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ORIGINAL PAPER

Detection of quorum sensing molecules in *Burkholderia cepacia* culture supernatants with enzyme-linked immunosorbent assays

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Abstract The *Burkholderia cepacia* complex (Bcc) employs a quorum sensing (QS) mechanism which is a cell density-dependent bacterial communication system to regulate certain gene expressions. As with many other Gram-negative bacteria, *Burkholderia cepacia* species use (*N*-acyl-)homoserine lactones (AHLs or HSLs) as signalling molecules. Because of the essential role of QS in bacterial behavior, the aim of this study was to demonstrate the applicability of our in-house-developed enzyme-linked immunosorbent assays (ELISAs) for the detection of bacterial activities via HSLs in *B. cepacia* strain LA3 culture supernatants. For this purpose the previously developed monoclonal antibodies (mAbs) HSL1/2-2C10 and HSL1/2-4H5 were exploited. *N*-3-Oxo-decanoyl-L-homoserine lactone (3-oxo-C10-HSL) was used as main analyte throughout all experiments. With the bacterial culture medium (named ABC medium) a matrix effect in both ELISAs was visible (slight increase in optical density, shift in test midpoints (IC_{50}) and working ranges). For example, ELISA with mAb HSL1/2-2C10 and enzyme tracer HSL3-HRP (HSL derivative conjugated to horseradish peroxidase) had an IC_{50} of 120 μg L^{-1} for 3-oxo-C10-HSL in phosphate-buffered saline versus 372 μg L^{-1} in ABC medium. A significant increase of HSLs in *B. cepacia* strain LA3 culture supernatants after 12 h to 48 h of growth was observed. Although the analytical result of these immunoassays cannot distinguish HSLs from homoserines (HSs), the appearance of these compounds can be easily followed. Hydrolysis and spiking experiments were carried out with these biological samples. According to our knowledge, these are the first immunoassays for the detection of quorum sensing molecules in biological culture supernatants. This study provides a cost-effective, fast, and sensitive analytical method for detection of HSLs/HSs in biological samples without complex sample preparation and will offer a quick idea about *B. cepacia* activities. The low sample amount requirement (less than 1 mL) constitutes a tremendous advantage for many analytical questions with biological samples.

Keywords Quorum sensing · (*N*-acyl-)homoserine lactone (AHL or HSL) · (*N*-acyl-)homoserine · *Burkholderia cepacia* · Enzyme-linked immunosorbent assay (ELISA) · Monoclonal antibodies · Biological samples

Abbreviations

AHL or HSL	(*N*-acyl-)homoserine lactone
ACN	acetonitrile
AIs	autoinducers
Bcc	*Burkholderia cepacia* complex
CF	cystic fibrosis
CR	cross reactivity
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase

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HS	*N*-acyl-homoserine
IC_{50}	inhibitory concentration 50%, test mid-point of the standard curve
LOD	limit of detection
mAb	monoclonal antibody
NaOH	sodium hydroxide
OD	optical density
3-oxo-C10-HSL	*N*-3-oxo-decanoyl-L-homoserine lactone
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
Protein G	Protein G from *Streptococcus*
RT	room temperature
QS	quorum sensing
TMB	3,3′,5,5′-tetramethylbenzidine

Introduction

The *Burkholderia cepacia* complex (Bcc) consists of 17 closely related species [1], which were isolated from different natural habitats, such as plant rhizosphere, soil, and river water [2, 3]. They were also found in some urban environments like playgrounds and athletic fields [4–10]. Furthermore, they are able to promote plant growth, enhance plant disease resistance [3, 11, 12], and were applied for bioremediation [13, 14]. However, in contrast to these beneficial functions, the Bcc species are known to act as human opportunistic pathogens, e.g., in cystic fibrosis (CF) [2]. The Bcc species employ quorum sensing (QS), using *N*-acyl-homoserine lactones (AHL or HSL) as auto-inducers (AIs). QS is a mechanism that enables bacteria to communicate by using diffusible chemical signalling molecules, called AIs. When the amount of QS molecules in their environment and the cell cytoplasm reaches a certain threshold level, a receptor protein–AI complex is formed and the transcription of specific bacterial genes is activated, which is, e.g., responsible for biofilm formation, virulence factor secretion, and bioluminescence [15, 16]. Most members of the *Burkholderia cepacia* complex were found to use the CepIR QS system [17]. Numerous homoserine lactones (HSLs) with different side chain length and functional groups at the C3 position were confirmed as AIs for Bcc species [18]. Furthermore, the evidence of interspecies communication between *B. cepacia* and *Pseudomonas aeruginosa* in CF patients was also suggested to be related to QS and this correlation may cause a higher risk for the infected patients [19, 20]. The monitoring of HSLs is essential, because the presence and amount of HSLs play an important role for bacterial behavior. To date, different methods have been successfully developed for HSLs analysis. In general, there are two major methods: conventional analysis using chromatography and spectroscopy [21–26] or bioassays depending on bioreporters [27–32]. However, the former is usually quite cost and time consuming, and the latter method is limited by the specificity of the employed bioreporter.

The strain *B. cepacia* LA3 was isolated from the rhizosphere of traditional Indian rice cultivar [3, 21] and characterized to produce *N*-octanoyl-homoserine lactone (C8-HSL) and *N*-decanoyl-homoserine lactone (C10-HSL) as main AIs [21, 33]. In this paper, recently developed mAbs, namely HSL1/2-2C10 and HSL1/2-4H5 [34], were used in enzyme-linked immunosorbent assays (ELISAs) to detect HSLs (and HSs) in *B. cepacia* LA3 bacterial culture supernatants. Based on the fact that HSLs can be easily hydrolyzed under natural conditions, a comparison of determinations before and after chemical hydrolysis of samples from the culture supernatants has been carried out and evaluated.

Material and methods

The HSL standards, *N*-octanoyl-L-homoserine lactone (C8-HSL, MW 227) and *N*-decanoyl-L-homoserine lactone (C10-HSL, MW 255) were purchased from Cayman Chemical (Michigan, USA). The main analyte *N*-3-oxo-decanoyl-L-homoserine lactone (3-oxo-C10-HSL, MW 269) was purchased from Sigma-Aldrich (Taufkirchen, Germany). The homoserine molecules (HS), the hydrolysis products of HSLs, were prepared with the addition of NaOH [34].

The enzyme tracers HSL1-HRP and HSL3-HRP were produced according to the active ester method [34]. For storage at −20 °C, HSL3-HRP was diluted 1:3 in Super-Freeze peroxidase stabilizer (Pierce/Perbio now Thermo Scientific, Bonn, Germany).

For ELISAs, Protein G from *Streptococcus* (Protein G), hydrogen peroxide (H_2O_2), and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Steinheim, Germany). Dimethylsulfoxide (DMSO), 99%, was purchased from Fluka (now Sigma-Aldrich). Buffer salts (sodium dihydrogen phosphate monohydrate, di-sodium hydrogen phosphate dehydrate, and sodium chloride, sodium acetate, sodium carbonate, citric acid monohydrate), Tween 20, and sulfuric acid, 95–97%, were purchased from Merck (Darmstadt, Germany). Ultra pure water, which was used for buffers and all other solutions, was prepared by a Milli-Q filter system from Millipore (Eschborn, Germany). Nunc Maxisorp 96-well microtiter plates and lids were purchased from Thermo Fischer Scientific (Schwerte, Germany). Washing steps in microtiter plates were performed with an automated microtiter plate washer from Bio-Tek Instruments (Bad Friedrichshall, Germany). Absorbance was read by a multi-detection reader Spectra Max M5^e from Molecular Devices (Palo

Alto, USA, now part of Danaher Corporation, Washington, DC, USA). A heating-shaking-mixing system, Heidolph incubator 1000 from Metrohm (Herisau, Switzerland), was used for incubation or shaking.

Preparation of *Burkholderia cepacia* culture supernatants

Burkholderia cepacia strain LA3, an isolate from the rhizosphere of rice [3], was cultivated in ABC minimal medium modified according to Clark and Maaloe [35] with the following composition for A, B, and C. Part A: 2 g $(NH_4)_2SO_4$, 6 g Na_2HPO_4, 3 g KH_2PO_4, and 3 g NaCl dissolved in 100 mL of distilled H_2O; part B: 1 M $MgSO_4 \cdot 6H_2O$ (2 mL), 0.5 M $CaCl_2 \cdot 2H_2O$ (0.2 mL), and 0.01 M $FeCl_3 \cdot 6H_2O$ (0.3 mL) dissolved in 900 mL of distilled H_2O. Both parts were autoclaved separately and were afterwards mixed together. Part C: glucose was added as C-source (10 mL of a sterile 20% (w/v) glucose solution). The pH was adjusted to 6.8 (pH meter, pH 523, WTW, Weilheim, Germany).

Two baffled flasks A and B (100 mL, Schott AG, Mainz, Germany), each containing 50 mL of the medium, were separately inoculated 1:1,000 with an overnight culture. The flasks were incubated and shaken at 30 °C and 175 rpm using an incubator shaker (Innova 4200, New Brunswick Scientific, Edison, NJ, USA). Cell cultures were sampled directly after inoculation and after 4, 8, 12, 24, 32, and 48 h from A and B in parallel. The turbidimetry was determined at 436 nm with a spectrophotometer (CE3021, Cecil Instruments Ltd., Cambridge, UK) at the sampling time points. To obtain cell-free supernatants, samples were centrifuged at 4 °C at 15,000 *g* for 30 min (Sorvall Evolution RC, Thermo Fisher Scientific, Bonn, Germany). Subsequently, the supernatants were filtered through nitrocellulose filters (filter type 0.22 μm GSWP, Millipore, Schwalbach, Germany), frozen at −80 °C for 1 h, and stored at −20 °C until measurement. These two inoculates were defined as setups A and B and were used for the determinations by ELISA.

The hydrolysis of *B. cepacia* samples was performed by the addition of NaOH. Briefly, 100 μL of 1 M NaOH was added to 800 μL sample in a glass tube, which then was shaken for 15 min at RT. The solution was neutralized to pH 7 by titration of 1 M HCl and ABC medium was added to get a final volume of 1 mL, taking the amount of HCl used for titration into consideration. All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

ELISA in the enzyme tracer format: determination of cross reactivities

Protein G solution with a concentration of 2 μg mL^{-1} was used for coating of the microtiter plate (200 μL $well^{-1}$ in 50 mM carbonate buffer, pH 9.6, 4 °C, overnight). The next day, the plate was washed and 150 μL $well^{-1}$ of mAb solution (concentrations of mAbs, see Table 1) was added for 2 h at RT. The plate was washed again, and 100 μL $well^{-1}$ of 3-oxo-C10-HSL standard solutions in 40 mM PBS buffer (0 μg L^{-1} (only 40 mM PBS buffer), and 0.5 to 20,000 μg L^{-1}), were added and incubated for 1 h. Then, 50 μL $well^{-1}$ of the enzyme tracer solutions, HSL1-HRP or HSL3-HRP (dilutions, see Table 1), respectively, were directly added to the plate, which still contained the analyte standard solutions. The mixture was shaken at 200 rpm for 30 min, and the plate was washed. Then 150 μL $well^{-1}$ of TMB substrate (200 μL 1% H_2O_2, 800 μL TMB solution (6 mg mL^{-1} TMB diluted in DMSO) in 50 mL sodium acetate buffer, pH 5.5, was used for the enzymatic reaction. Finally, the enzyme reaction was stopped after 15 min using 50 μL $well^{-1}$ of 2 M H_2SO_4. The absorbance measurements were performed with a Spectra Max M5^e (Molecular Devices) microplate reader at 450 nm (reference 650 nm). A blank, consisting of all buffers, was subtracted from all values.

The wash program included washing three times with 200 μL $well^{-1}$ 4 mM PBST buffer (40 mM PBS 1:10 diluted plus 0.05% Tween 20, pH 7.2). The incubation temperature was 25±3 °C. The optimized concentrations of all described solutions were determined by 2D titrations (results not shown).

Curve fitting of the standard curve was performed with SOFTmax Pro using the four-parameter fit according to

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} + D \quad (1)$$

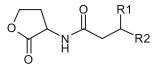
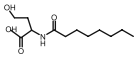
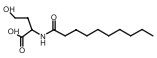
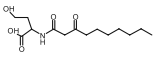
In standard curves, the parameters are as follows: A, maximum adsorption; B, slope; C, IC_{50} (test midpoint of curve); D, minimum adsorption; R^2, fitting rate. Standard curves were then normalized to % control values (using SigmaPlot (Systat Software, Inc., Chicago, IL, USA)), according to the formula:

$$\text{Control}(\%) = (A/A_0) \times 100, \quad (2)$$

where A is the absorbance value for each standard concentration and A_0 is the value of absorbance for the zero standard (only buffer). From these normalized standard curves, the limit of detection (LOD) of the assays was determined from 15% to 85% control [36].

Using the optimized immunoassays, CRs of *B. cepacia*-relevant HSL substances were determined. On each microtiter plate, the main analyte (3-oxo-C10-HSL) was used as reference in duplicate determination, whereas cross reactants were determined in duplicate or triplicate. Standards for the cross reactants were set up in 40 mM PBS, pH 7.6, ranging from 0 (only PBS buffer) and 0.5 to 50,000 μg mL^{-1}.

Table 1 Cross reactivities (CR) and test midpoints (IC_{50}) for relevant HS(L) molecules in *Burkholderia cepacia* culture supernatants using mAbs HSL1/2-2C10 and HSL1/2-4H5 (in part from [34])

			MAb HSL1/2-2C10 [400 ng mL^{-1}] Enzyme-Tracer HSL1-HRP 1:1,000			MAb HSL1/2-4H5 [200 ng mL^{-1}] Enzyme-Tracer HSL3-HRP 1:400		
Analyte (Short form)	**R1**	**R2**	**CR [%]**	**IC_{50} [µg L^{-1}]**	**n**	**CR [%]**	**IC_{50} [µg L^{-1}]**	**n**
C8-HSL	H	$(CH_2)_4CH_3$	71	215±8	4	156	146±8	2
C10-HSL	H	$(CH_2)_6CH_3$	41	369±82	4	56	404±29	2
3-oxo-C10-HSL	O	$(CH_2)_6CH_3$	**100**	134±30	53	**100**	253±44	19
C8-HS			7276	1.7±0.2	2	----	----	----
C10-HS			1531	9±3	4	1667	16±1	3
3-oxo-C10-HS			1813	7±1	4	2268	14±4	3

CR% was calculated using the formula:

$$CR(\%) = (IC_{50} \text{ for the main analyte}) / (IC_{50} \text{ for the cross reactant}) \times 100 \quad (3)$$

Standard curves in ABC medium: matrix effects

Since the LA3 supernatant samples were prepared in ABC medium, but the assays on the other hand were initially developed in 40 mM PBS buffer, IC_{50} values of the main analyte (3-oxo-C10-HSL) were determined in both matrices. The assays were carried out as described in "ELISA in the enzyme tracer format: determination of cross reactivities". For the determination of the matrix effect, standard solutions of the main analyte (3-oxo-C10-HSL) were set up in ABC medium though, ranging from 0 (only ABC medium), 0.5 to 20,000 µg L^{-1}. For better comparison of both assays (with mAbs HSL1/2-2C10 and HSL1/2-4H5, respectively), the same enzyme tracer, namely HSL3-HRP, was used here, diluted 1:600 and 1:400, respectively. A blank, consisting of all different buffers, was subtracted from all values. Normally, this blank value was in the same absorbance range as the highest standard concentration of the standard curve (signal is inversely proportional to analyte concentration). The change of OD, and the shifting of IC_{50} and working ranges between immunoassays in PBS and in ABC medium indicate the matrix effect of the assays.

Standard curves in ABC medium: determination of HSL *in B. cepacia* LA3 supernatants

As described in "Standard curves in ABC medium: matrix effects", different concentrations of 3-oxo-C10-HSL in ABC medium were used for the setup of the standard curve for *B. cepacia* LA3 supernatants measurements. Concentrations of 3-oxo-C10-HSL and each LA3 supernatant sample were determined in two to four wells on one plate, and the mean values with standard deviations were taken for final results. Depending on the working range of the assays, the samples were used undiluted or prepared in a dilution of 1:5, 1:10, or 1:20 before and after hydrolysis to obtain the best reliable results. For control purpose, 50 µg L^{-1} (and 25 µg L^{-1}) of 3-oxo-C10-HS was spiked into each hydrolyzed sample. The biological samples were calculated by SOFTmax Pro and results were expressed as 3-oxo-C10-HSL equivalents.

Results and discussion

Matrix effects

Initially, the assays were developed in PBS buffer. Biological samples though have usually very different background substances, called the matrix, which can contribute to changes in immunoassay performance (e.g., interference with antibody–analyte binding, enzyme tracer). In extreme cases the matrix can entirely inhibit the assays and thus it has to be considered carefully [37]. In this study, the matrix effects were determined by comparing the 3-oxo-C10-HSL standard curves in 40 mM PBS buffer and ABC medium. A slight OD increase (about 0.2) in ABC medium was observed for both ELISAs, but the signal was still in an acceptable range in both buffers. A shift of standard curves to the right (to higher concentration) was clearly visible and is shown in Fig. 1. Both ELISAs performed with a lower sensitivity in ABC medium than in PBS buffer. The ABC/

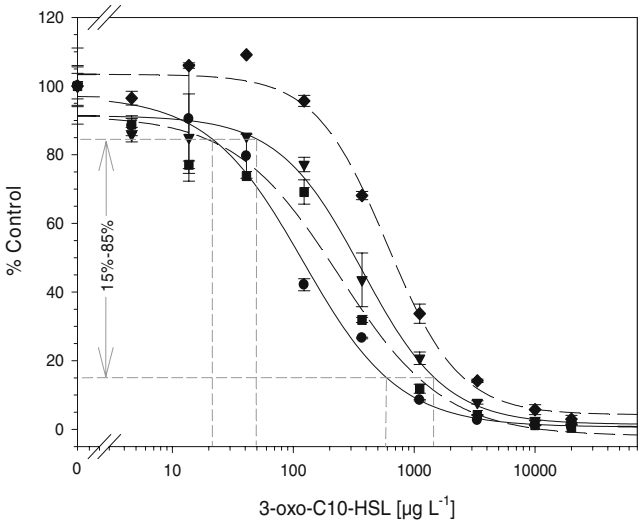


Fig. 1 Matrix effects in ABC medium. Standard curves for 3-oxo-C10-HSL with mAbs HSL1/2-2C10 and HSL1/2-4H5 in PBS buffer and in ABC medium using HSL3-HRP as enzyme tracer. Assay conditions: Protein G 2 µg mL^{-1}; HSL1/2-2C10 400 ng mL^{-1}, HSL3-HRP 1:600; HSL1/2-4H5 200 ng mL^{-1}, HSL3-HRP 1:400. Four-parameter curve fitting data are given in parentheses: *solid line, circles* 3-oxo-C10-HSL in PBS with HSL1/2-2C10 (*A* 97.4, *B* 1.1, *C* 120 µg L^{-1}, *D* 0.72, R^2 0.991); *solid line, inverted triangles* 3-oxo-C10-HSL HSL in ABC medium with HSL1/2-2C10 (*A* 91.4 , *B* 1.25, *C* 372 µg L^{-1}, *D* 1.45, R^2 0.989); *dashed line, squares* 3-oxo-C10-HSL in PBS with HSL1/2-4H5 (*A* 90.1, *B* 1.04, *C* 243 µg L^{-1}, *D* −1.53, R^2 0.979); *dashed line, diamond* 3-oxo-C10-HSL in ABC medium with HSL1/2-4H5 (*A* 103.4, *B* 1.45, *C* 600 µg L^{-1}, *D* 4.3, R^2 0.992)

PBS factor of IC_{50} for 3-oxo-C10-HSL is 3.1 using mAb HSL1/2-2C10 and 2.5 using mAb HSL1/2-4H5. Noticing this matrix effect, the standard curve of 3-oxo-C10-HSL was set up in ABC medium as a reference for culture supernatant samples. The working range was taken from 15% to 85% control of the standard curve [38]. According to the standard curves it was 21–585 µg L^{-1} (78–2,170 nM) in the assay with PBS buffer and 50–1,500 µg L^{-1} (186–5,576 nM) in the assay with ABC medium (Fig. 1; mAb HSL1/2-2C10, enzyme tracer HSL3-HRP). This implies for the assay in ABC medium that this assay has the lowest relative error between 50 and 1,500 µg L^{-1} and correspondingly lower accuracy outside of this range [36].

Assay comparison with two mAbs and two setups with *B. cepacia* LA3 culture supernatants

Our previous study had confirmed that the mAbs HSL1/2-2C10 and HSL1/2-4H5 have a very similar specificity pattern toward HSLs and HSs [34]. As listed in Table 1, mAb HSL1/2-2C10 has a lower IC_{50} for 3-oxo-C10-HSL (134±30 µg L^{-1}) than HSL1/2-4H5 (253±44 µg L^{-1}), and the recognition of C8-HSL and C10-HSL was slightly different as well. In general, these antibodies showed significantly better recognition of the hydrolyzed form of HSLs (HSs). Using mAb HSL1/2-2C10, C8-HS has a CR of about 7,280%, C10-HS of 1,531%, and 3-oxo-C10-HS of 1,813%. The similar high recognition for HSs was revealed with mAb HSL1/2-4H5.

Undiluted *B. cepacia* culture supernatants were then analyzed individually in two setups (A and B, see "Preparation of *Burkholderia cepacia* culture supernatants") by ELISAs in the enzyme tracer format using both mAbs. Although C8- and C10-HSL were expected to be the main AIs for *B. cepacia* [39, 40], in these experiments 3-oxo-C10-HSL was used as the reference standard, because this was set as the main analyte in our optimized assays. Results were then expressed as 3-oxo-C10-HSL equivalents. This strategy can be used in immunoassays, especially when several compounds show higher cross reactivities (≥50%). All four assays (2 mAbs, 2 setups) showed very similar results for these *B. cepacia* culture supernatants (Fig. 2). In summary, no signal was detected from samples taken from cultures grown less than 12 h, and the HSL concentrations increased continuously from 12 h to 48 h. The maximum concentration of about 3,500 µg L^{-1} of 3-oxo-C10-HSL equivalents was detected at 48 h. When these samples were analyzed for C8-HSL and C10-HSL by UPLC-MS, the trends of value development were exactly the same (start of HSL appearance at 12 h, highest values at 48 h) but the determined concentrations were lower (A. Fekete, unpublished results). The concentrations (ca. 120 µg L^{-1}) were similar to those determined earlier for culture supernatants of *B. cepacia* strain JA-8 [39]. The reason for the higher concentrations determined by immunoassays might be the presence of HSL lactonase in the culture supernatants, which

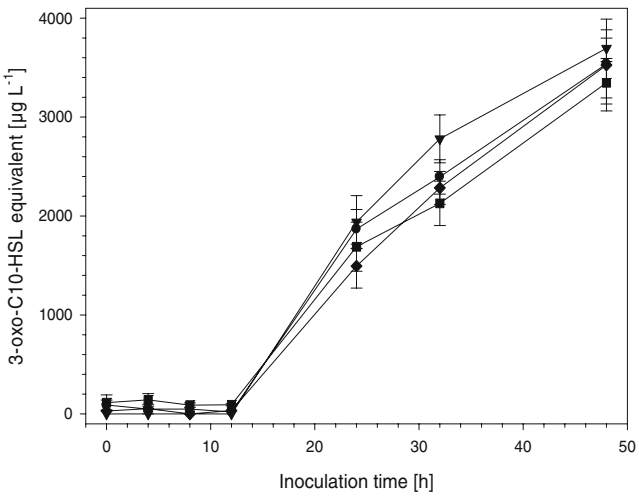


Fig. 2 Comparison of two ELISAs with different antibodies and setups A and B of undiluted *B. cepacia* culture supernatants. MAb HSL1/2-2C10 (400 ng mL^{-1}) and enzyme tracer HSL3-HRP (1:600): *circles* setup A ; *inverted triangles* setup B. MAb HSL1/2-4H5 (200 ng mL^{-1}), HSL3-HRP (1:400): *squares* setup A; *diamond* setup B

can hydrolyze the HSLs to HSs (see also "Hydrolysis and spiking experiments").

Due to the fact that these two setups and two assays had reproducible results, the further experiments were performed only with mAb HSL1/2-2C10 and setup A.

Dilution control of *B. cepacia* LA3 culture supernatants

As mentioned before, the maximum concentration detected in *B. cepacia* LA3 culture supernatants at 48 h was about 3,500 μg L^{-1} of 3-oxo-C10-HSL equivalents. This was outside the assay's working range and therefore considered as not reliable (Fig. 1). For this reason the samples were diluted 1:5, 1:10, and 1:20 in ABC medium for the HSL determinations. Samples diluted 1:5 had the highest concentrations, and the 1:10 and 1:20 diluted samples showed correspondingly lower concentrations (Fig. 3). Again, it was noticed that the LOD range of the assays had an effect on the accuracy of the detection, particularly the samples with higher concentrations. Thus, attention had to be paid to sample dilutions with careful consideration of the detection ranges of the assays. From the results shown in Fig. 3, the 1:10 dilution was selected to be the most suitable for these measurements, because most of the values were within the working range. Additionally, the hydrolyzed samples were also diluted with the same factors as described for the untreated ones and the final results were very comparable (data not shown).

Hydrolysis and spiking experiments

In natural environments, HSLs are supposed to be hydrolyzed and HSs will be produced by lactone ring opening. This reaction can take place at alkaline pH and RT, but also in the presence of HSL lactonase in the samples. The presence of C10-HS in *B. cepacia* LA3 culture supernatants was in addition observed earlier by Englmann et al. [40]. The analytical result of our ELISAs though for HSLs in *B. cepacia* LA3 culture supernatants will not distinguish HSLs from HSs. In order to find out whether HSLs *B. cepacia* culture supernatants were already hydrolyzed or not, a measurement was carried out with the original sample before and after hydrolysis (Fig. 4, circles, original sample; inverted triangles, samples after chemical hydrolysis). In addition, the hydrolyzed samples were then spiked with 50 μg L^{-1} of 3-oxo-C10-HS. Figure 4 shows only the 1:10 dilution of culture supernatants, but the dilutions 1:5 and 1:20 provided comparable results. The data points (squares) in Fig. 4 illustrate the concentration of hydrolyzed samples which were spiked with 50 μg L^{-1} of 3-oxo-C10-HS, respectively. The spike of 50 μg L^{-1} of 3-oxo-C10-HS resulted in an average concentration of 1,497±60 μg L^{-1} of 3-oxo-C10-HSL equivalents (0 to 12 h). This higher determination reflects the higher CR to 3-oxo-C10-HS. For control purposes, the whole process was repeated with a spiking concentration of 25 μg L^{-1} of 3-oxo-C10-HS. Here, the spiked concentration of 25 μg L^{-1} of 3-oxo-C10-HS was analyzed as an average concentration of 669± 76 μg L^{-1} of 3-oxo-C10-HSL equivalents (0 to 12 h).

Assuming that C8- and C10-HSL were present in the *B. cepacia* LA3 culture supernatants (after 12 h), the spiked concentration of 3-oxo-C10-HS was still clearly visible.

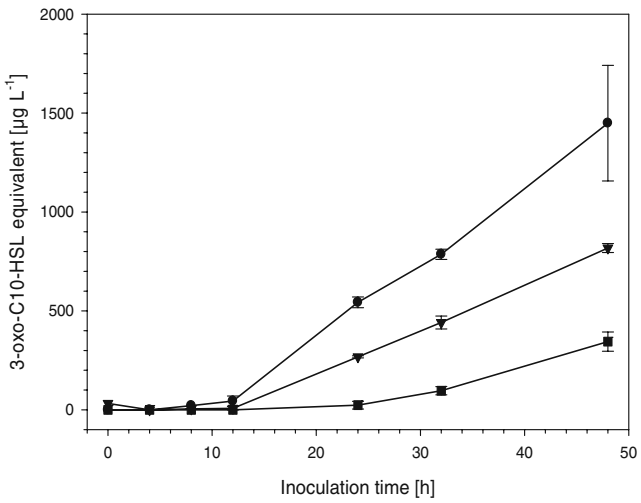


Fig. 3 Dilution controls in ELISA using mAb HSL1/2-2C10 (400 ng mL^{-1}) and enzyme tracer HSL3-HRP (1:600). *Circles* 1:5 dilution; *inverted triangles* 1:10 dilution; *squares* 1:20 dilution

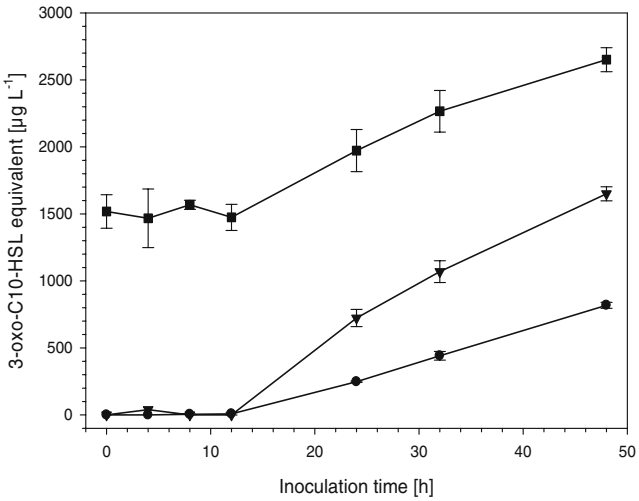


Fig. 4 Hydrolysis and spiking experiments with 1:10 diluted *B. cepacia* culture supernatants. ELISA with mAb HSL1/2-2C10 400 ng mL^{-1} and enzyme tracer HSL3-HRP 1:600. *Circles* 1:10 diluted culture supernatants. *Inverted triangles* 1:10 diluted culture supernatants after hydrolysis of HSLs. *Squares* 1:10 diluted culture supernatants after hydrolysis of HSLs and with a spiked concentration of 50 μg L^{-1} of 3-oxo-C10-HS to each sample

The spike of 50 μg L^{-1} of 3-oxo-C10-HS resulted in an average concentration of 1,149±131 μg L^{-1} of 3-oxo-C10-HSL equivalents (difference between measurements of 1:10 diluted culture supernatants after hydrolysis, without and with spike, 24–48 h). The determined concentrations though were probably slightly influenced by the presence of C8-HS and C10-HS.

Although we could not give the distribution of HSLs and HSs in the *B. cepacia* culture supernatants, a clear increase of these compounds during the bacterial growth could be followed over time.

Conclusions and outlook

The appearance of HSLs (or HSs) in the nanomolar to low micromolar range in *B. cepacia* strain LA3 culture supernatant could be successfully followed with our recently developed ELISAs. Using mAbs HSL1/2-2C10 and HSL1/2-4H5 the continuous production of the HSLs was very clearly observed from 12 h to 48 h. By comparing the detected concentration before and after hydrolysis, it was clearly visible that the HSLs in the samples were still not all in the hydrolyzed form. The dilutions, spiking, and repetition of the experiments demonstrated the reliability of these assays.

There are some important factors of these immunoassays that need to be considered. First are the matrix effects, which showed effects on the assays, e.g., the changing of absorbance and working range. The second is the dilution of the samples. Because the concentration in biological samples is not known, different dilutions should be tested to obtain the best results. HSLs and HSs cannot be distinguished in these ELISAs. However, this could be regarded as an advantage, because this method will determine both forms in biological samples directly and without the need for sample preparation.

We demonstrate that our established immunoassays are the first immunochemical test systems for direct HSL detection in biological samples at low micromolar to nanomolar range. As advantages, in comparison to conventional analysis, immunoassays are very cost effective, fast, require low amounts of sample (less than 1 mL), and no or less sample preparation. The appearance of HSLs/HSs in biological samples can be detected faster, thus giving a quick idea about bacterial activities. With a fast chemical hydrolysis as sample preparation, one could focus on the analysis of HSs alone, which would be even more sensitive (picomolar range). Other applications in the quorum quenching field and other immunochemical technologies, including immunoaffinity columns and in situ tests of HSLs/HSs, will be of interest in the future.

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