Microbiological Research 168 (2013) 547–554

Contents lists available at SciVerse ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

N-acylhomoserine lactone-degrading bacteria isolated from hatchery bivalve larval cultures

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ARTICLE INFO

Article history:
Received 24 October 2012
Received in revised form 26 April 2013
Accepted 27 April 2013
Available online 3 June 2013

Keywords:
Quorum sensing
Aquaculture
Quorum quenching
Marine bacteria

ABSTRACT

Quorum sensing (QS) systems, which depend on *N*-acylhomoserine lactone (AHL) signal molecules, mediate the production of virulence factors in many pathogenic microorganisms. One hundred and forty-six bacterial strains, isolated from a bivalve hatchery, were screened for their capacity to degrade five synthetic AHLs [*N*-butyryl-DL-homoserine lactone (C_4-HSL), *N*-hexanoyl-DL-homoserine lactone (C_6-HSL), *N*-octanoyl-DL-homoserine lactone (C_8-HSL), *N*-decanoyl-DL-homoserine lactone (C_{10}-HSL) and *N*-dodecanoyl-DL-homoserine lactone (C_{12}-HSL)] using well diffusion agar-plate assays with three biosensors, *Chromobacterium violaceum* CV026, *C. violaceum* VIR07 and *Agrobacterium tumefaciens* NTL4 (pZLR4). The results of these assays led to our choosing four strains (PP2-67, PP2-459, PP2-644 and PP2-663) that were able to degrade all five synthetic AHLs, thus showing a wide spectrum of quorum quenching (QQ) activity. We subsequently confirmed and measured the QQ activity of the four strains by high-performance liquid chromatography plus mass-spectrometry analysis (HPLC–MS). One of the strains which showed the highest AHL-degrading activity, PP2-459, identified as being a member of the genus *Thalassomonas* was chosen for further study. Finally, using thin-layer chromatography (TLC), we went on to confirm this strain's capacity to degrade the AHLs produced by other non-pathogenic and pathogenic bacteria not taxonomically related.

1. Introduction

Bacterial diseases in bivalve hatcheries are a considerable limiting factor in commercial aquaculture and result in substantial economic losses. For many years treatment with antibiotics has been the only viable strategy to tackle the problem. Nevertheless, their use does nothing to guarantee the ultimate success of larval cultures and their overuse has resulted in the development of resistance. The seriousness of the situation has recently promoted the exploration of novel strategies to control marine pathogens, but only some of them, such as the use of probiotics, are applicable to bivalve aquaculture (Defoirdt et al. 2007a, 2011a; Prado et al. 2010; Verschuere et al. 2000). One of the most promising alternatives is based on the inhibition of the expression of virulence genes, regulated in many aquaculture pathogens by bacterial cell-to-cell signalling, known as quorum sensing (QS) (Bjarnsholt et al. 2011; Dong et al. 2007; González and Keshavan 2006; Natrah et al. 2011). QS is a population-density-dependent gene-expression mechanism which involves the production of signal molecules known as autoinducers, a ubiquitous phenomenon in bacteria (González and Marketon 2003). The most thoroughly characterized Gram-negative, bacterial intraspecific autoinducers are *N*-acylhomoserine lactones (AHLs), which have been reported to accumulate in the culture medium as the density of the population increases, and on reaching a critical threshold concentration bind to an AHL-receptor protein belonging to the LuxR family of transcriptional regulators. The activated LuxR/AHL complex then binds specific DNA sequences, resulting in the activation or repression of target genes, including in many cases the activation of important virulence phenotypes (Eberhard et al. 1991; Fuqua et al. 1994; Natrah et al. 2011).

It has been reported that some aquatic organisms such as micro-algae, macro-algae, invertebrates and also other bacteria have the potential to disrupt QS by means of various different mechanisms (Natrah et al. 2011). One such mechanism involves the production of compounds that interfere with the detection of signal molecules; these compounds, known as quorum sensing inhibitors (QSIs), were first described in the red marine alga *Delisea*

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http://dx.doi.org/10.1016/j.micres.2013.04.011

pulchra. This alga synthesizes halogenated furanones, which protect both fish and shrimp from vibriosis (Givskov et al. 1996; Rasch et al. 2004). The same mechanism that inhibits QS in aquaculture pathogens has been described in the marine bacterium *Halobacillus salinus* (Teasdale et al. 2008). A second strategy, known as quorum quenching (QQ), is the enzymatic inactivation of AHLs by the production of acylases or lactonases, as described elsewhere with reference to the fish pathogen *Tenacibaculum maritimum* (Defoirdt et al. 2011b; Romero et al. 2010, 2011; Tait et al. 2005). Thus, the use of AHL-degrading bacteria have obtained success when cultures from the intestinal tract of healthy shrimp and fish enriched in AHL-degrading enzymes increase the survival rate of turbot larvae (*Scophthalmus maximus*) (Tinh et al. 2008) and of giant freshwater prawns (*Macrobrachium rosenbergii*) (Nhan et al. 2010).

Within this context we have studied the capacity of a number of marine bacteria isolated from a bivalve hatchery to disrupt AHL-mediated QS systems in bivalve pathogens.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The 146 strains tested in this study were isolated from successful larval cultures of different bivalve species at a bivalve hatchery in Galicia (NW Spain) and belong to the culture collection of the research group of the University of Santiago de Compostela involved in this work (Table 1). They are all Gram-negative rods with respiratory metabolism and grow best at about 2.5–3% NaCl (w/v), classified according to Kushner and Kamekura (1988) as marine bacteria.

These 146 marine bacteria and *Vibrio anguillarum* ATCC 19264^T were routinely cultured at 20 °C in marine broth (MB, Difco). *Halomonas anticariensis* FP35^T (CECT 5854^T) (Martínez-Cánovas et al. 2004) was cultured at 32 °C in MY medium (3 g malt extract, 3 g yeast extract, 10 g glucose and 5 g peptone per litre) (Haynes et al. 1955; Moraine and Rogovin 1966) modified with a balanced mixture of sea salts (Rodríguez-Valera et al. 1981). *Agrobacterium tumefaciens* NTL4 (pZLR4) (Shaw et al. 1997; Chilton et al. 1974) was cultured at 32 °C in Luria Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl per litre) supplemented with 2.5 mmol l^{-1} $CaCl_2$ and 2.5 mmol l^{-1} $MgSO_4$ (LB/MC), in MGM minimal medium (11 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1 mg biotin, 27.8 mg $CaCl_2$ and 246 mg $MgSO_4$ per litre) or AB medium (3 g K_2HPO_4, 1 g $Na_2H_2PO_4$, 1 g NH_4Cl, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.15 g KCl, 0.01 g $CaCl_2$, 0.0025 g $FeSO_4{\cdot}7H_2O$ and 5 g glucose per litre) containing 50 μg gentamycin ml^{-1}. *Chromobacterium violaceum* CV026 (McClean et al. 1997) and *C. violaceum* VIR07 (Morohoshi et al. 2008) were grown at 32 °C in LB medium supplemented with kanamycin (50 μg ml^{-1}).

2.2. Screening for AHL-degradation activity using synthetic AHLs

The potential QQ activity of the 146 isolates was tested by well diffusion agar-plate assays as described elsewhere (Romero et al. 2011). Briefly, synthetic AHLs were added at 10 μmol l^{-1} to 500 μl of an overnight culture of each marine bacterial strain in MB medium and incubated for 24 h at 20 °C and 150 rpm rotary shaking. The same quantities of AHLs were added to 500 μl of cell-free MB media and incubated for 24 h at 20 °C as negative controls. In parallel, each marine bacterial strain was also cultivated in MB medium with no exogenous AHLs added for 24 h at 20 °C to detect its own production of AHLs. The AHLs in each overnight culture were detected on agar plates covered with 5 ml of semi-solid LB agar containing 500 μl of overnight cultures of indicator strains.

Table 1
Marine bacteria showing QQ activity against all synthetic AHLs tested.

Origin	No. of strains tested	Isolates showing QQ activity	C_4-HSL (10 μmol l^{-1})	C_6-HSL (10 μmol l^{-1})	C_8-HSL (10 μmol l^{-1})	C_{10}-HSL (10 μmol l^{-1})	C_{12}-HSL (10 μmol l^{-1})	Phylogenetically related species	% identity
Pod razor clam (*Ensis siliqua*)	15	PP2-67	+	+	+	+	+	*Alteromonas marina*	99.44
Carpet-shell clam (*Venerupis decussata*)	61	PP2-459	+	+	+	+	+	*Thalassomonas agariperforans*	96.65
		PP2-644	+	+	+	+	+	*Alteromonas stellipolaris*	99.93
Manila clam (*V. philippinarum*)	36	PP2-663	+	+	+	+	+	*Paracoccus homiensis*	97.43
Pullet carpet shell (*V. corrugata*)	27	–							
Groover razor clam (*Solen marginatus*)	7	–							