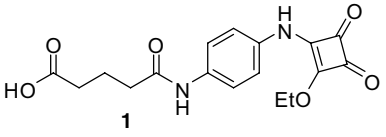

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Figure 1. Squaric monoester monoamide hapten **1**.

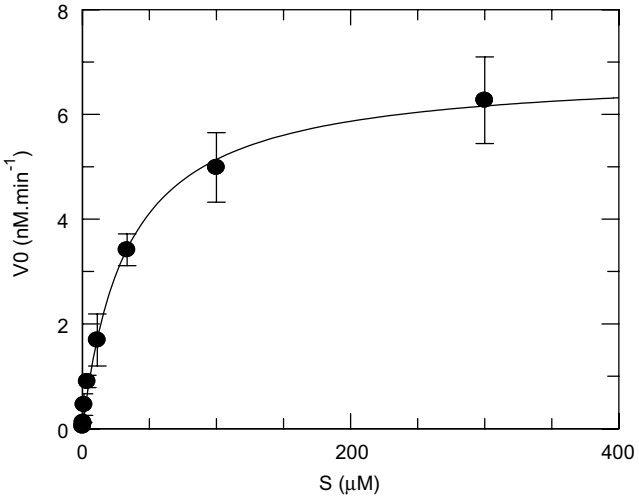


Figure 2. Hydrolysis of 3-oxo-C_{12}-AHL by mAb XYD-**11G2**.

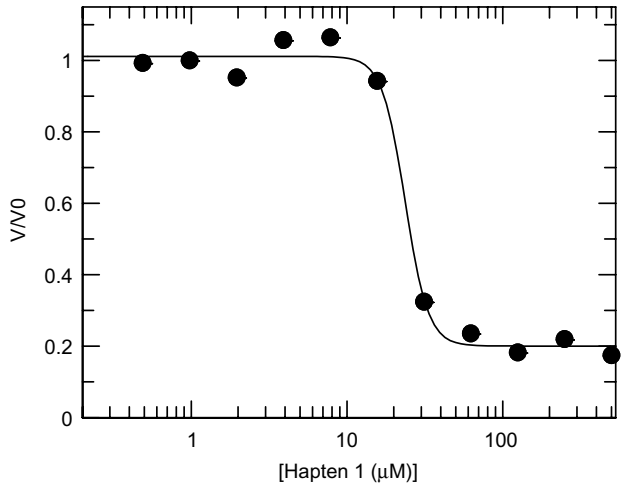
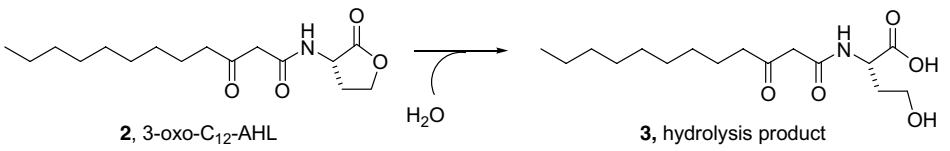


Figure 3. Inhibition of 3-oxo-C_{12}-AHL hydrolysis by hapten **1**.



2, 3-oxo-C_{12}-AHL **3,** hydrolysis product

Scheme 1. Hydrolysis of 3-oxo-C_{12}-AHL.

virulence factors in *P. aeruginosa*. The synthesis of pyocyanin by these bacteria has been shown to be regulated by 3-oxo-C_{12}-AHL, and in our previous studies, we have demonstrated that antibodies that bind 3-oxo-C_{12}-AHL can decrease the production of pyocyanin in *P. aeruginosa*.[32] Wild-type strain PAO1 was cultured overnight at 37 °C in LB medium, after which 1:2000 dilutions (200 μL, LB) were added to serial dilutions of mAb XYD-**11G2** (800 μL, LB) or control mAb 19G2 (an anti-stilbene binding antibody) that were dialyzed extensively against LB-medium in order to eliminate potential effects of the initial solvent (phosphate-buffered saline) on bacterial growth. Cultures were incubated for 12 h, after which concentrations of pyocyanin were determined following procedures described by Smith et al.[30] While the highest concentration of antibody had no significant effect on PAO1 growth, 10 μM and 2 μM concentrations of mAb XYD-**11G2** did show a small but significant effect on pyocyanin production (Fig. 4). All experiments were performed in triplicate.

In conclusion, our findings demonstrate that antibody catalysis of AHLs is possible. We note, although, our hapten was not a designed mimic of the transition state for AHL hydrolysis, antibody XYD-**11G2** can catalyze the hydrolysis of the *P. aeruginosa* quorum sensing molecule 3-oxo-C_{12}-AHL with moderate activity. We envision that the catalytic efficiency of antibodies for AHL hydrolysis can be improved markedly by utilizing new reactive immunization haptens that carry more structural similarity to homoserine lactones, and will be the subject of future reports.

K_m and k_{cat} determination. The reactions were carried out in phosphate buffered saline (PBS, pH 7.4) at 25 °C. Antibody-catalyzed reactions contained 1 μM mAb XYD-**11G2** with varying concentrations of substrate (from 150 μM to 0.2 μM). Assays were conducted by reverse phase LC–MS analysis (Agilent Zorbax column, 5 μm, 300SB-C8, 4.6 × 50 mm) with gradients of MeCN–H_2O–0.1% formic acid (from 0 to 1 min: 5% MeCN, from 1 min to 5.5 min: gradient of 5% MeCN to 98% MeCN, and from 5 to 8.5 min: 98% MeCN) allowing for quantification of 3-oxo-C_{12}-AHL degradation and the formation of the ring-opened hydrolysis product by detection of the following ions: 298 (3-oxo-C_{12}-AHL + H^+), 316 (hydrolyzed **3** + H^+), 320 (3-oxo-C_{12}-AHL + Na), and 338 (hydrolyzed **3** + Na). After a reaction time of 4 h, the reaction mixture was quenched into 25 μL of a 50% acetic acid solution in water. The ring-opened hydrolysis product concentrations were determined by calculating the area corresponding to the difference between the product formed with antibody and the background (without antibody and PBS instead). The complete hydrolysis of 3-oxo-C_{12}-AHL (100 μL of a 100 μM solution in methanol) in the presence of 10 μL of a solution of 1 N NaOH was also performed to correlate the amount of product formed in terms of area obtained by LC–MS and its concentration (μM). Kinetic parameters were obtained by fitting experimental data with non-linear regression analysis using GraFit 5.0 (Erathicus Software, Ltd).

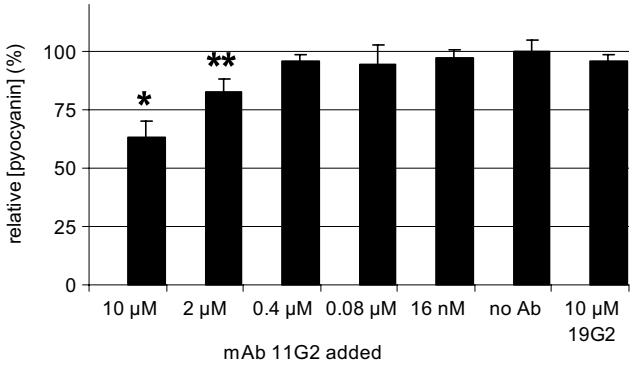


Figure 4. Inhibition of pyocyanin production in wild-type *Pseudomonas aeruginosa* (PAO1) after 12-h incubation in the presence of various concentrations of mAb XYD-**11G2** or control mAb EP2-19G2. *$p \leqslant 0.001$ versus no Ab, **$p \leqslant 0.01$ versus no Ab.

Inhibition constant determination. The assays were performed as above but with a fixed concentration of 3-oxo-C_{12}-AHL (100 μM), and concentrations of inhibitor **1** varying from 505 μM to 0.25 μM. Analyses were conducted as described above. After a reaction time of 4 h, the reaction mixture was quenched into 25 μL of a 50% acetic acid solution in water.

Acknowledgments

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