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Cloning, expression and characterization of two S-ribosylhomocysteine lyases from *Lactobacillus plantarum* YM-4-3: Implication of conserved and divergent roles in quorum sensing

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ABSTRACT

Quorum sensing (QS) is a means of cell-to-cell communication that regulates, via small signalling molecules, expression of a series of genes and controls multicellular behaviour in many bacterial species. The enzyme S-ribosylhomocysteine lyase (LuxS) transforms S-ribosylhomocysteine (SRH) into 4, 5-dihydroxy-2, 3-pentanedione (DPD), the precursor of the interspecies QS signalling molecule autoinducer-2 (AI-2). In this study, two LuxS-coding genes, *luxS1* and *luxS2*, with 70% sequence identity were isolated from *Lactobacillus plantarum* YM-4-3, and overexpressed in *Escherichia coli* BL21 (DE3), and the protein products were purified successfully. After incubation of LuxS1 or LuxS2 with SRH, the reaction products were able to induce *Vibrio harveyi* BB170 bioluminescence, clearly demonstrating that both LuxS1 and LuxS2 synthesize AI-2 from SRH *in vitro*. Ellman's assay results revealed optimal temperatures for LuxS1 and LuxS2 of 45 and 37 °C, respectively, and their activities were stimulated or inhibited by several metal ions and chemical reagents. In addition, enzyme kinetics data showed that K_m, V_{max} and K_{cat} value of LuxS1 for the substrate (SRH) were higher than that of LuxS2. These results suggest that LuxS1 and LuxS2 mediate QS in a temperature-dependent manner and may play conserved roles in AI-2 synthesis but exhibit different activities in response to external environmental stress. To our knowledge, this paper is the first report of two *luxS* genes present in one bacterial genome and the subsequent comparative elucidation of their functions in AI-2 production. Collectively, our study provides a solid basis for future research concerning the AI-2/LuxS QS system in *L. plantarum* YM-4-3.

1. Introduction

Quorum sensing (QS) is a process that allows bacteria to communicate with each other, and its function relies on production, release, detection and group-level response to extracellular signalling molecules called autoinducers (AIs) [1]. As the density of a bacterial population increases, the autoinducer concentration in the external environment also increases [2], followed by activation or repression of a target sensor kinase or response regulator, which facilitates expression of QS-dependent genes, once a critical threshold of AIs has been reached [3]. Evidence indicates that many physiological processes, such as biofilm formation, virulence factor production, cell adhesion, competence development and stress adaptation, are regulated by the QS system [4]. In 1979, Nealson and Hastings found that bioluminescence of the marine bacterium *Vibrio fischeri* was dependent on cell density [5], a finding that initiates research in the QS field. The following three types of QS signalling molecules have been reported to date: acylated homoserine lactone, typically produced by Gram-negative bacteria; an oligopeptide termed autoinducing peptide, typically utilized by Gram-positive bacteria; and the autoinducer-2 (AI-2) signalling molecule, a furanosyl borate diester found in both Gram-negative and -positive bacteria and is therefore considered to be a universal signalling molecule allowing interspecies bacterial communication [4,6,7]. In most environmental niches, bacteria exist in complex communities, and production and consumption of AI-2 by bacteria should have reciprocal effects on gene regulation [8], causing the AI-2 mediated QS system to be highly important.

AI-2 is produced from S-adenosylmethionine (SAM) in three enzymatic steps [9]. Briefly, SAM donates a methyl group and is converted to S-adenosylhomocysteine (SAH). The toxic SAH is then quickly removed by a nucleosidase to produce S-ribosylhomocysteine (SRH). Finally, SRH is transformed to homocysteine and 4, 5-dihydroxy-2, 3-pentanedione (DPD), which is the precursor of AI-2 that forms as a result of the spontaneous rearrangement of DPD, by S-

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ribosylhomocysteine lyase (LuxS) [10]. The gene encoding LuxS, *luxS*, which has been found in both Gram-negative and -positive bacteria, is highly conserved among many different bacterial species [11]. As homocysteine is a key component of the cell activated methyl cycle (AMC), an important metabolic process in bacterial methionine metabolism [12], LuxS plays important roles in both bacterial communication and metabolism.

AI-2 regulates biofilm formation and virulence in opportunistic bacteria, including *Pseudomonas aeruginosa* PAO1 and *Escherichia coli* O157:H7 [13,14]. There are also reports that *luxS* has an important impact on probiotics with respect to tolerance to the various stress conditions of the human gastrointestinal (GI) tract, conferring colonization resistance against pathogens in the GI tract through competitive adhesion or via direct inhibition through production of antimicrobial compounds, such as lactic acid, hydrogen peroxide and bacteriocins [15–17]. Although AI-2-mediated QS systems have drawn much attention, there is little information regarding AI-2 from *Lactobacillus plantarum*, a versatile and widespread microorganism that is found in materials and environments ranging from vegetables, dairy products and meat fermentation products to the human GI tract [18]. Indeed, many *L. plantarum* strains are recognized as probiotics with claims of health benefits to the consumer, and their probiotic effects possibly regulated by AI-2 need to be determined.

In this study, bioinformatic analysis revealed two *luxS* genes in the genome of *L. plantarum* YM-4-3. To our knowledge, this is the first report of two different *luxS* genes present in one organism. To explore the functions of these two *luxS* genes, heterologous expression and protein purification were performed, and activity on AI-2 synthesized *in vitro* using a reporter strain *V. harveyi* BB170 bioassay was determined. Finally, the effects of temperature, various metal ions and chemical reagents on enzyme activities, and the enzyme kinetic parameters were assessed.

2. Materials and methods

2.1. Bacterial strains and culture media

The bacterial strains, plasmids and primers used in this study are listed in Table 1. *L. plantarum* YM-4-3, a putative probiotic organism isolated from Douchi, a traditional Chinese fermented soybean food, was cultivated in de Man, Rogosa and Sharpe (MRS) medium. *E. coli* DH5α and BL21 (DE3) were cultured in Luria Broth (LB) liquid medium or plated on LB agar. When necessary, 100 μg/mL ampicillin (Amp) (Sigma, Shanghai, China) or 50 μg/mL kanamycin (Kan) (Sigma, Shanghai, China) were used for screening *E. coli* transformants. *V. harveyi* BB170 was obtained from American Type Culture Collection (ATCC, Manassas, USA), cultivated in autoinducer bioassay (AB) medium and used for the AI-2 bioluminescence assay [19].

2.2. Cloning of luxS genes

The nucleotide sequence of one *luxS* gene from *L. plantarum* WCFS1 was chosen as a query, and two LuxS-coding genes, named *luxS1* and *luxS2*, in the *L. plantarum* YM-4-3 genome, were obtained by a local BLAST search with E-values $< 1e^{-5}$. Primer sets (luxS-F and luxS1-R) and (luxS-F and luxS2-R) (Table 1) were designed based on the *L. plantarum* YM-4-3 genomic DNA sequence. *L. plantarum* YM-4-3 total genomic DNA was extracted using a DNAprep Pure Bacteria Kit (Bioteke, Beijing, China) and used as the template in a polymerase chain reaction (PCR) under the following conditions: 95 °C for 3.0 min; 35 cycles at 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 50 s; a final extension of 5 min at 72 °C. The products were purified using TIANgel Midi Purification Kit (Tiangen, Beijing, China), cloned into the pMD18-T vector (Takara, Dalian, China) and sequenced.

2.3. Sequence and phylogenetic analysis of luxS1 and luxS2

The theoretical isoelectric points and molecular weights (MWs) of the deduced proteins of *luxS1* and *luxS2* were calculated using the pI/MW tool (http://web.expasy.org/compute_pi/). An online homology search was performed using BLAST algorithms in NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST), and LuxS1 and LuxS2 homologous sequences from different bacteria were downloaded. Then, the amino acid sequences of LuxS1, LuxS2 and other LuxS proteins from different bacteria were aligned using the DNAman software package (Lynnon Biosoft, San Ramon, USA). The putative three-dimensional (3D) structures were predicted based on homology modelling with the LuxS protein crystal structure of *Streptococcus suis* HA9801 (PDB code 4XCH, http://www.rcsb.org/pdb/explore/explore.do?structureId=4xch) [20] using the Clustal X2.0 and ESPript/ENDscript 3.0 software package (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). A phylogenetic tree was constructed using the Neighbour-Joining (NJ) method with MEGA version 7.0 [21]. The GenBank accession nos. for LuxS1 and LuxS2 are ETF12584 and ETF12641, respectively.

2.4. Expression of luxS1 and luxS2

The *luxS1* and *luxS2* gene fragments were digested with *Hin*d III and *Xho* I to obtain products with sticky ends, which were then cloned into the pET28-A vector digested with the same restriction enzymes. The recombinant plasmids pET28-*luxS1* and pET28-*luxS2* were transformed into competent *E. coli* BL21 (DE3) cells. Putative positive transformants were selected by plating on LB agar containing 50 μg/mL Kan and used for expression as described by Zhang et al. [22]. Single positive pET28-*luxS1* and pET28-*luxS2* transformants were cultured in 5 mL LB medium for 12 h at 37 °C in an agitating incubator (175 rpm/min). The cells were further cultured in 200 mL LB medium in a 500 mL shaking flask under the same conditions until the culture reached an OD_{600} value of 0.4–0.6. Recombinant protein expression was induced by adding

Table 1
Characteristics of bacterial strains, plasmids and primers used in this study.

Strain, plasmid and primer		Relevant characteristic or sequence (5′-3′)[a]	Source
Strains	*L. plantarum* YM-4-3	Wild type	This study
	E. coli DH5α	F^-, *endA1*, *glnV44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *deoR*, *nupG*, Φ80d*lacZ* ΔM15, Δ(*lacZYA-argF*) U169, *hsdR17*(rK^-, mK^+), λ^-	Takara
	E. coli BL21 (DE3)	F^-, *ompT*, *gal*, *dcm*, *lon*, *hsdSB*(rB^-, mB^-), λ(DE3 [*lacI*, *lacUV5-T7* gene 1, *ind1*, *sam7*, *nin5*])	Takara
	V. harveyi BB170	$AI\text{-}1^-$, $AI\text{-}2^+$	ATCC
Plasmids	pMD18-T	Cloning vector, Amp^R	Takara
	pET28-A	Prokaryotic expression vector, Kan^R	Takara
	pET28-*luxS1*	pET28-A containing the *luxS1* of *L. plantarum* YM-4-3, Kan^R	This study
	pET28-*luxS2*	pET28-A containing the *luxS2* of *L. plantarum* YM-4-3, Kan^R	This study
Primers	luxS-F	CGAAGCTTTGGCTAAAGTAGAAAGTT	This study
	luxS1-R	ATTCTCGAGCTATTCAACGACTTTGCG	This study
	luxS2-R	ATACTCGAGCTAGATAACGTTACGGTTG	This study

[a] The underlined nucleotides indicate restriction enzyme sites for *Hin*d III (AAGCTT) or *Xho* I (CTCGAG).