Fast, Continuous, and High-Throughput (Bio)Chemical Activity Assay for *N*-Acyl-L-Homoserine Lactone Quorum-Quenching Enzymes

Daniel Last, Georg H. E. Krüger, Mark Dörr, Uwe T. Bornscheuer

Institute of Biochemistry, Department of Biotechnology and Enzyme Catalysis, Greifswald University, Greifswald, Germany

ABSTRACT

Quorum sensing, the bacterial cell-cell communication by small molecules, controls important processes such as infection and biofilm formation. Therefore, it is a promising target with several therapeutic and technical applications besides its significant ecological relevance. Enzymes inactivating *N*-acyl-L-homoserine lactones, the most common class of communication molecules among Gram-negative proteobacteria, mainly belong to the groups of quorum-quenching lactonases or quorum-quenching acylases. However, identification, characterization, and optimization of these valuable biocatalysts are based on a very limited number of fundamentally different methods with their respective strengths and weaknesses. Here, a (bio)chemical activity assay is described, which perfectly complements the other methods in this field. It enables continuous and high-throughput activity measurements of purified and unpurified quorum-quenching enzymes within several minutes. For this, the reaction products released by quorum-quenching lactonases and quorum-quenching acylases are converted either by a secondary enzyme or by autohydrolysis to L-homoserine. In turn, L-homoserine is detected by the previously described calcein assay, which is sensitive to α -amino acids with free N and C termini. Besides its establishment, the method was applied to the characterization of three previously undescribed quorum-quenching lactonases and variants thereof and to the identification of quorum-quenching acylase-expressing *Escherichia coli* clones in an artificial library. Furthermore, this study indicates that porcine aminoacylase 1 is not active toward *N*-acyl-L-homoserine lactones as published previously but instead converts the autohydrolysis product *N*-acyl-L-homoserine.

IMPORTANCE

In this study, a novel method is presented for the identification, characterization, and optimization of quorum-quenching enzymes that are active toward *N*-acyl-L-homoserine lactones. These are the most common communication molecules among Gram-negative proteobacteria. The activity assay is a highly valuable complement to the available analytical tools in this field. It will facilitate studies on the environmental impact of quorum-quenching enzymes and contribute to the development of therapeutic and technical applications of this promising enzyme class.

The bacterial cell-cell communication by small molecules known as quorum sensing (QS) enables these single-celled organisms to coordinate their gene expression among a local population, depending on environmental conditions (1–4). Important processes such as infection and biofilm formation are controlled by QS. Hence, QS is a highly relevant target for naturally occurring and human-made interference termed quorum quenching (QQ), and it is mediated in particular by small-molecule inhibitors and QQ enzymes (5). From an ecological point of view, QQ has a significant influence on the interactions between bacteria (6, 7) and also bacteria and eukaryotes (8). Prospective therapeutic applications of QQ include the prevention and treatment of bacterial infections in humans, animals, and plants (5, 9–11). Technical applications are, for instance, the reduction of biofilm formation on medical devices and technical membranes and the reduction of food spoilage (12–14).

N-Acyl-L-homoserine lactones (AHL) are the most common type of communication molecules among Gram-negative proteobacteria (5). They consist of a strictly conserved L-homoserine lactone (HSL) moiety linked to a variable acyl side chain via an amide bond (all chemical structures relevant to this study and the overall scheme of the established activity assay are shown in Fig. 1). The acyl side chains are usually unbranched fatty acids, which mainly differ in length and substitution at the third carbon atom.

Nevertheless, AHL with more unusual acyl side chains like branched-chain or unsaturated fatty acids and *p*-coumaric acid are also present in nature (15, 16).

AHL-converting enzymes mainly belong to the groups of QQ lactonases and QQ acylases besides the rarely occurring oxidoreductases (17). QQ lactonases hydrolyze the HSL ring of AHL with low acyl side chain specificity, thereby forming *N*-acyl-L-homoserine (AH) (Fig. 1) (18). QQ acylases preferentially act on the amide bond of AHL with medium to long acyl side chains, thereby releasing HSL and the respective carboxylic acid (Fig. 1). However, for the identification, characterization, and optimization

Received 15 March 2016 Accepted 25 April 2016

Accepted manuscript posted online 6 May 2016

Citation Last D, Krüger GHE, Dörr M, Bornscheuer UT. 2016. Fast, continuous, and high-throughput (bio)chemical activity assay for *N*-acyl-L-homoserine lactone quorum-quenching enzymes. *Appl Environ Microbiol* 82:4145–4154. doi:10.1128/AEM.00830-16.

Editor: R. E. Parales, University of California—Davis

Address correspondence to Uwe T. Bornscheuer, uwe.bornscheuer@uni-greifswald.de.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.00830-16>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

FIG 1 Reaction scheme of the established assay for the measurement of QQ lactonase and QQ acylase activities. The autohydrolysis rates of AHL and HSL strongly depend on the reaction conditions, in particular an increase in the pH or the temperature strongly accelerates the autohydrolysis (28, 29). Due to the stabilization effect of the variable acyl side chain, AHL are much more stable than HSL (28, 29).

tion of these valuable biocatalysts, only a very limited number of fundamentally different methods are described in the literature with their respective strengths and weaknesses. Unfortunately, the combination of AHL structure and specificity profiles of the QQ enzymes prevents any obvious chromogenic or fluorogenic AHL derivatization; this approach would otherwise enable easy monitoring of the reaction progress (19). Hence, QQ activity measurements have to be performed indirectly, preferentially by the detection of a reaction product or, alternatively, the remaining AHL.

Biosensors are by far the most widely used AHL detection systems, work in the physiological concentration range (low nanomolar to low micromolar in planktonic cell cultures), and are cost-effective and suitable for high-throughput screening (20). In turn, these biological systems often require several hours to form a detectable signal, are usually restricted to certain types of AHL, and monitor the remaining AHL in an endpoint manner. For instance, one of the most frequently used biosensors, *Chromobacterium violaceum* strain CV026 (biosafety level 2 organism), produces the pigment violacein upon exposure to AHL with short acyl side chains during overnight incubation (21). At least for some biosensors, including CV026, technical tricks have to be applied in order to obtain semiquantitative results instead of simple yes or no answers concerning the absence or presence of QQ enzymes (22). In addition to this, more laborious but application-oriented methods are available, for instance, the measurement of the AHL-dependent formation of pathogenicity factors by *Pseudomonas aeruginosa* (23–25).

High-performance liquid chromatography (HPLC) and gas chromatography (GC) are much less frequently used than biosensor-based methods (15, 25, 26). Although providing quantitative data, both systems are working in the nonphysiological range (high micromolar to low millimolar) unless samples are concentrated or derivatized. Furthermore, they are restricted to endpoint measurements and low throughput.

Here, we present an alternative method for the fast and continuous high-throughput measurement of QQ enzyme activities, perfectly complementing the assays described above. Our method

was verified for the characterization of three previously undescribed QQ lactonases as well as for a QQ acylase screening in an artificial library. Moreover, in contrast to previous publications, our results indicate that porcine aminoacylase 1 (pAcy1) is not active toward AHL but rather toward the corresponding autohydrolysis product AH.

MATERIALS AND METHODS

Consumables. All chemicals and the commercial pAcy1 preparation (grade II, 500 to 1,500 U/mg protein) were purchased from Sigma-Aldrich (Steinheim, Germany). The BC assay quantitation kit obtained from Interchim (Montlucon, France) was used to determine the protein contents; bovine serum albumin served as the standard.

Syntheses and heat-accelerated autohydrolyses of N-acyl-L-homoserine lactones. Syntheses of N-hexanoyl-L-homoserine lactone (C₆-HSL) and N-octanoyl-L-homoserine lactone (C₈-HSL) were performed according to the method of Hodgkinson et al. (27). Stock solutions of C₆-HSL and C₈-HSL (400 mM) were prepared in anhydrous dimethyl sulfoxide (DMSO) containing 0.2% glacial acetic acid and stored in aliquots at –20°C.

N-Hexanoyl-L-homoserine (C₆-HS) was obtained by heat-accelerated autohydrolysis of C₆-HSL (95°C, 500 rpm, 20 min) in sodium phosphate buffer (SPB) (50 mM, pH 7.5); C₆-HSL and related compounds were previously described to be sensitive to higher temperatures (28, 29). The conversion was monitored with a LaChrom HPLC (Merck, Darmstadt, Germany) at a wavelength of 205 nm. It was equipped with a LiChrospher 100 RP-18e column (5 µm, 250 mm by 4 mm; Merck). The solvent (74.75% MilliQ water, 25% acetonitrile, 0.25% glacial acetic acid) was used in an isocratic mode with a flow rate of 1 ml/min. Elution times were 3.4 min (C₆-HS) and 7.0 min (C₆-HSL).

Expression vectors. The expression plasmids for pAcy1 and for the QQ acylase QqaR originated from previous studies (23, 30). The synthetic and codon-optimized gene of hAcy1 (GenBank accession no. KU922759) was provided already cloned into pET28b(+) by GenScript (Piscataway, NJ, USA). The synthetic and codon-optimized genes *aiiM* (KU922758), *qqlB* (KU922760), *qqlG* (KU922761), and *qqlM* (KU922762) were ordered from Life Technologies (Regensburg, Germany) and cloned into pET28b(+). The complete coding DNA sequences were deposited in GenBank as indicated. All constructs were confirmed by sequencing (Eu-

TABLE 1 Enzymes relevant for this study

Enzyme	Activity	Natural origin	Source	Reference or source
pAcy1	Aminoacylase	<i>Sus scrofa</i>	Recombinant from <i>E. coli</i> or commercial	30
hAcy1	Aminoacylase	<i>Homo sapiens</i>	Recombinant from <i>E. coli</i>	This study
AiiM	QQ lactonase	<i>Microbacterium testaceum</i>	Recombinant from <i>E. coli</i>	38
AidH	QQ lactonase	<i>Ochrobactrum</i> sp. T63	Not practically used	40
QqlB	QQ lactonase	<i>Paraburkholderia glathei</i>	Recombinant from <i>E. coli</i>	This study
QqlG	QQ lactonase	<i>Geminicoccus roseus</i>	Recombinant from <i>E. coli</i>	This study
QqlM	QQ lactonase	<i>Mesorhizobium ciceri</i>	Recombinant from <i>E. coli</i>	This study
QqaR	QQ acylase	<i>Deinococcus radiodurans</i> R1	Recombinant from <i>E. coli</i>	23

rofin, Ebersberg, Germany). An overview of all of the enzymes relevant for this study is provided in Table 1.

Transformation, protein expression, cell lysis, and protein purification. Chemocompetent *E. coli* BL21(DE3) cells were transformed by the heat shock method (31). Cells were grown in lysogenic broth (LB) medium (10 g/liter tryptone, 10 g/liter sodium chloride, 5 g/liter yeast extract; supplemented with 50 µg/ml kanamycin or 100 µg/ml ampicillin after autoclaving) at 37°C and 180 rpm until the optical density at 600 nm (OD_{600}) reached 0.6 ± 0.1 . Then protein expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG); cultivation was continued at 20°C and 180 rpm for 18 h. The aminoacylase expression cultures were additionally supplemented with chloramphenicol (25 µg/ml) and L-arabinose (2 mg/ml) for chaperone coexpression (30). Cell cultures were centrifuged (15 min, $4,000 \times g$, 4°C), washed with washing/lysis buffer (pAcy1, hAcy1, and AiiM: 100 mM Tris-HCl, 150 mM NaCl [pH 8.0]; all other enzymes: SPB, 250 mM NaCl [pH 7.25]), centrifuged again, and stored as cell pellets at −20°C. These were resuspended in washing/lysis buffer followed by two French press passages (each $1,500 \times psi$) and cell debris removal (45 min, $10,000 \times g$, 4°C). Proteins were purified with gravity flow columns of Strep-Tactin Sepharose (pAcy1, hAcy1, and AiiM; IBA, Göttingen, Germany) or Co-IDA resin (all other enzymes; Carl-Roth, Karlsruhe, Germany). Elution was mediated by the respective washing/lysis buffer additionally containing 2.5 mM desthiobiotin (pAcy1, hAcy1, and AiiM) or 300 mM imidazole (all other enzymes). Imidazole was removed from His tag elution fractions by dialysis overnight (12,000 molecular weight cutoff [MWCO]; >1,000-fold dilution) against SPB.

Purification of pAcy1 from the crude commercial preparation was achieved by size exclusion chromatography with a Superdex 75 column (150 ml) on an ÄKTApurifier (GE Healthcare Europe, Freiburg, Germany), which was run with SPB as the buffer solution.

The crude *E. coli* cell lysates as used for the CV026 assay, for instance, were prepared from the respective cultivations as follows. The number of harvested cells per aliquot was normalized by the formula 5 absorption units per OD_{600} in milliliters. Cells were centrifuged (15 min, $4,000 \times g$, 4°C), washed with SPB, centrifuged again, and stored at −20°C. The cell pellets were resuspended in 500 µl SPB and sonicated on ice (Bandelin Sonopuls HD 2070; 1 min, 50% pulse, 50% power), followed by cell debris removal by centrifugation (5 min, $17,000 \times g$, 4°C).

Biocatalyses and GC-MS analyses. Biocatalysis reactions (0.75 ml in SPB) contained 2 mM C₆-HSL or C₆-HS as the substrate and 100 µg/ml of the specified aminoacylase. AiiM was added in purified form (10 µg/ml) or as a crude cell lysate (15 µl) if applicable. Reactions were carried out at 37°C and 750 rpm for the indicated periods of time and stopped by the addition of 50 µl concentrated hydrochloric acid. Each sample was extracted two times with 0.3 ml hexane containing 5 mM external standard methyl benzoate. The combined organic phases were dried with anhydrous sodium sulfate. For the derivatization of the reaction product hexanoic acid, 10 µl N,O-bis(trimethylsilyl)trifluoroacetamide was added to 90 µl of the organic phase followed by incubation at 60°C and 500 rpm for 20 min. Samples were analyzed by gas chromatography-mass spectrometry (GC-MS) (Shimadzu GC2010 coupled to a QP2010S MS device)

equipped with a BPX-5 column (25 m, 0.25 mm), and the following program parameters: injector, 250°C; pressure, 38.3 kPa; column flow rate, 0.93 ml/min; MS ion source temperature, 200°C; MS interface temperature, 220°C; and column oven temperature profile, 0 min at 60°C, 5 min at 60°C, 15 min at 220°C, and at 20 min at 220°C.

Calcein assay. The calcein assay was performed according to the method of Dean et al. (32). Briefly, 5- to 20-µl samples were filled up to 200 µl with preincubated substrate/sensor solution [SPB, 1 to 2 mM substrate, 1 µM calcein, 5 µM copper(II) chloride]. For the fluorescence measurements (excitation, 450 nm; emission, 530 nm; incubation temperature, 37°C), an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland) was used.

Biosensor assay with *Chromobacterium violaceum* strain 026. In order to gain more information than a simple yes or no answer concerning the absence or presence of QQ enzyme activity, the CV026 assay was performed in a modified and technically easier way than described elsewhere (21, 22). For this, samples were mixed in equal amounts with serial C₆-HSL dilutions (0 to 800 µM in LB medium) in the wells of a microtiter plate. Following the biocatalysis reactions (4 h, 750 rpm, 37°C), 20 µl of each sample was transferred to a new microtiter plate filled with 80 µl of a CV026 culture per well (1:50 dilution of an overnight culture in LB medium; stored at 4°C until use). After overnight incubation at 750 rpm and 30°C, the violacein color formation was determined.

Screening for the QqaR expression strain in an artificial library. The challenge of this experiment was to identify the *E. coli* expression strain of the QQ acylase QqaR in an artificial library with an excess of negative-control clones (visualized in Fig. S1 in the supplemental material). The *E. coli* expression strain of the QQ lactonase AiiM served as a negative control as it possess the same plasmid backbone and therefore the same antibiotic resistance and burden after the induction by IPTG as the QqaR expression strain. In detail, separated 4-h-old cultures of the uninduced QqaR and AiiM expression strains were separately diluted with LB medium to an OD_{600} of 0.5, mixed in a volumetric ratio of 3:93 (aiming for a high excess of the negative control over the positive control), and plated on agar plates. On control agar plates prepared from nonmixed cultures, the AiiM expression strain showed about 4 times higher CFU than the QqaR expression strain. Hence, the overall probability to find the QqaR expression strain within the library was estimated to be about 1:125. Clones were transferred with a 96-pin head microbial colony picker QPix 420 (Molecular Devices, Sunnyvale, CA, USA) from agar plates into the wells of microtiter plates (MTPs). One positive (QqaR expression strain) and one negative control (AiiM expression strain) were positioned manually in the middle of each MTP. The resulting master MTPs (150 µl LB medium per well) were incubated for 14 h at 37°C and 700 rpm, supplemented with glycerol (90%, 50 µl per well) and stored at −80°C. Preexpression MTPs (200 µl LB medium per well) were inoculated and incubated for 5 h at 37°C and 700 rpm. The subsequently inoculated expression MTPs filled with 200 µl of Zyp-5052 autoinduction medium per well (33) were loaded onto an automated robotic platform (34), which carried out all of the following screening steps. The expression MTPs were incubated for 6 h at 37°C and 700 rpm, followed by another 16 h at 20°C and 700 rpm. Cells were centrifuged (20 min, $5,000 \times g$, 4°C), washed with

FIG 2 Investigations on the conversion of C₆-HS and C₆-HSL (each 2 mM) by aminoacylases (each 100 μg/ml). (A) Quantification of hexanoic acid formation by GC-MS analysis. The three measurements involving C₆-HSL are hidden by the curve of hAcy1 E147A/C₆-HS as no hexanoic acid formation was found for all of them. (B) Continuous monitoring of HS formation by the calcein assay normalized to the respective starting fluorescence values. The error bars indicate the standard deviations ($n = 3$).

SPB, and centrifuged for a second time. Cell lysis was mediated by resuspension in a 100-fold dilution of BugBuster 10× protein extraction reagent (Merck, Darmstadt, Germany) in SPB and incubation for 2 h at 30°C and 700 rpm, followed by a final centrifugation step. The calcein assay was performed as described above with C₈-HSL (1 mM) as the substrate but using a Varioskan microplate reader (Thermo Scientific, Waltham, MA, USA). It was assumed that the signal formation by clones expressing QqaR would take too long for individual and continuous activity measurements of one MTP after another. Therefore, all six MTPs were screened in parallel with a single point fluorescence measurement after 4 h of biocatalysis at 37°C and 700 rpm.

Nucleotide sequence accession numbers. The complete coding DNA sequences for the synthetic and codon-optimized genes were deposited in GenBank under accession no. [KU922758](#) to [KU922762](#).

RESULTS

Overall concept of the activity assay for quorum-quenching enzymes. The method presented here is based on L-homoserine (HS), which is present in the strictly conserved HSL moiety of each AHL (Fig. 1). The respective biocatalysis products of QQ lactonases and QQ acylases are converted either by a secondary enzyme or by autohydrolysis to HS. This compound is finally detected with the previously described calcein assay, which is sensitive to α-amino acids with free N and C termini (32).

At first, the sensitivity of the calcein assay toward HS was determined by a standard curve mimicking a biocatalysis reaction with *N*-hexanoyl-L-homoserine lactone (C₆-HSL) as the model AHL. Thus, the concentrations of the final cascade reaction products HS and hexanoic acid were increased, while the concentration of C₆-HSL was decreased proportionally. Under the conditions tested, the calcein assay was sensitive in the micromolar range and

linear up to 2 mM HS (see Fig. S2 in the supplemental material). At this point, it should be mentioned that exact quantifications based on relative fluorescence units should be handled with care due to the strong impact of the sample composition and reaction conditions and the inner-filter effects and photo bleaching. Here, the calcein assay was established and used as a semiquantitative method only. For this reason, the data obtained were not converted to concentrations or activity units.

Cascade reaction to monitor quorum-quenching lactonase activity. The conversion of AHL to AH by QQ lactonases does not lead to signal formation in the calcein assay due to the blocked N terminus of HS (Fig. 1). Therefore, a cascade reaction was conceived, additionally involving an aminoacylase that cleaves the amide bond of AH but not that of AHL. Thereby, the detectable HS and the respective carboxylic acid are released.

For this purpose, the activities of recombinant porcine aminoacylase 1 (pAcy1) and recombinant human aminoacylase 1 (hAcy1) toward the model AH C₆-HS were tested. This AH was anticipated to be at least an acceptable substrate for both aminoacylases with regard to its amino acid and acyl moieties (35, 36) and was obtained by heat-accelerated autohydrolysis of C₆-HSL (see Fig. S3 in the supplemental material). On the other hand, no detectable conversion of C₆-HSL was expected due to its unusual amino acid structure. The inactive hAcy1 variant E147A served as a negative control (37).

Indeed, pAcy1 and hAcy1 selectively converted C₆-HS but not C₆-HSL, which was determined by GC-MS analysis of hexanoic acid formation (Fig. 2A). The results for the calcein assay were similar to those for the GC-MS analysis (Fig. 2B).

FIG 3 Cascade reactions involving AiiM (as purified enzyme or as crude cell lysate) combined with the most active aminoacylase pAcy1 or the negative-control hAcy1 E147A (each 100 μg/ml) with C₆-HSL (2 mM) as the substrate. (A) Quantification of hexanoic acid formation by GC-MS analysis. (B) Continuous monitoring of HS formation by the calcein assay normalized to the respective starting fluorescence values. The error bars indicate the standard deviations ($n = 3$).

In order to perform the cascade reaction with the physiologically relevant substrate C₆-HSL, the most active aminoacylase, pAcy1, was combined with the model QQ lactonase AiiM (9, 38). Both forms of AiiM, purified and crude cell lysate, led to the expected increase in calcein fluorescence within a few minutes (Fig. 3). Here, the secondary enzyme pAcy1 was the limiting factor of the two-step cascade reaction as 1:20 dilutions of purified AiiM gave similar results in the calcein assay (data not shown). All of these results show that the new method enables fast activity measurements of purified and unpurified QQ lactonases when combined with a suitable aminoacylase.

Finally, a commercial and inexpensive pAcy1 preparation derived from porcine kidney was tested as a potential substitute for the recombinant and purified one. However, also without the addition of the substrate, a strong increase in fluorescence was observed. This background activity was removed completely by size exclusion chromatography (see Fig. S4 to S6 in the supplemental material) but only partially by dialysis (12,000 MWCO; data not shown). The chromatographically purified pAcy1 was used for the subsequent experiment.

Application to the characterization of three previously undescribed quorum-quenching lactonases. AidH is one of the three known QQ lactonases with an α/β -hydrolase fold (17, 39, 40). Based on its amino acid sequence (GenBank accession no. [GQ849010](#)) and active-site residues (39), three homologous open reading frames were identified by a NCBI BLAST search. These belong to the two soil- and plant-associated bacteria, *Paraburkholderia glathei* (QqlB) ([KDR41687.1](#); 49% amino acid identity; annotated as epoxide hydrolase) and *Mesorhizobium ciceri* (QqlM) ([WP_013530214.1](#); 68% amino acid identity; annotated

as α/β -hydrolase), and to the salt water-associated bacterium *Geminicoccus roseus* (QqlG) ([WP_027134646.1](#); 66% amino acid identity; annotated as α/β -hydrolase) (41–45). Recombinantly expressed QqlB, QqlG, and QqlM showed QQ activity in the modified CV026 assay with crude cell lysates (see Fig. S7 and S8 in the supplemental material).

All 13 active-site residues described for AidH were also found in QqlG and QqlM. QqlB was selected as the third enzyme as it differed in 7 of these 13 positions. From biochemical and structural studies on AidH, Gao et al. (39) concluded that Y160 plays a key role in biocatalysis by hydrogen bond formation via its hydroxyl group. However, QqlB possesses a phenylalanine at the homologous position and therefore no such hydroxyl group. In order to investigate the importance of the position and especially of the hydroxyl group, all variants of QqlB, QqlG, and QqlM with tyrosine and phenylalanine at the homologous positions (QqlB, QqlB F160Y, QqlG, QqlG Y160F, QqlM, QqlM Y160F) were generated, recombinantly expressed (see Fig. S7 in the supplemental material), purified via His tag (see Fig. S9), and dialyzed. In order to unequivocally measure the activities of the QQ lactonases, the secondary enzyme of this cascade reaction, pAcy1, was necessarily provided in excess; dilutions of the QQ lactonases showed lower slopes but the same tendencies (data not shown). The calcein assay revealed no differences between the respective phenylalanine and the tyrosine variants of QqlG and QqlM (Fig. 4). QqlB F160Y was even less active than the natural phenylalanine variant. It has to be mentioned that both QqlB variants were less well expressed (see Fig. S7) and less stable than those of QqlG and QqlM. QqlB and QqlB F160Y partially formed precipitates during dialysis, which were removed by centrifugation.

FIG 4 Calcein assay for the comparison of phenyl and tyrosine variants of QqLB, QqLG, and QqLM at the homologous position of AidH Y160 (each 30 $\mu\text{g/ml}$) normalized to the respective starting fluorescence values. C_6 -HSL (2 mM) served as the substrate. Standard deviations (average: 3%; individual maximum: 8%) are not indicated for a better clarity.

Cascade reaction to monitor quorum-quenching acylase activity. In order to monitor QQ acylase activity with the calcein assay, the biocatalysis product HSL has to be converted to HS in a second step (Fig. 1). For this purpose, one might simply exploit the autohydrolysis of the HSL ring, which increases disproportionally with decreasing lengths of the acyl side chains of AHL (29). Thus, HSL should be much more susceptible to autohydrolysis than AHL as it lacks such a stabilization effect. In par-

ticular, AHL with medium to long acyl side chains would be suitable as these are also strongly preferred by QQ acylases (23, 25, 26). *N*-Octanoyl *L*-homoserine lactone (C_8 -HSL) served as the model AHL, and QqaR was used as the model QQ acylase (23).

Indeed, the calcein assay showed an increase in fluorescence, depending on the concentration of QqaR (Fig. 5). It is also obvious that imidazole, derived from the elution buffer of the His tag pu-

FIG 5 Continuous calcein assay monitoring the activities of the indicated concentrations of purified and dialyzed QQ acylase QqaR normalized to the respective starting fluorescence values. Nondialyzed QqaR directly obtained from His tag purification was also tested, showing no increase in fluorescence despite a high enzyme concentration. This indicates a strong quenching (so-called inner filter) effect by imidazole. C_8 -HSL (1 mM) served as the substrate. The error bars indicate the standard deviations ($n = 3$).

FIG 6 Identification of three *E. coli* clones with QQ acylase activity in an artificial library presented as an assembly of all clones of the six MTPs and normalized to the respective negative control. C₈-HSL (1 mM) was used as the substrate.

rification (300 mM; final assay concentration, ≤ 30 mM), strongly quenches the fluorescence of calcein.

Application to the screening for quorum-quenching acylases in an artificial library. The method described might be used, for instance, to identify QQ acylases in a metagenome-derived *E. coli* library, which would be a classical application for biosensors (24). In order to obtain an artificial metagenome library, the *E. coli* expression strains for Qqar and AiiM were mixed in a ratio of about 1:125 CFU (the entire screening procedure is visualized in Fig. S1 in the supplemental material). In total, 576 clones, including 6 clones of the positive (Qqar) and 6 clones of the negative (AiiM) controls were screened in the MTP format on a fully automated robotic platform (34). Indeed, 3 active clones were found with the calcein assay (Fig. 6), which is a statistically plausible number with regard to the Gaussian distribution (theoretically, four to five hits were expected). Their sequencing revealed that all three belonged to the Qqar expression strain. Hence, the proposed method was working in a reliable manner and is a useful alternative to biosensor-based screening.

DISCUSSION

In the present study, a (bio)chemical activity assay for the identification, characterization, and optimization of QQ lactonases and QQ acylases, which is based on the strictly conserved HSL moiety of AHL, was established. The respective biocatalysis products are converted either by a secondary enzyme or by autohydrolysis to HS. This compound is detected by the previously described calcein assay, which is sensitive only to α -amino acids with free N and C termini (32).

As QQ lactonases convert AHL to AH, a secondary enzyme that specifically cleaves the amide bond of AH and releases HS is required. For this purpose, hAcy1 and pAcy1 were tested, and the latter enzyme showed the higher activity (Fig. 2A and B). pAcy1 is especially suitable as a secondary enzyme as it can be stored at 4°C for several days to few weeks (data not shown) or in solution with

50% glycerol at -20°C for several months (according to commercial suppliers) with acceptable or even no loss in activity. Unfortunately, commercial preparations of recombinantly expressed and purified pAcy1 are too expensive for the intended purpose. In order to obtain pAcy1 in large amounts and without possessing the recombinant expression strain, an inexpensive commercial preparation derived from porcine kidney was tested. However, this preparation led to a fast increase in the calcein assay even without the addition of a substrate (see Fig. S6 in the supplemental material). Hence, commercial pAcy1 preparations should be tested properly as amino acids, metal chelators, and fluorescence-quenching compounds like imidazole (Fig. 5) might interfere with the calcein assay. Here, pAcy1 was purified to an appropriate degree in only one step by size exclusion chromatography (see Fig. S4 to S6). Probably other unspecific protein purification methods like hydrophobic interaction or ion-exchange chromatography would be suitable as well. If the specific activity of pAcy1 is too low toward a certain type of AH, most likely those with very bulky or branched acyl side chains (35), and this cannot be overcome by higher enzyme concentrations, one should consider the use of pAcy1 variants (35) or even other aminoacylases instead. Moreover, cells that should be examined for QQ enzymes have to be washed with a buffer solution prior to cell disruption. This step removes amino acids and other interfering compounds that might be present in the growth medium.

In order to obtain the GC-MS data shown in Fig. 2A and 3A, it took about 4 days of lab work to perform the biocatalysis reactions, to extract and derivatize the samples, and to analyze them. In contrast to this, the calcein assay measurements visualized in Fig. 2B and 3B were performed in less than 2 h. Hence, our method enables much faster and less expensive activity measurements than the GC-MS method. Biosensor-based methods require several additional hours of signal formation as well as forward planning as a suitable preculture is needed. The continuous and high-throughput character of the method presented also

facilitates the ability to find a suitable combination of enzyme concentration and biocatalysis time; several enzyme dilutions can be tested in parallel, and the measurements can be stopped once the signal formation is sufficient. In contrast to this, the GC-MS method is more or less a trial-and-error approach in this regard due to the measurement of one endpoint sample after another.

Another aspect that has to be discussed here in detail is that pAcy1 was previously described to be active toward AHL (46). For this, Xu et al. (46) monitored the formation of an amine by *o*-phthaldialdehyde (OPA) derivatization. However, the highest activities toward C₄-HSL (the least stable AHL) were found at high pH values (optimum, 10) and at high temperatures (optimum, 76°C). Both conditions are known to accelerate the autohydrolysis of AHL to AH (28, 29). An increase in pH from 7.85 to 8.5 reduces the half-life of the much more stable C₈-HSL from 7 h to only 30 min (28). As shown in this study, 10 min of incubation at 95°C led to almost full autohydrolysis of C₆-HSL (see Fig. S3 in the supplemental material). Xu et al. (46) performed their biocatalysis reactions over 2 h. In addition to this, pAcy1 was found here to be highly active toward C₆-HS, while no conversion was found for C₆-HSL (Fig. 2). Moreover, the supposed high temperature optimum of 76°C contradicts the relation of thermostability and activity of pAcy1 (47). While 80% of pAcy1 is inactivated after only 2.5 min at 70°C, it retains its activity almost completely after 10 min at 50°C. As the activities of pAcy1 are quite similar at both temperatures according to Wang et al. (47), one should expect that the QQ performance of pAcy1 over the 2 h of biocatalysis should have been much better at 50°C than at 70°C. Taken together, these results indicate that Xu et al. (46) have measured the activity of pAcy1 toward AH but not toward AHL as claimed. Not only in the course of this study but also in the literature, doubts concerning the QQ activity of pAcy1 have been mentioned (5) or no QQ activity was found at all (48). Perhaps the antibiofilm activity of the pAcy1 preparation, which was linked by Xu et al. (46) to its supposed QQ activity, was (i) due to the activity of pAcy1 toward another biofilm component, (ii) due to another enzyme like paraoxonase (49, 50), or (iii) due to low-molecular-weight compounds. The very low purity of such crude commercial pAcy1 preparations is exemplarily shown in Fig. S4. Nevertheless, pAcy1 did not show any measurable QQ activity under the tested conditions and thus can be used as a secondary enzyme for the method presented.

Following its establishment, our method was used to study the three previously undescribed QQ lactonases QqlB, QqlG, and QqlM originating from *Paraburkholderia glathei* (QqlB), *Geminicoccus roseus* (QqlG), and *Mesorhizobium ciceri* (QqlM). The open reading frames were identified on the basis of their homologies to the amino acid sequence and active-site residues of AidH, one of the three known QQ lactonases with an α/β -hydrolase fold (39, 40). The QQ activities of the recombinantly expressed proteins were confirmed by a modified CV026 assay (see Fig. S8 in the supplemental material).

Based on AidH crystal structures, Gao et al. (39) highlighted that the hydroxyl group of the amino acid Y160 is crucial for biocatalysis. Interestingly, QqlB possesses phenylalanine at the homologous position and therefore no such hydroxyl group. For this reason, the activities of the respective phenylalanine and tyrosine variants of QqlB, QqlG, and QqlM were compared by means of the calcein assay (Fig. 4). Indeed, the amino acid sub-

stitutions did not affect the activities of QqlG and QqlM, whereas the mutation F160Y further decreased the activity of QqlB. These results at least reveal that the importance of the hydroxyl group of Y160 in AidH cannot be generalized for homologous QQ lactonases. With biosensors, it would have been difficult or even impossible to obtain activity measurements with such a high resolution as shown here with the modified calcein assay.

In addition to QQ lactonases, the calcein assay was used for studies on a QQ acylase, which belongs to the other important class of AHL QQ enzymes. Here, the fast autohydrolysis of the biocatalysis product HSL to HS was exploited as the second step of the cascade reaction. This approach offers maximum flexibility concerning the acyl side chain of AHL, which is of particular interest for AHL with unusual acyl side chains, thereby circumventing the laborious development of specific biosensor strains (16, 18). The activity measurements took only several minutes and in the case of the screening a few hours (Fig. 5 and 6). Nevertheless, the autohydrolysis can be the rate-limiting step, depending on the concentration and the activity of the QQ acylase. For this reason, one might search for a lactonase as a secondary enzyme that hydrolyzes HSL, but not AHL (such HSL-hydrolyzing enzymes were not investigated here). Alternatively, one might perform the calcein assay in an endpoint manner with temporary incubation at a high temperature. This should strongly accelerate the autohydrolysis of HSL as shown here for the much more stable C₆-HSL (see Fig. S3 in the supplemental material).

In general, our method is also suitable for screening applications with crude cell lysate as shown by the reliable identification of clones expressing the QQ acylase QqaR in an artificial library (Fig. 6). It took only 4 h to distinguish between active and inactive clones. While the calcein assay enables the direct monitoring of the biocatalysis reactions, several additional hours of signal formation would have been required with biosensors subsequent to the biocatalysis reactions. On the other hand, the calcein assay requires much higher AHL concentrations than biosensor methods, which is of particular relevance for large-scale screening applications. Due to the high commercial price, one should consider that AHL synthesis is quite easy, robust, and inexpensive (27).

All in all, our method enables fast, continuous, and high-throughput activity measurements of QQ lactonases and QQ acylases. Therefore, it perfectly complements the few and fundamentally different methods described so far in this field.

ACKNOWLEDGMENTS

We are grateful to Putri Dwi Utari, Ronald van Merkerk, and Wim J. Quax (all Department of Pharmaceutical Biology, University of Groningen, The Netherlands) for the research stay of Daniel Last in their lab and for providing us with the QqaR expression plasmid. Additionally, we thank Daniela Zühlke and Katharina Riedel (both Institute of Microbiology, University of Greifswald, Germany) for providing us with the CV026 biosensor strain. We also appreciate the kind assistance of Ayad W. H. Dawood during the purification of pAcy1 by size exclusion chromatography.

We thank the Studienstiftung des Deutschen Volkes (Bonn, Germany) for a stipend to Daniel Last.

Uwe Bornscheuer is a cofounder of the company Enzymicals AG, Greifswald.

FUNDING INFORMATION

This work, including the efforts of Daniel Last, was funded by Studienstiftung des Deutschen Volkes (Studienstiftung).

The funding institution had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

- West SA, Winzer K, Gardner A, Diggle SP. 2012. Quorum sensing and the confusion about diffusion. *Trends Microbiol* 20:586–594. <http://dx.doi.org/10.1016/j.tim.2012.09.004>.
- Redfield RJ. 2002. Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol* 10:365–370. [http://dx.doi.org/10.1016/S0966-842X\(02\)02400-9](http://dx.doi.org/10.1016/S0966-842X(02)02400-9).
- Hense BA, Kuttler C, Müller J, Rothballer M, Hartmann A, Kreft JU. 2007. Does efficiency sensing unify diffusion and quorum sensing? *Nat Rev Microbiol* 5:230–239. <http://dx.doi.org/10.1038/nrmicro1600>.
- Popat R, Cornforth DM, McNally L, Brown SP. 2015. Collective sensing and collective responses in quorum-sensing bacteria. *J R Soc Interface* 12:20140882. <http://dx.doi.org/10.1098/rsif.2014.0882>.
- Defoirdt T, Boon N, Bossier P, Verstraete W. 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. *Aquaculture* 240:69–88. <http://dx.doi.org/10.1016/j.aquaculture.2004.06.031>.
- Teasdale ME, Liu J, Wallace J, Akhlaghi F, Rowley DC. 2009. Secondary metabolites produced by the marine bacterium *Halobacillus salinus* that inhibit quorum sensing-controlled phenotypes in gram-negative bacteria. *Appl Environ Microbiol* 75:567–572. <http://dx.doi.org/10.1128/AEM.00632-08>.
- Dong YH, Zhang XF, Xu JL, Zhang LH. 2004. Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. *Appl Environ Microbiol* 70:954–960. <http://dx.doi.org/10.1128/AEM.70.2.954-960.2004>.
- Givskov M, de Nys R, Manefield M, Gram L, Maximiliani R, Eberl L, Molin S, Steinberg PD, Kjelleberg S. 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *J Bacteriol* 178:6618–6622.
- Migiyama Y, Kaneko Y, Yanagihara K, Morohoshi T, Morinaga Y, Nakamura S, Miyazaki T, Hasegawa H, Izumikawa K, Kakeya H, Kohrogi H, Kohno S. 2013. Efficacy of AiiM, an *N*-acylhomoserine lactonase, against *Pseudomonas aeruginosa* in a mouse model of acute pneumonia. *Antimicrob Agents Chemother* 57:3653–3658. <http://dx.doi.org/10.1128/AAC.00456-13>.
- Wahjudi M, Murugappan S, van Merkerk R, Eissens AC, Visser MR, Hinrichs WL, Quax WJ. 2013. Development of a dry, stable and inhalable acyl-homoserine-lactone-acylase powder formulation for the treatment of pulmonary *Pseudomonas aeruginosa* infections. *Eur J Pharm Sci* 48:637–643. <http://dx.doi.org/10.1016/j.ejps.2012.12.015>.
- Helman Y, Chernin L. 2015. Silencing the mob: disrupting quorum sensing as a means to fight plant disease. *Mol Plant Pathol* 16:316–329. <http://dx.doi.org/10.1111/mpp.12180>.
- Kisch JM, Utpatel C, Hilterhaus L, Streit WR, Liese A. 2014. *Pseudomonas aeruginosa* biofilm growth inhibition on medical plastic materials by immobilized esterases and acylase. *ChemBioChem* 15:1911–1919. <http://dx.doi.org/10.1002/cbic.201400023>.
- Oh HS, Yeon KM, Yang CS, Kim SR, Lee CH, Park SY, Han JY, Lee JK. 2012. Control of membrane biofouling in MBR for wastewater treatment by quorum quenching bacteria encapsulated in microporous membrane. *Environ Sci Technol* 46:4877–4884. <http://dx.doi.org/10.1021/es204312u>.
- Bai AJ, Rai VR. 2011. Bacterial quorum sensing and food industry. *Compr Rev Food Sci Food Ind* 10:183–193. <http://dx.doi.org/10.1111/j.1541-4337.2011.00150.x>.
- Thiel V, Kunze B, Verma P, Wagner-Döbler I, Schulz S. 2009. New structural variants of homoserine lactones in bacteria. *ChemBioChem* 10:1861–1868. <http://dx.doi.org/10.1002/cbic.200900126>.
- Schaefer LM, Greenberg EP, Oliver CM, Oda Y, Huang JJ, Bittan-Banin G, Peres CM, Schmidt S, Juhaszova K, Sufrin JR, Harwood CS. 2008. A new class of homoserine lactone quorum-sensing signals. *Nature* 454:595–599. <http://dx.doi.org/10.1038/nature07088>.
- Romero M, Mayer C, Muras A, Otero A. 2015. Silencing bacterial communication through enzymatic quorum-sensing inhibition, p 219–236. In Kalia VC (ed), *Quorum sensing vs quorum quenching: a battle with no end in sight*. Springer (India), New Delhi, India.
- Momb J, Yoon D-W, Fast W. 2010. Enzymic disruption of *N*-aroyl-L-homoserine lactone-based quorum sensing. *ChemBioChem* 11:1535–1537. <http://dx.doi.org/10.1002/cbic.201000191>.
- Reymond JL, Fluxa VS, Maillard N. 2009. Enzyme assays. *Chem Commun (Camb)* 1:34–46. <http://dx.doi.org/10.1039/b813732c>.
- Rai N, Rai R, Venkatesh KV. 2015. Quorum sensing biosensors, p 173–183. In Kalia VC (ed), *Quorum sensing vs quorum quenching: a battle with no end in sight*. Springer (India), New Delhi, India.
- McClellan KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* 143(Part 12):3703–3711. <http://dx.doi.org/10.1099/00221287-143-12-3703>.
- Zhang HB, Wang LH, Zhang LH. 2007. Detection and analysis of quorum-quenching enzymes against acyl homoserine lactone quorum-sensing signals. *Curr Protoc Microbiol* Chapter 1:Unit 1C.3. <http://dx.doi.org/10.1002/9780471729259.mc01c03s05>.
- Koch G, Nadal-Jimenez P, Cool RH, Quax WJ. 2014. *Deinococcus radiodurans* can interfere with quorum sensing by producing an AHL-acylase and an AHL-lactonase. *FEMS Microbiol Lett* 356:62–70. <http://dx.doi.org/10.1111/1574-6968.12479>.
- Schipper C, Hornung C, Bijtenhoorn P, Quitschau M, Grond S, Streit WR. 2009. Metagenome-derived clones encoding two novel lactonase family proteins involved in biofilm inhibition in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 75:224–233. <http://dx.doi.org/10.1128/AEM.01389-08>.
- Wahjudi M, Papaioannou E, Hendrawati O, van Assen AH, van Merkerk R, Cool RH, Poelarends GJ, Quax WJ. 2011. PA0305 of *Pseudomonas aeruginosa* is a quorum quenching acylhomoserine lactone acylase belonging to the Ntn hydrolase superfamily. *Microbiology* 157:2042–2055. <http://dx.doi.org/10.1099/mic.0.043935-0>.
- Sio CF, Otten LG, Cool RH, Diggle SP, Braun PG, Bos R, Daykin M, Camara M, Williams P, Quax WJ. 2006. Quorum quenching by an *N*-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. *Infect Immun* 74:1673–1682. <http://dx.doi.org/10.1128/IAI.74.3.1673-1682.2006>.
- Hodgkinson JT, Galloway WRJD, Casoli M, Keane H, Su X, Salmond GPC, Welch M, Spring DR. 2011. Robust routes for the synthesis of *N*-acylated-L-homoserine lactone (AHL) quorum sensing molecules with high levels of enantiomeric purity. *Tetrahedron Lett* 52:3291–3294. <http://dx.doi.org/10.1016/j.tetlet.2011.04.059>.
- Byers JT, Lucas C, Salmond GP, Welch M. 2002. Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. *J Bacteriol* 184:1163–1171. <http://dx.doi.org/10.1128/jb.184.4.1163-1171.2002>.
- Yates EA, Philipp B, Buckley C, Atkinson S, Chhabra SR, Sockett RE, Goldner M, Dessaux Y, Camara M, Smith H, Williams P. 2002. *N*-Acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect Immun* 70:5635–5646. <http://dx.doi.org/10.1128/IAI.70.10.5635-5646.2002>.
- Wardenga R, Hollmann F, Thum O, Bornscheuer U. 2008. Functional expression of porcine aminoacylase 1 in *E. coli* using a codon optimized synthetic gene and molecular chaperones. *Appl Microbiol Biotechnol* 81:721–729. <http://dx.doi.org/10.1007/s00253-008-1716-7>.
- Chung CT, Niemela SL, Miller RH. 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci U S A* 86:2172–2175. <http://dx.doi.org/10.1073/pnas.86.7.2172>.
- Dean KE, Klein G, Renaudet O, Reymond JL. 2003. A green fluorescent chemosensor for amino acids provides a versatile high-throughput screening (HTS) assay for proteases. *Bioorg Med Chem Lett* 13:1653–1656. [http://dx.doi.org/10.1016/S0966-894X\(03\)00280-4](http://dx.doi.org/10.1016/S0966-894X(03)00280-4).
- Studier FW. 2005. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif* 41:207–234. <http://dx.doi.org/10.1016/j.pep.2005.01.016>.
- Dörr M, Fibinger MP, Last D, Schmidt S, Santos-Aberturas J, Böttcher D, Hummel A, Vickers C, Voss M, Bornscheuer UT. 3 February 2016. Fully automatized high-throughput enzyme library screening using a robotic platform. *Biotechnol Bioeng* <http://dx.doi.org/10.1002/bit.25925>.

35. Lindner HA, Alary A, Wilke M, Sulea T. 2008. Probing the acyl-binding pocket of aminoacylase-1. *Biochemistry* 47:4266–4275. <http://dx.doi.org/10.1021/bi702156h>.
36. Lindner HA, Tafler-Naumann M, Röhm KH. 2008. *N*-acetyl amino acid utilization by kidney aminoacylase-1. *Biochimie* 90:773–780. <http://dx.doi.org/10.1016/j.biochi.2007.12.006>.
37. Lindner HA, Alary A, Boju LI, Sulea T, Menard R. 2005. Roles of dimerization domain residues in binding and catalysis by aminoacylase-1. *Biochemistry* 44:15645–15651. <http://dx.doi.org/10.1021/bi051180y>.
38. Wang WZ, Morohoshi T, Ikenoya M, Someya N, Ikeda T. 2010. AiiM, a novel class of *N*-acylhomoserine lactonase from the leaf-associated bacterium *Microbacterium testaceum*. *Appl Environ Microbiol* 76:2524–2530. <http://dx.doi.org/10.1128/AEM.02738-09>.
39. Gao A, Mei GY, Liu S, Wang P, Tang Q, Liu YP, Wen H, An XM, Zhang LQ, Yan XX, Liang DC. 2013. High-resolution structures of AidH complexes provide insights into a novel catalytic mechanism for *N*-acyl homoserine lactonase. *Acta Crystallogr D Biol Crystallogr* 69:82–91. <http://dx.doi.org/10.1107/S0907444912042369>.
40. Mei GY, Yan XX, Turak A, Luo ZQ, Zhang LQ. 2010. AidH, an alpha/beta-hydrolase fold family member from an *Ochrobactrum* sp. strain, is a novel *N*-acylhomoserine lactonase. *Appl Environ Microbiol* 76:4933–4942. <http://dx.doi.org/10.1128/AEM.00477-10>.
41. Lindemann SR, Moran JJ, Stegen JC, Renslow RS, Hutchison JR, Cole JK, Dohnalkova AC, Tremblay J, Singh K, Malfatti SA, Chen F, Tringe SG, Beyenal H, Fredrickson JK. 2013. The epsomitic phototrophic microbial mat of Hot Lake, Washington: community structural responses to seasonal cycling. *Front Microbiol* 4:323. <http://dx.doi.org/10.3389/fmicb.2013.00323>.
42. Foessel BU, Gössner AS, Drake HL, Schramm A. 2007. *Geminicoccus roseus* gen. nov., sp. nov., an aerobic phototrophic *Alphaproteobacterium* isolated from a marine aquaculture biofilter. *Syst Appl Microbiol* 30:581–586. <http://dx.doi.org/10.1016/j.syapm.2007.05.005>.
43. Cockell CS, Rettberg P, Rabbow E, Olsson-Francis K. 2011. Exposure of phototrophs to 548 days in low Earth orbit: microbial selection pressures in outer space and on early earth. *ISME J* 5:1671–1682. <http://dx.doi.org/10.1038/ismej.2011.46>.
44. Nandasena K, Yates R, Tiwari R, O'Hara G, Howieson J, Ninawi M, Chertkov O, Detter C, Tapia R, Han S, Woyke T, Pitluck S, Nolan M, Land M, Liolios K, Pati A, Copeland A, Kyrpides N, Ivanova N, Goodwin L, Meenakshi U, Reeve W. 2014. Complete genome sequence of *Mesorhizobium ciceri* bv. *biserrulae* type strain (WSM1271^T). *Stand Genomic Sci* 9:462–472. <http://dx.doi.org/10.4056/signs.4458283>.
45. Coenye T, Vandamme P. 2003. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ Microbiol* 5:719–729. <http://dx.doi.org/10.1046/j.1462-2920.2003.00471.x>.
46. Xu F, Byun T, Deussen HJ, Duke KR. 2003. Degradation of *N*-acylhomoserine lactones, the bacterial quorum-sensing molecules, by acylase. *J Biotechnol* 101:89–96. [http://dx.doi.org/10.1016/S0168-1656\(02\)00305-X](http://dx.doi.org/10.1016/S0168-1656(02)00305-X).
47. Wang HJ, Bai JH, Liu DS, Zhang T, Zhou HM. 1999. Preparation and properties of immobilized pig kidney aminoacylase and optical resolution of *N*-acyl-DL-alanine. *Appl Biochem Biotechnol* 76:183–191. <http://dx.doi.org/10.1385/ABAB:76:3:183>.
48. Lin YH, Xu JL, Hu J, Wang LH, Ong SL, Leadbetter JR, Zhang LH. 2003. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Mol Microbiol* 47:849–860. <http://dx.doi.org/10.1046/j.1365-2958.2003.03351.x>.
49. Draganov DI, Teiber JF, Speelman A, Osawa Y, Sunahara R, La Du BN. 2005. Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J Lipid Res* 46:1239–1247. <http://dx.doi.org/10.1194/jlr.M400511-JLR200>.
50. Teiber JF, Horke S, Haines DC, Chowdhary PK, Xiao J, Kramer GL, Haley RW, Draganov DI. 2008. Dominant role of paraoxonases in inactivation of the *Pseudomonas aeruginosa* quorum-sensing signal *N*-(3-oxododecanoyl)-L-homoserine lactone. *Infect Immun* 76:2512–2519. <http://dx.doi.org/10.1128/IAI.01606-07>.