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THE PROBIOTIC *Shewanella putrefaciens* PDP11 TARGET VIRULENCE FACTORS BY MODULATING QUORUM SENSING INHIBITION

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Introduction

Bacteria communicate with each other by producing signal molecules and regulating the production of virulence factors that have importance in pathogenicity. Quorum sensing (QS) is a bacterial communication mechanism based on the perception of population density and secretion of determining signal molecules called autoinducers (AI) such as the case of Acylhomoserine lactones (AHLs) (Borges et al. 2019). AHLs-mediated QS processes seem to be common in the marine environment and among marine pathogenic bacteria, which pathogenesis could be mitigated by probiotics (Kuebutornye et al. 2020), among others. Probiotics are defined as live microbial cells that confer health benefits to the host and some of their mechanisms include the production of antagonistic compounds that are inhibitory toward pathogens (Zhou et al. 2018). In this sense, Shewanella putrefaciens Pdp11, a strain described as a probiotic for use in aquaculture, has been analysed to mediate QS processes by quorum-quenching assays using synthetic AHLs.

Material and methods

The detection of AHLs was done as previously described (Torres et al. 2016). *S. putrefaciens* Pdp11 was cultured in tryptone soy agar plates supplemented with NaCl (1.5%) (TSAs) at 23ºC for 24h. Then, the probiotic Pdp11 was grown in 2 mL of tryptone soja broth supplemented with NaCl (1.5%) (TSBs) at 23ºC for 24h under agitation (120 rpm). After 24h, a stock solution of N-butyryl- DL-homoserine lactone (C4-HSL, Sigma-Aldrich), N-hexanoyl- DL-homoserine lactone (C6-HSL, Sigma-Aldrich), N-octanoyl- DL-homoserine lactone (C8-HSL, Sigma-Aldrich), N-decanoyl- DL-homoserine lactone (C10-HSL, Sigma-Aldrich), N-dodecanoyl- DL-homoserine lactone (C12-HSL, Sigma-Aldrich), N-(3-Oxodecanoyl)-L-homoserine lactone (3-oxo-C8-HSL, Sigma-Aldrich), N-(3-decanoyl)-L-homoserine lactone (3-oxo10-C-HSL, Sigma-Aldrich) and N-(3-Oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL, Sigma-Aldrich) were added to achieve a final concentration of 2μg/mL (10μM), and incubated for further 24h. At the same time, an overnight culture of the biosensor *Chromobacterium violaceum* CV026 and VIR07, maintained on Luria-Bertani (LB) plates supplemented with 50μg/mL of kanamycin, and *A. tumefaciens* NTL4 (pZLR4), maintained on LB plates supplemented with 50μg/mL of gentamycin, was diluted to 1:10 in 5 mL of LB soft 0.7% (w/v) agar and poured respectively onto LB agar plates for CV026 and VIR07, and LB medium supplemented with X-gal for NTL4. Then, 6-mm-diameter wells were hollowed in the medium with the back of a sterile Pasteur pipette and 100 µL-aliquots of each Pdp11 culture were loaded in the wells. Simultaneously, 2mL of TSBs were incubated containing AHLs as positive control. The plates were incubated at 28ºC for 24h to check for the presence or absence of a coloured halo around the wells.

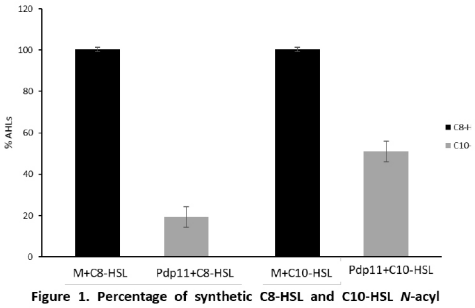

Figure 1. Percentage of synthetic C8-HSL and C10-HSL *N*-acyl homoserine lactones (AHL) measured in the cell-free culture media by degradation of the strain *S. putrefaciens* Pdp11 after 24h. Cell-free medium (M) added HSLs present 100% activity and was used as negative control. Initial AHL concentration was 10 mM. Error bars represent one standard deviation.

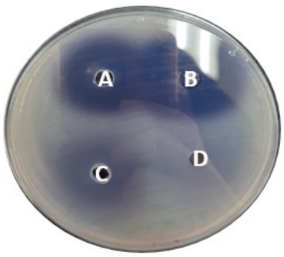

Figure 2. Recovery of synthetic C8-HSL after acidification visualized on agar plate assay by means of the biosensor strain *Chromobacterium violaceum* (CV026). Cell-free medium added with synthetic C8-HSL AB), synthetic C8-HSL extracted by pH7 C) and pH2 D). Scale bar, 1cm.

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To determine whether the QQ activity was related, C8-HSL was added as above in overnight cultures of the degrading strain Pdp11, which was incubated for a further 24h at 23ºC under shaking (120 rpm). The mixtures were then centrifuged at 10000g for 2min, one supernatants were extracted with equal volumes of acetonitrile (1:1) and another supernatants were acidified with HCl 1N to pH 2 and incubated for 24h at 23ºC on shaking. Finally, the remaining AHLs were suspended in methanol 70% (v/v) and detected by well diffusion agar-plate assays using sterile discs and the corresponding biosensor as above. At the same time, to confirm AHL degradation, the final concentration of it in the culture media was evaluated using HPLC-MS.

Results and discussion

Figure 1 shows the degradative activity of *S. putrefaciens* Pdp11 of the synthetic C8- and C10-HSL after 24h. The enzymatic activity is estimated at around 80% and 30% for C8- and C10-HSL, respectively, while the rest of AHLs tested were not degraded by the Pdp11 strain. It would be an interesting feature of the probiotic Pdp11 strain since these AHLs are related to facilitating microbial adhesion by promoting biofilm formation among other virulence factors related to pathogens (Fan et al. 2019).

On the other hand, a distinctive feature of AHL inactivated by lactonase is that it can be reactivated by acid treatment (Shaheer et al. 2021). In this way, little C8-AHL was recovered when it is extracted to pH2 (Figure 2), which indicates the enzyme activity is not derived from the hydrolysis of the lactone ring derived from the action of lactonases (Yates et al., 2002), suggesting the enzyme activity in Pdp11 could be an AHL-acylase. The potential QQ activity of Pdp11 was unknown so, these preliminary studies led to a further as another promising probiotic QQ tool for aquaculture.

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