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ORIGINAL ARTICLE

Detection of different quorum-sensing signal molecules in a virulent *Edwardsiella tarda* strain LTB-4

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Abstract

Aims: The aim of this study was to elucidate the potential quorum-sensing (QS) signal molecules of an emerging pathogen (*Edwardsiella tarda* strain LTB-4) of cultured turbot (*Scophthalmus maximus*).
Methods and Results: A sensitive and rapid double-layer plate method using biosensor strain *Agrobacterium tumefaciens* KYC55 was developed to detect the N-acylhomoserine lactone (AHL)-related compounds in bacteria. LTB-4 was found to have two QS systems, one was based on the AHLs and the other was based on the autoinducer-2 (AI-2). The AI-2 activity produced by LTB-4 was growth phase dependent and topped at OD_{600} of 1·0. The protocol to detect cholerae autoinducer 1 (CAI-1) activity in bacteria was modified, lowering the background luminescence of biosensor strain *Vibrio harveyi* JAF375. CAI-1 activity could not be detected in LTB-4.
Conclusion: *Edwardsiella tarda* LTB-4 produced at least four kinds of AHLs during its whole growth phase. In comparison with the AHL-inducing QS, AI-2 may be the first predominant signal, functioning at early exponential phase. LTB-4 did not produce any CAI-1 activity.
Significance and Impact of the Study: Different QS signal molecules of *Edw. tarda* LTB-4 were clarified by improved bioassays. In contrast to earlier studies detecting two types of AHLs, strain LTB-4 produced at least four kinds of AHLs, which seemed to be C_4-HSL, C_6-HSL, 3-oxo-C_6-HSL and an uncharacterized AHL molecule.

Introduction

Edwardsiella tarda is considered as an important pathogen of fish and has been isolated from a variety of cultured fishes, including eel, channel catfish, mullet, seabream, chinook salmon, tilapia, tropical fish black skirt tetra and angelfish (Austin and Austin 2007; Mohanty and Sahoo 2007). Recently, *Edw. tarda* has caused serious loss of marine cultured fish such as turbot (*Scophthalmus maximus*) and Japanese flounder (*Paralychthys olivaceus*), in mainland of China. A virulent *Edw. tarda* strain (designated as LTB-4) was obtained from the brain of diseased turbot in a local fish farm in Qingdao, and identified by phenotypic tests and 16S rDNA sequencing. Pathogenicity assays revealed that the isolated *Edw. tarda* strain was virulent to turbot and zebrafish (*Danio rerio*) (Lan *et al.* 2008).

Bacterial quorum sensing (QS) is mediated by small, structurally diverse, diffusible signal molecules that regulate gene expression in response to changes in cell population density. Many Gram-negative bacteria use AHLs as QS signal molecules. An AHL-QS system is most commonly mediated by two proteins belonging to the LuxI–LuxR protein families, which originate from the LuxI-AHL synthase and LuxR-AHL-response regulator involved in regulating bioluminescence in *Vibrio fischeri* in relation to cell density (Steindler and Venturi 2007). Recently, many fish and shrimp pathogens in aquaculture, such as *V. harveyi*, *Aeromonas hydrophila* and *A. salmonicida*, were reported to control virulence factor expression by QS system (Swift *et al.* 1997; Henke and Bassler 2004b; Defoirdt *et al.* 2005). *Vibrio harveyi* used a three-channel QS system that functions in parallel to regulate several virulence factors such as a type III secretion

system (Henke and Bassler 2004a), extracellular toxin (Manefield *et al.* 2000) and a siderophore (Lilley and Bassler 2000). The first channel of the system is mediated by the harveyi autoinducer 1 (HAI-1), an AHL for intra-species communication; the second channel is mediated by AI-2, which is a furanosyl borate diester for interspecies communication; the chemical structure of the third autoinducer, called cholerae autoinducer 1 (CAI-1), has recently been identified as (S)-3-hydroxytridecan-4-one (Henke and Bassler 2004b; Higgins *et al.* 2007). A previous study demonstrated that *Edw. tarda* strain NUF251 from diseased flounder had the ability to produce two kinds of AHL molecules, which seemed to be N-hexanoyl-L-homoserine lactone (C_6-HSL) and N-heptanoyl-L-homoserine lactone (C_7-HSL) (Morohoshi *et al.* 2004). Zhang *et al.* (2008) suggested that *Edw. tarda* strain TX1 isolated from diseased fish had a LuxS/AI-2-mediated signal transduction pathway that regulates the production of virulence-associated elements (i.e. the expression of genes associated with the type III secretion system and biofilm formation). It is possible that the QS system of *Edw. tarda* controls the production of various virulent factors or the infection of host cells, and disruption/manipulation of QS in *Edw. tarda* might be a powerful therapeutic strategy to control edwardsiellosis in aquaculture.

In this study, *Edw. tarda* strain LTB-4, isolated from diseased turbot, was monitored for the production of different QS signal molecules during the entire growth cycle using different biosensors. Effective methods to detect AHL-related compounds and CAI-1 activity in bacteria were developed, and the specificity of the AHL fingerprint of the strain LTB-4 was determined.

Materials and methods

Bacterial strains, media and cultivation conditions

Bacterial strains that were used in this study are listed in Table 1. *Edwardsiella tarda* LTB-4 was identified by phenotypic tests and 16S rDNA sequencing in a previous study (Lan *et al.* 2008). *Edwardsiella tarda* strains, *Vibrio* strains and *Chromobacterium violaceum* CV026 were grown at 28°C under constant agitation in Luria–Bertani (LB) medium (supplemented with 1·5% (w/v) NaCl (LBN) for *Edw. tarda* strains and *Vibrio* strains) overnight with subculturing every 3–7 days, except that CV026 was grown in LB medium with 20 μg ml^{-1} of kanamycin. *Agrobacterium tumefaciens* KYC55 (pJZ372)(pJZ384)(pJZ410) was grown at 28°C under constant agitation in AT minimal broth (especially for the culture of *Ag. tumefaciens*) with appropriate antibiotics (Tempe *et al.* 1977). Antibiotic concentrations used for KYC55 were as follows: 100μg ml^{-1} of gentamycin, 100 μg ml^{-1} of spectinomycin and 1 μg ml^{-1} of tetracycline. *Vibrio harveyi* mutant stains were grown in

Table 1 Bacterial strains used in this study

Strain	Relevant characteristic(s)	Source
Edwardsiella tarda LTB-4	Isolated from the brain of diseased turbot, China	Lan *et al.* (2008)
Edw. tarda NCIMB2034	Pathogenic to fish (unknown species)	NCIMB
Edw. tarda LMG2793	=ATCC 15947; isolated from the human faeces	LMG
Edw. tarda NUF251	Wild-type *Edw. tarda* isolated in 1986 from diseased Japanese flounder in Nagasaki Prefecture, Japan	Morohoshi *et al.*(2004)
Vibrio anguillarum VIB72	=LMG 4437; isolated from cod (*Gadus morhua*), ulcerous lesion, Norway	LMG
V. harveyi VIB295	=LMG4044; isolated from dead amphipod (*Talorchestia* sp.), USA	LMG
V. parahaemolyticus VIB304	=LMG2850; isolated from patient suffering from 'Shirashu' food poisoning, Japan	LMG
V. harveyi BB120	Wild type, from which strains BB152, BB170, MM77 and JAF375 are derived	Bassler *et al.* (1997)
V. harveyi BB152	Mutation in *luxM* (AHL synthase) (Km^r; *luxLM*::Tn5; AI-1$^-$; AI-2$^+$) (ATCC® BAA-1119)	Bassler *et al.* (1993)
V. harveyi BB170	Mutation in *luxN* (AHL receptor that detects the AHL produced by *LuxM*) (Km^r; *luxN*::Tn5; AI-1$^+$ AI-2$^+$) (ATCC® BAA-1117)	Bassler *et al.* (1993)
V. harveyi MM77	Mutation in *luxM* and *luxS* (AHL and AI-2 synthase) (Km^r; *luxLM*::Tn5, *luxS*:: Tn5; AI-1$^-$; AI-2$^-$)	Mok *et al.* (2003)
V. harveyi JAF375	mutation in *luxN* and *luxQ* (HAI-1 and AI-2 receptor) (*luxN*::Cmr, *luxQ*::Tn5)	Freeman and Bassler (1999)
Ag. tumefaciens KYC55 (pJZ372)(pJZ384)(pJZ410)	For detection of N-Acylhomoserine Lactone-type quorum-sensing (QS) molecules	Zhu *et al.* (2003)
C. violaceum CV026	For detection of N-Acylhomoserine Lactone-type quorum sensing molecules	McClean *et al.* (1997)
Escherichia coli DH5α	As a negative control in the bioassay of cholerae autoinducer 1 activity	Henke and Bassler (2004b)

LMG, Laboratory of Microbiology collection (Ghent, Belgium); NCIMB, National Collections of Industrial, Marine and Food Bacteria (Scotland, UK).

autoinducer bioassay (AB) medium prepared according to Greenberg *et al.* (1979). O-nitrophenyl-β-D-galactoside (ONPG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and AHL standards were obtained commercially from Sigma. Spectrophotometry (WFJ2100 Visible Spectrophotometer; UNICO, Shanghai, China) at OD_{600} was used to measure growth, and bioluminescence was measured with a BPCL-4 Ultra-Weak Luminescence Analyzer (Institute of Biophysics of Chinese Academy of Sciences, China) at emission of 474 nm.

Preparation of cell-free culture fluids

Ten millilitres of overnight culture of LTB-4 was centrifuged at 8000 ***g*** for 5 min to collect the cells from the LB growth medium. In order to remove the pregrowth culture fluids (which may contain some signalling molecules), cells were re-suspended in 10 ml of sterile 0·9% (w/v) saline and centrifuged to collect cells. Finally, the cells were re-suspended in 10 ml of fresh LB medium to be used as bacterial pre-inoculum. Two millilitres of bacterial pre-inoculum was inoculated in 200 ml fresh LB medium, in triplicates, and were cultured at 28°C under constant agitation over a period of 21 h. Aliquots of 20 ml were taken every 3 h and centrifuged at 12 000 ***g*** for 5 min to remove the cells from the growth medium. After filtration (0·22 μm), the obtained cell-free fluids were stored at −20°C for further examination. The pH values of the bacterial culture supernatant were measured at various time (growth) points as indicated. The type strains of *Edw. tarda* and *Vibrio* were incubated for 18 h at 28°C. The cell-free culture fluids were prepared as LTB-4.

Detection of AHLs in *Edw. tarda* LTB-4

Screening for AHLs in agar system

Double-layer plate method with Ag. tumefaciens *KYC55.*

A double-layer plate method was established to detect AHL-related compounds by using the ultrasensitive AHL biosensor strain KYC55 (overproducing the AHL receptor TraR by T7 expression system). LTB-4 was spread on LB agar and incubated at 28°C for 15h. The detecting culture mixture was prepared by combining 50 ml of overnight culture of KCY55 with 50 ml of 2 × AT buffer and 50 ml of 1·5% water agar (maintained at 45°C). X-Gal was added to a final concentration of 60 μg ml^{-1}. The detecting culture mixture was overlaid evenly to a thickness of 0·3 cm on LB agar inoculated with LTB-4. After the upper layer of agar had solidified, the double-layer agar plate was incubated at 28°C for 12–18 h, and then examined for blue colouration (blue X-Gal hydrolysis). Fifty microlitres of N-3-oxooctanoyl-l-homoserine lactone (oxo-C_8-HSL) with the concentration of 50 μg ml^{-1} was spread on LB agar and used as the positive control; LB agar without supplements was used as the negative control.

Cross-streaking method with C. violaceum *CV026*

Cross-streaking method with CV026 on LB agar (McClean *et al.* 1997) was used to assay the AHLs activity. LTB-4 was cross-streaked perpendicular to a horizontal streak of CV026 on LB agar plates, incubated at 28°C for 24–48 h, and then examined for the presence of violacein.

Growth phase–dependent analysis of AHLs production in liquid system

The level of AHLs in cell-free culture fluids of LTB-4 at various time points was quantified by examining the ability of samples to activate TraR in KYC55. Briefly, KYC55 was inoculated into an AT minimal medium without antibiotics plus the desired amount (up to 10%) of cell-free culture fluids of LTB-4. The culture mixtures were incubated at 28°C for 16 h under constant agitation, until the OD_{600} reached 0·2 to 1. The samples were then subjected to analysis of β-galactosidase activity using the Miller assay (1972). Fifty microlitres of oxo-C_8-HSL with the concentration of 50 μg ml^{-1} was used as a positive control, while LB media was the negative control.

Extraction of cell-free culture fluids for thin-layer chromatography

C_{18} reverse-phase thin-layer chromatography (TLC) as described by Shaw *et al.* (1997) was performed essentially to visualize the AHLs produced by *Edw. tarda* LTB-4. Each 5 ml of cell-free culture fluids from LTB-4 (at various time points), NUF251, LMG2793 and NCIMB2034 (harvested at OD_{600} of 2·5 after 18 h incubation at 28°C) was extracted twice with equal volumes of ethyl acetate and then dried in a fume hood. The residues of extraction were then dissolved in 50 μl of HPLC-grade ethyl acetate. Extraction samples with a 100-fold dilution (to avoid the smear of AHLs), in volumes of 0·3 μl, were applied to reversed-phase TLC plates (TLC aluminium sheets 20 cm × 20 cm, RP-18 F_{254S}, 1·05559, Merck 64271 Darmstadt, Germany) for the detection with KYC55. Extraction samples were applied in volumes of 12 μl for the detection with CV026. AHLs standards with various retention factors (R_f) were used as references.

Detection of AI-2 in *Edw. tarda* LTB-4

The protocol for the AI-2 bioassay with *V. harveyi* BB170, as described by Vilchez *et al.* (2007), was used. Sterile culture supernatants from *V. harveyi* BB152 and MM77

harvested at OD_{600} around 2·0 served as positive and negative controls respectively. Luminescence was measured every hour until the luminescence with fresh medium was minimal (after 3–4 h). Measurements were taken in triplicate. AI-2 activities were presented as fold induction over the luminescence of the sterile AB medium.

Detection of CAI-1 in *Edw. tarda* LTB-4

The protocol for the CAI-1 bioassay with *V. harveyi* JAF375 was modified from Vilchez *et al.* (2007) and Defoirdt *et al.* (2008). Our modified protocol reduced the background luminescence of *V. harveyi* JAF375 from 45% (Defoirdt *et al.* 2008) to 6·0% and improved its sensitivity. Briefly, *V. harveyi* JAF375 was cultured in AB medium to an OD_{600} of 0·9. The cultures were served as inoculums to AB–Fe medium, which allowed the cultures to grow to an OD_{600} of 1·0–1·1 after 1·5 h. The resulting inoculum culture was then diluted 1 : 50 000 in AB medium (working solution). Then, 1·5 ml of the diluted reporter cultures were mixed with each 1·5 ml cell-free culture fluids of LTB-4 (at various time points) or overnight culture of *Edw. tarda* NCIMB2034 and LMG2793, *V. anguillarum* VIB72, *V. harveyi* VIB295 and *V. parahaemolyticus* VIB304 in 15 ml test tubes. Overnight-culture supernatant of *V. harveyi* BB120 and *Escherichia coli* DH5α served as a positive control and a negative control respectively. Luminescence was measured every hour until the luminescence with fresh medium was minimal (after 2–3 h). Statistical analysis was performed using the SPSS software, ver. 12.0.

Result

Detection of AHLs in *Edw. tarda* LTB-4

Screening for AHLs in agar system

Blue colouration by *Ag. tumefaciens* KYC55 showed that LTB-4 produced AHL molecules. LTB-4 could induce a purple pigment formation with *C. violaceum* CV026. Both assays proved that LTB-4 produced AHL molecules.

Growth phase–dependent analysis of AHLs production in liquid system

LTB-4 produced AHL molecules during its whole growth phase (Fig. 1a), as also observed with *Edw. tarda* NCIMB2034 (data not shown). The level of AHLs recognized by KYC55 increased up to stationary phase (after 15 h at OD_{600} around 2·2). When the OD_{600} was maximal around 2·7 at 18 h, the level of AHLs as detected by KYC55, decreased sharply and subsequently recovered (Fig. 1a,b), resulting in a '*N*'-shaped AHLs production trend in each of the replicates (Fig. 1b). The pH values of bacterial cultures were determined at intervals throughout the growth curve and found to range from 7·1 at the point of inoculation to 7·9 after 21 h of growth.

Thin-layer chromatography

LTB-4 was found to produce three kinds of AHL molecules as detected with KYC55. The first spot (R_f value 0·620) migrating fastest on the TLC plate could be 3-oxo-C_6-HSL (R_f value 0·614), the second spot (R_f value 0·458) migrated as fast as C_4-HSL (R_f value 0·465) and the third spot was uncharacterized (Fig. 2a). KCY55-detectable AHLs profiles of LTB-4 at 15, 18 and 21 h were identical on the TLC plate (Fig. 2b). Two spots detected by CV026 with R_f value of 0·701 and 0·519 were similar to the R_f value of C_4-HSL (0·705) and the R_f value of C_6-HSL (0·513). The AHLs profile of LTB-4 on TLC plate by using KYC55 and CV026 corresponded with the profile of *Edw. tarda* LMG2793, NUF251 and NCIMB2034, and revealed that LTB-4 produced at least four kinds of AHL molecules.

Growth phase dependence of AI-2 synthesis in *Edw tarda* LTB-4

LTB-4 produced AI-2 activity that was growth phase–dependent. No autoinducer activity was observed when OD_{600} was lower than 0·2, as shown in Fig. 3. At an OD_{600} 1, the accumulation of AI-2 activity in LTB-4 culture supernatants was maximal, while the pH value of bacterial culture was the lowest (6·8). In stationary phase, when pH increased to 7·5, the autoinducer activity was significantly reduced ($P < 0·05$) and maintained a 3·5-fold lower level when compared with the log phase (OD_{600} 1).

Detection of CAI-1 in *Edw. tarda* LTB-4

Data analysed statistically showed that *V. anguillarum* VIB72, *V. harveyi* VIB295 and *V. parahaemolyticus* VIB304 significantly induced bioluminescence in *V. harveyi* JAF375 ($P < 0·05$; Table 2), and thus had CAI-1 activities. *Edw. tarda* LTB-4, LMG2793 and NCIMB2034 did not produce any activity.

Discussion

The QS processes of many Gram-negative bacteria were mediated with AHLs, which share the core homoserine lactone moiety, but distinct acyl side chains. In this study, *Edw. tarda* LTB-4, as well as strains NUF251, LMG2793 and NCIMB2034, were found to produce at least four kinds of AHLs. TLC chromatogram in combination with KYC55-based detection, showed three kinds of AHL molecules (C_4-HSL, 3-oxo-C_6-HSL and one uncharacterized

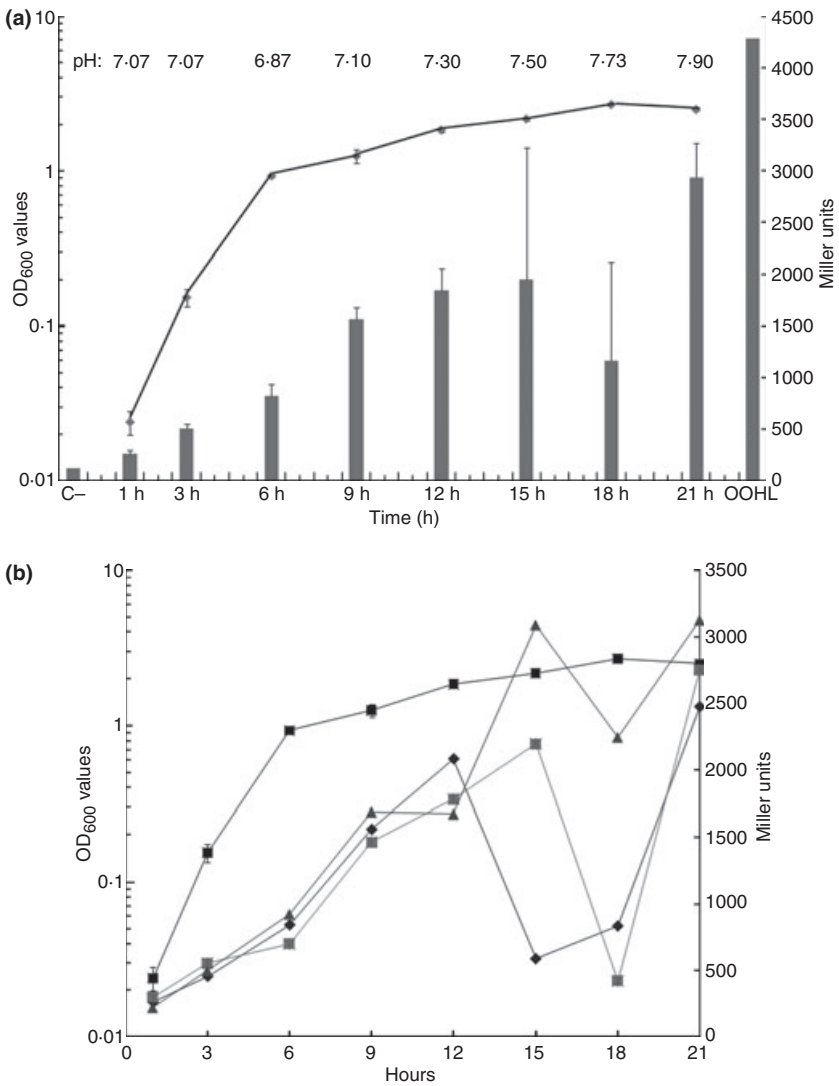

Figure 1 N-acylhomoserine lactones production by *Edwardsiella tarda* LTB-4 during the entire growth cycle. (a) LTB-4 produced AHL molecules during its whole growth phase (C−: negative control; OOHL: positive control; bars = SD). The pH value of the bacterial culture increased from 7·1 to 7·9 over the growth curve. (■) Miller unit; (–◆–) OD600. (b) A common '*N*'-shaped trend of AHLs production in LTB-4 was observed during its whole growth phase in each replicate. (–■–) OD600 values; (–◆–) Replicate 1; (–■–) Replicate 2; (–▲–) Replicate 3.

AHL molecule) in LTB-4, while two kinds of AHL molecules (C_4-HSL, C_6-HSL) were detected by CV026. The production of different AHL molecules has also been reported by other Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*, and these bacteria were verified to possess two homologues of the LuxI family of AHL synthases and two members of the LuxR family of AHL-dependent response regulators (Pearson *et al.* 1995; Atkinson *et al.* 2006). For instance, the *P. aeruginosa* QS network consists of two LuxIR circuits, termed LasIR and RhlIR. LasI, a LuxI homologue, produces 3-oxo-C_{12}-homoserine lactone (Pearson *et al.* 1994) and RhlI produces C_4-homoserine lactone (Pearson *et al.* 1995). The different AHL molecules in *Edw. tarda* could be the result of the presence of two luxI-like synthases. The NUF251 AHL production profile as detected with CV026 was different from the profile reported in a previous study (Morohoshi *et al.* 2004) and demonstrated that *Edw. tarda* NUF251 had the ability to produce two kinds of AHL molecules, C_6-HSL and C_7-HSL. However, the edwI/edwR gene sequences in LTB-4 were identical to the reported edwI/edwR gene sequences in NUF251 (data not shown). The cause for this difference in AHL profile is unknown.

For a complete coverage of AHLs produced by a certain bacterium, multiple biosensors must be used (Cha *et al.* 1998). McClean *et al.* (1997) constructed CV026 based on the LuxR homologues CviR protein that is able to detect AHLs having acyl chains of C_4–C_8 in length resulting in the production of purple pigment violacein. The biosensor strain *Ag. tumefaciens* KYC55 based on the TraI/R QS system, which consists of a three-plasmid system (pJZ372)(pJZ384)(pJZ410), has an increased detection range of AHLs (C_4–C_{18}) with great sensitivity and a

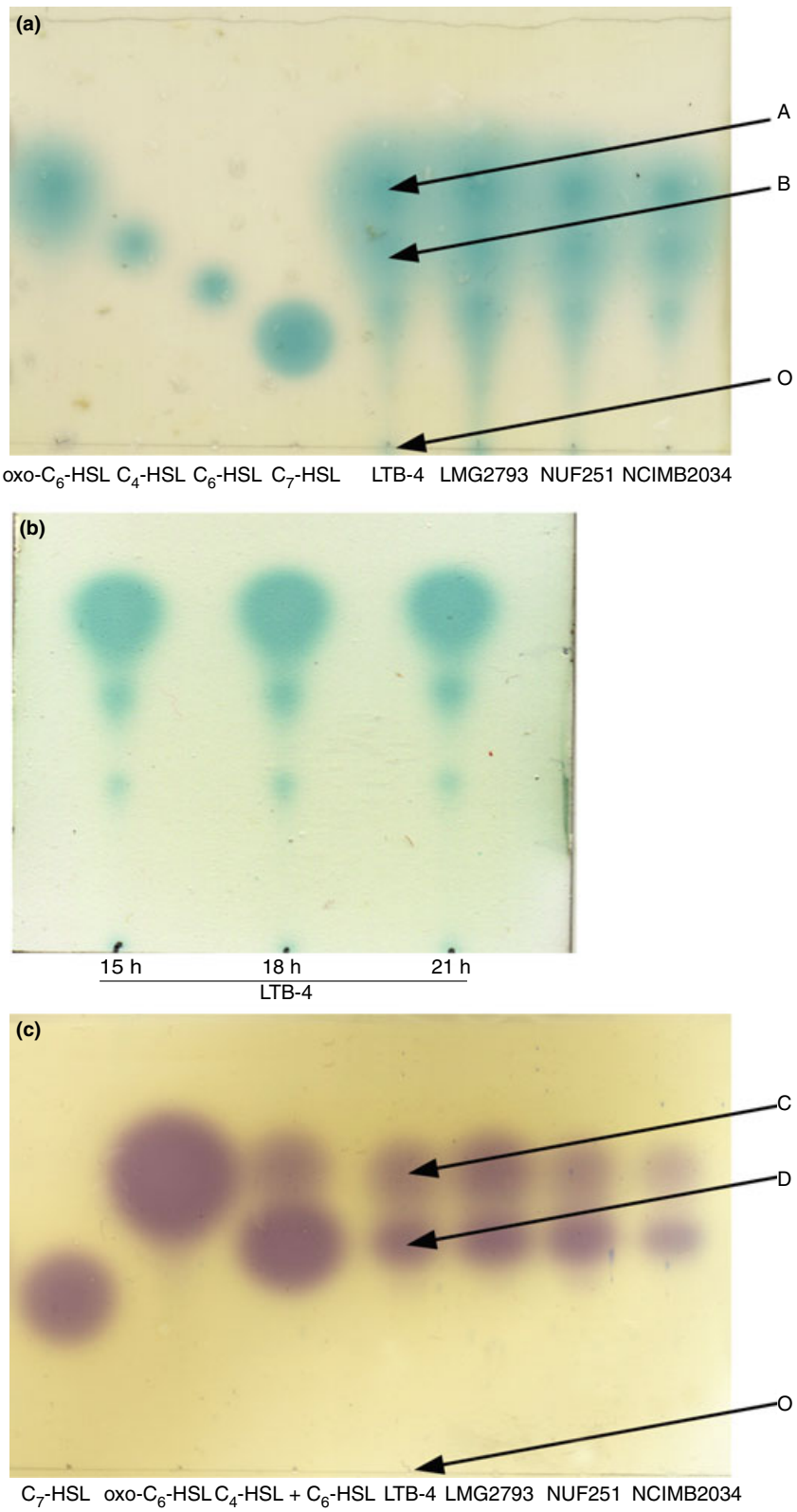

Figure 2 AHL molecules produced by *Edwardsiella tarda* LTB-4, separated by thin-layer chromatography (TLC) and detected with *Ag. tumefaciens* KYC55 (a & b) and *C. violaceum* CV026 (c). The origin (o) of TLC was marked. (a): LTB-4 and *Edw. tarda* (NUF251, LMG2793 and NCIMB2034) were tested with *Ag. tumefaciens* KYC55. Position A had the same retention factor as oxo- C_6-HSL, Position B had the same retention factor as C_4-HSL. Synthetic standards: C_4-HSL (0·5 μg), C_6-HSL (0·08 μg), oxo-C_6-HSL (0·0001 μg) and C_7-HSL (0·025 μg). (b): Identical N-acylhomoserine lactones profiles of LTB-4 extracts at 15h, 18h and 21h. (samples at 15h, 0·3μl; samples at 18h, 0·5 μl and samples at 21h, 0·3 μl). (c) LTB-4 and *Edw. tarda* (NUF251, LMG2793 and NCIMB2034) were tested with *C. violaceum* CV026. Position C and D had the same R_f value as C_4-HSL and C_6-HSL respectively. Synthetic standards: C_4-HSL (8 μg), C_6-HSL (0·8 μg), oxo-C_6-HSL (10 μg) and C_7-HSL (3 μg).

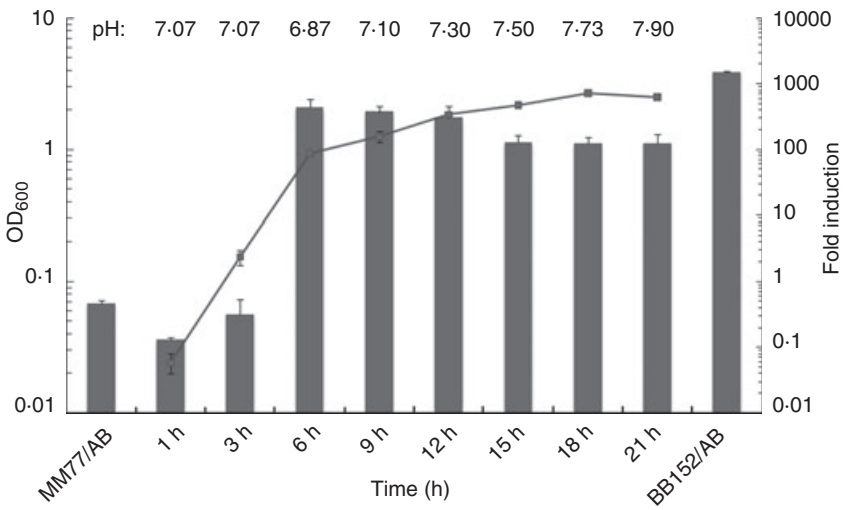

Figure 3 Induction of bioluminescence in *Vibrio harveyi* BB170 by the cell-free fluids of *Edwardsiella tarda* LTB-4. Results presented as fold induction were obtained by dividing the luminescence of the samples by the luminescence of the sterile AB medium (MM77: negative control; BB152: positive control; bars = SD). (■) AI-2 activity; (–■–) OD600.

Table 2 Induction of bioluminescence in the cholerae autoinducer 1 (CAI-1) biosensor strain *Vibrio harveyi* JAF375 by cell-free culture fluids prepared from *Edwardsiella tarda* and *Vibrio* (mean ± standard deviation of three replicates). Cell-free culture fluids from *V. harveyi* BB120 and *Escherichia coli* DH5α served as a positive control and a negative control respectively

Strain	CAI-1 activity†
Edw. tarda LTB-4 (at OD_{600} 1·84)	1·60 ± 0·07
Edw. tarda LTB-4 (at OD_{600} 2·17)	1·93 ± 0·23
Edw. tarda LTB-4 (at OD_{600} 2·69)	1·70 ± 0·19
Edw. tarda LTB-4 (at OD_{600} 2·50)	1·61 ± 0·07
Edw. tarda LMG2793	2·38 ± 0·30
Edw. tarda NCIMB2034	2·80 ± 0·09
V. anguillarum VIB72	5·95 ± 0·22*
V. harveyi VIB295	3·18 ± 0·12*
V. parahaemolyticus VIB304	3·73 ± 0·22*
V. harveyi BB120	16·80 ± 0·48*
E. coli DH5α	2·24 ± 0·19

*Significantly different from bioluminescence of *V. harveyi* JAF375 induced by cell-free culture fluids of *E. coli* DH5α ($P < 0·05$).
†CAI-1 activity is defined as the fold increase in the induction of bioluminescence that occurs in *V. harveyi* JAF375 in the presence of 50% cell-free culture fluid over that occurs when medium alone is added.

different detection threshold for different AHLs (Cha *et al.* 1998; Zhu *et al.* 2003). Here, because of the high concentration of other AHL molecules in the sample, C_6-HSL dropped below the detection limit of KYC55 at the dilution necessary to obtain clean spots for other AHLs. The 3-oxo-C_6-HSL spot, which migrated faster than the C_4-HSL molecule on the KYC55-based assay, was missing in the CV026-based assay, probably because of overlap between the 3-oxo-C_6-HSL molecule and the C_4-HSL molecule. Those two molecules were also reported to appear as one spot on the CV026 overlay by Van *et al.* (2007), as a consequence of the very small difference in R_f between those two compounds. Also, high concentration of 3-oxo-C_6-HSL could lead to a slower migration in the CV026-based assay. As TLC is not a useful technique to assign structures, analytical HPLC–mass spectrometry will be used to further determine the structure of these four AHLs produced by LTB-4.

In this study, a double-layer plate method using KYC55 was developed to detect AHL-related compounds in bacteria, and the production of blue colour quickly showed that LTB-4 produced AHL molecules. It appeared to be simple, sensitive and rapid to detect HSL-related compounds in bacteria without the necessity to prepare any cell-free culture fluids or AHLs extractions. In addition, it does not require that the biosensor strain and the strains to be tested are grown on the same medium and is independent of growth phase–linked production of AHLs by the strains tested. This method is now successfully applied to detect the AHLs production by other strains of interest in our laboratory.

Recently, it has been suggested that the growth rate of bacteria may not only be determined by nutritional and environmental factors but might also be 'self-controlled' by QS (Lazazzera 2000; Nackerdien *et al.* 2008). Quorum-sensing may play a more central role in bacterial physiology, for instance by slowing down its growth rate, suggesting that QS pathways converge with starvation-sensing pathways to regulate cell entry into stationary phase (Lazazzera 2000). LTB-4 accumulated AHL molecules during its whole growth phase, but a sharp decline of the AHLs level was observed when LTB-4 reached peak bacterial numbers (at OD_{600} 2·7), which was followed by a subsequent recovery, reaching even higher levels. The reason for this phenomenon, which was not seen in other

bacteria, is unclear and warrants further investigation. The common AHLs profiles of LTB-4 at various time points was observed on TLC plate with the activation of KYC55, which indicated that the AHL fingerprint did not change and these three kinds of AHL molecules may work together during different growth phases.

In this study, no AI-2 activity was observed when the OD_{600} was lower than 0·2. It was also reported by Surette and Bassler (1999) that the removal of the pregrowth culture fluid from these cells and re-suspension of the cells in fresh LB medium without glucose resulted in the termination of AI-2 production, or degradation of newly released AI-2 or both. Environmental factors (such as carbohydrates, pH and osmolarity) influence AI-2 production and degradation in *Salmonella typhimurium*. In LB medium, either carbohydrates or low pH is sufficient to induce the production of AI-2 in *Salm. typhimurium* LT2 (Surette and Bassler 1999). During the growth, the maximal AI-2 activity in LTB-4 was produced at OD_{600} 1 with the lowest pH (6·8). This corresponded with the result demonstrated by Zhang *et al.* (2008), in which both the AI-2 activity and the expression of $luxS_{Et}$ peaked in *Edw. tarda* TX1 at a cell density of OD_{600} 1.

Like in *Salm. typhimurium*, AI-2 activity in LTB-4 did not accumulate in stationary phase. The increasing pH value during stationary phase may be partially responsible for this phenomenon. In comparison with the AHL-induced QS, AI-2 could be the first predominant signal, functioning at early exponential phase (with the maximal level at OD_{600} 1). In *Clostridium perfringens*, the timing of toxin production, regulated by AI-2/LuxS, was critical for virulence. Interestingly, this coincides with the timing of maximal AI-2 production (Ohtani *et al.* 2002). Hence, it will be interesting to verify in *Edw. tarda* whether the production of virulence factors coincides with maximal AI-2 production (at OD_{600} 1) in trying to establish any causal link.

CAI-1 differs from AI-1 and AI-2 by being detected at extremely low cell densities (Henke and Bassler 2004a; Nackerdien *et al.* 2008). CAI-1 production was assayed either by using *V. cholerae* bioassay strain MM920 (Miller *et al.* 2002) or by using *V. harveyi* JAF375 (Defoirdt *et al.* 2008). The CAI-1 channel of the *V. harveyi* QS system was active already at quite low cell densities and led to the high background luminescence (Defoirdt *et al.* 2008). In this study, a modified method was used with *V. harveyi* JAF375, *E. coli* DH5α served as the negative control. By using this method, *Edw. tarda* NCIMB2034 and LMG2793 were shown to produce no CAI-1 activities. Either CAI-1 type molecules were not produced by LTB-4 in lag, log and stationary phase or they were not recognized by *V. harveyi* JAF375. *Vibrio harveyi* VIB295 produced a lower CAI-1 activity than BB120, which suggested that CAI-1 activity had strain-specific difference. *Vibrio anguillarum* VIB72 and *V. parahaemolyticus* VIB304 produced CAI-1 activities, which is in accordance with previous reports (Henke and Bassler 2004a; Defoirdt *et al.* 2008).

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