New insight into the regulation of class II bacteriocin production by quorum sensing in *Lactobacillus pentosus* 31-1

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The biosynthesis of class II bacteriocins is frequently regulated by a quorum-sensing regulatory mechanism mediated by autoinducing peptide. Here, it was preliminarily confirmed that bacteriocin production by *Lactobacillus pentosus* 31-1 was controlled by a quorum sensing (QS) system mediated by peptide signal molecules in the cell-free supernatant (CFS). Effects of the growth medium and inoculum size on bacteriocin producing in *L. pentosus* 31-1 were investigated. Bacteriocin production could be detected even when *L. pentosus* 31-1 was diluted 10⁸-fold in MRS broth and incubated at 37°C for 24 h. However, by dilution of the culture 200-fold or more in skim milk medium, no bacteriocin activity could be detected after 60 h of incubation. Bacteriocin production could be switched on in these cultures after the addition of CFS from a bacteriocin-producing culture, and the active components in the CFS were shown to be peptides in nature. FII-22, one of the fractions purified from the CFS was identified as the putative inducing peptide (IP). Regulation of bacteriocin production in *L. pentosus* 31-1 by FII-22 was affected by the additional time and amount. The optimal induction of bacteriocin production effect was obtained by addition of 0.5 µg/ml (final concentration) FII-22 to the culture after 8 h of incubation. This study provided a novel perspective on screening for QS regulated class II bacteriocin producing lactic acid bacteria. This study may assist in a deeper understanding of how QS works on regulation of class II bacteriocin production.

Key words: Quorum sensing, class II bacteriocin, *Lactobacillus pentosus*, inducing peptide, skim milk medium.

INTRODUCTION

Class II bacteriocins are unmodified, heat-stable, low-molecular-mass (< 10 kDa), hydrophobic peptides, usually characterized by a G-G-Xaa processing site in the bacteriocin precursor. Class II bacteriocins produced by lactic acid bacteria (LAB) have been gaining attention as one of the most interesting and potential groups of antimicrobial peptides in food preservation (Cleveland et al., 2001; Drider et al., 2006; Zhang et al., 2010).

Recent studies exhibit increasing interests on the regulation systems of class II bacteriocins producing LAB, and quorum sensing (QS) system has been shown to be involved in the regulation of class II bacteriocins synthesis. The QS system which regulated class II bacteriocin production is composed of an induction peptide (IP), a histidine protein kinase (HPK) and a response regulator (RR) (Straume et al., 2007). IP is the indicator of cell density, functioning as the input signal. Upon reaching a critical threshold concentration, IP activates transcription of a defined set of genes (Nes et al., 1996). This QS mechanism has been found in several class II bacteriocin producing LAB, including
Carnobacterium piscicol (Axelsson and Holck, 1995; Kleerebezem et al., 2001; Quadri et al., 1997; Saucier et al., 1997), Lactobacillus plantarum (Brurberg et al., 1997; Diep et al., 1996; Maldonado-Barragán et al., 2009; Maldonado et al., 2004; Navarro et al., 2008; Ruiz-Barba et al., 2010), L. salivarius (Flynn et al., 2002), L. sake (Brurberg et al., 1997; Diep et al., 2000), Enterococcus faecium (Nilsen et al., 1998; O’Keeffe et al., 1999), Streptococcus thermophilus (Fontaine et al., 2007), L. acidophilus (Dobson et al., 2007; Tabasco et al., 2009) and C. maltaromaticum (Rohde and Quadri, 2006). However, the existence of QS system in L. pentosus has not yet been demonstrated.

Strategies used for identification of QS system vary greatly among species. N-acyl homoserine lactones (AHL)-mediated QS systems in most gram-negative bacteria are often identified by detecting AHL, and several methods to detect AHLS have been developed (Ahmer et al., 2007; Middleton et al., 2002; Steindler and Venturi, 2007). However, great difficulties were encountered in confirmation of IP-mediated QS in class II bacteriocins producing LAB, since the signal molecules involved in IP-mediated QS system have not yet been characterized adequately. The presence of IP was usually assessed using bacteriocin negative (Bac') phenotype, but the methods to obtain Bac' phenotype have not been well established. Generally, Bac' phenotype was obtained by starting a new culture with a highly diluted inoculum or incubating the culture under suboptimal conditions (Diep et al., 2000; Flynn et al., 2002; Quadri, 2003). Another method used in the identification of (putative) peptide-based QS systems was in silico screening described by Sturme et al. (2007). Nevertheless, traditional methods are still playing an irreplaceable role in screening of class II bacteriocin producing LAB for QS system.

L. pentosus 31-1, a class II bacteriocin producing strain (Liu et al., 2008; Zhang et al., 2009) which was isolated from Xuan-Wei Ham, a traditional Chinese fermented meat product, was used throughout this study. The aim of this study was to investigate how inoculums size and culture media affect bacteriocin producing in L. pentosus 31-1, obtain a Bac' culture, establish an approach for preliminarily unraveling the involvement of QS in the regulation of bacteriocin production in L. pentosus 31-1, and determine the effect of time of addition and the addition amount of inducing peptide on bacteriocin production in L. pentosus 31-1.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacteriocin-producing strain L. pentosus 31-1 was grown in MRS incubated at 37°C. L. plantarum PL2 used in this study was deposited in Applied Microbiology Laboratory China Agricultural University, and was isolated from fermented cabbage pickles. L. plantarum PL2 was cultivated in MRS medium at 37°C and was used as the indicator strain in bacteriocin activity assay.

Preparation of cell-free supernatant and assay for bacteriocin activity

The cells were removed by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant was withdrawn and adjusted to pH 6.5 with 2.5 mol/l NaOH, then sterilized by filtering through 0.22 μm-size filters to obtain a cell-free supernatant (CFS).

Determination of bacteriocin activity of the CFS was performed using agar-well diffusion method (Mayr-Hartling et al., 1972). Results were expressed as diameter of the inhibition zone, which was measured by a caliper. All experiments were done in quadruplicate.

Preliminary characterization of the inducers in CFS

Sensitivity to proteolytic enzymes was tested by treatment of the CFS with the following enzymes: pepsin (Sigma), trypsin (Sigma) or proteinase K (Sigma) for 2 h at 37°C at a final concentration of 1 mg/ml. The enzymes were inactivated by incubating at 80°C for 10 min. The thermal sensitivity of the inducers was tested by boiling the CFS for 20 min. After these treatments, the residual inducing activity of the CFS was determined as described below, and untreated CFS was used as control.

Preparation of peptide fractions

The peptide present in the CFS was precipitated with ammonium sulfate (400 g/l) by stirring for 24 h at 4°C, and pelleted by centrifugation (10,000 x g, 20 min, 4°C), then dissolved in buffer A (20 mM-sodium phosphate, pH7.0). The resultant crude peptides were fractionated using two different Ultra-filtration (UF) membranes with molecular weight of 3 and 1 kDa cut-offs (MWCO) (Millipore, Bellerica, MA, USA). The peptides which could not pass through the 3 kDa membrane were designated as FI, those could not pass through the 1 kDa membrane but could pass through the 3 kDa membrane were designated as FII. These fractions were lyophilized.

The peptide concentrations of these partial purified peptides were determined according to the method described by Lowry et al. (1951), using bovine serum albumin as a standard. The bacteriocin inducing activity was determined after each step. Fractions FI and FII were dissolved in buffer B (20 mM sodium phosphate, pH 7.0; 1 M (NH₄)₂SO₄) at a concentration of 5 mg/ml, and applied to a hydrophobic-interaction chromatography column (30 x 1.0 cm) of Octyl-Sepharose 4FF (RuiDaHengHui) (Eijssink et al., 1996). The sample (0.5 ml) was applied onto the column at a flow rate of 1.5 ml/min equilibrated with buffer B, and eluted with a linear gradient of 1.0 to 0.0 M (NH₄)₂SO₄ in buffer A. The absorption was measured at 220 nm to detect protein peaks. Fractions were collected separately according to the peptide elution profile. The fractions were lyophilized, and bacteriocin inducing activity was determined after desalting with 1 kDa cut-offs UF membranes. The fractions (0.5 ml) showed inducing activity were dissolved in buffer A at a concentration of 5mg/ml, and subjected to gel filtration chromatography on a Sephadex G-10 (Pharmacia) column (0.7 x 18.5 cm) at a flow rate of 0.3 ml/min equilibrated with buffer A. The column was washed with the same buffer. Fractions were collected separately according to the peptide elution profile. After desalting through a 1 kDa UF membrane, these fractions were lyophilized.

Bacteriocin induction assay

The Bac' culture was prepared by diluting a 10-h-old culture of L.
p. s. 31-1 400-fold into separate 100-ml aliquots of skim milk medium. In order to induce bacteriocin production, different amounts of CFS from a bacteriocin-producing culture was added to the Bac culture, and the mixture was incubated at 37°C for appropriate period of time, then the bacteriocin activity was tested. Bac cultures without addition of any inducer were used as controls. Also, MRS broth or the peptide fractions which were purified from the CFS of bacteriocin-producing cultures were used in induction experiments. The inducing activity was presented in terms of the diameter of the inhibition zone.

Effect of inoculums size on bacteriocin production of L. pentosus 31-1

A 10-h-old culture (2 × 10⁵ CFU/ml) of L. pentosus 31-1 was diluted in MRS broth or skim milk medium, and incubated at 37°C for an appropriate period of time. Bacteriocin activity was assayed as described above.

Effect of addition time and addition amount of FII-22 on bacteriocin production of L. pentosus 31-1

FII-22 was applied to the Bac culture of L. pentosus 31-1 at 0, 4, 8 or 12 h, respectively. The inhibition zone was detected every 2 h from the 48th to the 60th h.

FII-22 was added to the Bac culture at various peptide concentrations (0.1, 0.5, 2.5, 25 and 750 μg/ml) after it was cultured for 8 h. The inhibition zone was determined at 60 h.

The Bac cultures which were prepared by dilution a 10-h-old culture of L. pentosus 31-1 100-fold in skim milk medium without the addition of FII-22 and incubated at 37°C for the same period of time served as a control.

RESULTS

Effect of inoculums size on bacteriocin production of L. pentosus 31-1 in MRS broth

To study whether bacteriocin production by L. pentosus 31-1 was an inducible process, a 10-h-old culture of L. pentosus 31-1 was 10-fold serial diluted in MRS broth bacteriocin production could be detected at all inoculums levels, even when L. pentosus 31-1 was diluted up to 10⁶-fold (Ca. 10-100 CFU/ml), whereas small inoculums size (Ca. 10⁴ - 10⁶ CFU/ml) always led to spontaneously lose of bacteriocin synthesis in QS-regulated class II bacteriocin producing bacteria (Nes and Eijsink, 1999).

Effect of inoculums size on bacteriocin production of L. pentosus 31-1 in skim milk medium

To avoid signal interference from unidentified compounds in MRS medium, we used skim milk medium for further research. As shown in Table 2, bacteriocin production by L. pentosus 31-1 was associated with inoculums size when cultivated in skim milk medium. When a 10-h-old culture of L. pentosus 31-1 was diluted 50-fold or 100-fold in skim milk medium, bacteriocin production could be detected after 60 h of incubation at 37°C, whereas bacteriocin production was abolished when the culture was diluted 200-fold or 400-fold in skim milk medium.

Inducing bacteriocin production

CFS or MRS broth was added to the Bac culture, which was then incubated at 37°C for 60 h, followed by determination of bacteriocin activity. The results given in Table 3 showed that addition of ≥5 μl/ml CFS or ≥ 200 μl/ml MRS broth to Bac culture all regained its ability to produce bacteriocin. Influence of background bacteriocin activity from the CFS was also taken into account. Subculture of L. pentosus 31-1 Bac culture in skim milk medium could not lead to bacteriocin production. Complete inactivation of the inducers was observed after the CFS or MRS broth was treated with pepsin, trypsin or proteinase K, which therefore identified them as proteinaceous substances. The inducing activity was not significantly altered by boiling the CFS for 20 min, showing that the activity of the inducers were heat resistant. These results preliminarily confirmed that and incubated at 37°C for 24 h. As shown in Table 1, bacteriocin production by L. pentosus 31-1 is regulated by QS system, the inducer could be a peptide and it

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Table 1. Effect of inoculums size on bacteriocin production by L. pentosus 31-1 in MRS broth.

<table>
<thead>
<tr>
<th>Dilution fold</th>
<th>10³</th>
<th>10⁴</th>
<th>10⁵</th>
<th>10⁶</th>
<th>10⁷</th>
<th>10⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriocin activity</td>
<td>+</td>
<td>^a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

^a^ + = inhibition zone more than 10.00 mm in diameter  ^b^ + = inhibition zone between 6.00 and 10.00 mm in diameter.

Table 2. Bacteriocin production by L. pentosus 31-1 cultured in skim milk medium at different inoculums size.

<table>
<thead>
<tr>
<th>Dilution fold</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriocin activity</td>
<td>+</td>
<td>^a</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

|^a^ + = inhibition zone between 6.00 and 10.00 mm in diameter  ^-^ = no inhibition zone
Table 3. Induction of bacteriocin production of Bac<sup>c</sup> culture by CFS or MRS broth.

<table>
<thead>
<tr>
<th>Amount (µl/ml)</th>
<th>CFS</th>
<th>MRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriocin activity</td>
<td>-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> = inhibition zone between 6.00 and 10.00 mm in diameter  
<sup>b</sup> = inhibition zone more than 10.00 mm in diameter  
<sup>c</sup> = no inhibition zone.

Figure 1. Bacteriocin activity of four different treatment groups. A: Bac culture of L. pentosus 31-1 grown for 60 h in skim milk medium supplemented with 2.5 µg/ml FII-22 (final concentration); B: The control: Bac culture of L. pentosus 31-1 grown for 60 h in skim milk medium; C: The CFS of treatment B+2.5 µg/ml FII-22 (final concentration); D: FII-22 (2.5 µg/ml).

Screening of putative crude inducing peptides (IPs) from peptide fractions

Due to the complicated composition of the L. pentosus 31-1 CFS, the inducing effects could not be identified without excluding the nonsignaling molecules present in CFS. The CFS was fractionated into six fractions according to the method described above and namely: FI-1, FI-21, FI-22, FI-1, FI-21 and FI-22. The inducing activity of each fraction was tested. The Bac<sup>c</sup> culture of L. pentosus 31-1 could be induced to revert to its Bac<sup>c</sup> phenotype by FI-21 (2.5 µg/ml) or FII-22 (2.5 µg/ml) after 60 h of incubation, suggesting that the active component may present in FI-21 or FII-22 that functioned as inducers. FII-22 had a relatively low molecular weight ranged from 1 to 3 kDa, which was in agreement with the characterization of other inducing peptide reported previously (Belkum et al., 2007). Compared with FI-21, the inducing effect of FII-22 was stronger, and it was thus chosen for further investigation.

To shed more light on the role of FII-22, the bacteriocin activity of FII-22 and the synergistic antibacterial effect of FII-22 and the control was determined. As shown in Figure 1, the control (B), the CFS of the control plus FII-22 (C) as well as FII-22 (D) all exhibited no inhibitory activity. But the group (A) which was induced with FII-22 showed a clear inhibition zone in the indicator lawn. Therefore, the bacteriocin activity was not derived from FII-22 itself. Furthermore, it had nothing to do with the synergistic antibacterial activity of FII-22 and the CFS of the control. The results indicated that the bacteriocin synthesis in L. pentosus 31-1 was induced by FII-22.

Effect of time of addition of FII-22 on bacteriocin production in L. pentosus 31-1

Addition of FII-22 at any time resulted in earlier onset and much higher amount of bacteriocin production as compared to the control culture (Figure 2). But addition of FII-22 at different time points lead to different induction effect. When FII-22 was added at 0 h, bacteriocin activity could be detected after 48 h of incubation, which was 12 h faster than that in the control and was also faster than that in other groups. But comparatively weak induction effect was determined with prolonged incubation when compared with those groups that added at 4, 8 or 12 h. Adding FII-22 at 8 h (late log phase), significant differences were observed between the test group and the control group in inhibition zones. The optimal inducing effect was achieved by supplementing with FII-22 at 8 h, which exhibited a significantly larger inhibition zone of 12.06 ± 0.56 mm after 60 h of incubation, whereas only 10.35 ± 0.44 mm of inhibition zone was observed in the cultures that added FII-22 at 0 h.

Effect of the addition amount of FII-22 on bacteriocin production in L. pentosus 31-1

As shown in Figure 3, FII-22 with the concentration of 0.5 or 2.5 µg/ml significantly enhanced bacteriocin production. However, 0.1 and 25 µg/ml FII-22 exhibited no inducing effects. The results indicated that there was an optimal
amount of FII-22 added to the Bac⁺ culture to induce bacteriocin production in L. pentosus 31-1. In order to achieve ideal inducing effect, 0.5 μg/ml of FII-22 was selected as an optimal inducer concentration.

DISCUSSION

When strain 31-1 was diluted 200-fold or more in skim milk medium, spontaneously loss of bacteriocin production (Bac⁺) could be observed, whereas addition of CFS from bacteriocin production culture of L. pentosus 31-1 could restore bacteriocin production, which is consistence with the earlier research (Diep et al., 1995; Eijsink et al., 1996), suggesting bacteriocin production by L. pentosus 31-1 is regulated by QS system. Although QS controlled class II bacteriocin biosynthesis has previously been found in various LAB strains, to our knowledge, this is the first report of QS regulated class II bacteriocin synthesis in L. pentosus.

A general strategy for obtaining a Bac⁺ phenotype in class II bacteriocin producing LAB is diluting a Bac⁺ culture many fold in fresh medium (Diep et al., 1995; Eijsink et al., 1996). But a Bac⁺ phenotype could not be obtained even by extremely dilution (10⁵-fold) of L. pentosus 31-1 in MRS broth. Similar phenomenon was observed for Lactobacillus sakei B706 (Axelsson and Holck, 1995; Diep et al., 2000). Our results showed that the peptides in De Man, Rogosa and Sharpe (MRS) broth might have acted as the inducers and somehow masked the truth, since very little inducing peptide is needed for inducing bacteriocin production (Eijsink et al., 1996). Peptide pheromone is able to activate bacteriocin production at nanomolar level (Belkum et al., 2007). Furthermore, subculture of L. pentosus 31-1 Bac⁺ culture into MRS broth also demonstrated that these media may contain some undefined components which may act as inducing peptide. Therefore, it is crucial to select an appropriate medium without disturbing substance to investigate QS. LAB was commonly cultivated in complex growth media, such as MRS, ELB, Ml7 and APT (Carolissen-Mackay et al., 1997). These media all contained significant amounts of peptides. So it might be quite difficult to screen QS system using these media, and this may be one of the reasons that make bacteriocin-producing LAB lag behind in QS research.

Incubation in skimmed milk medium led to low growth rate and insufficient bacteriocin production of L. pentosus 31-1 as compared with those in MRS broth, which is consistence with Avonts et al. (2004), similar finding was reported earlier for L. salivarius subsp. salivarius UCC118 grown in wort (Flynn et al., 2002). On the contrary, Streptococcus macedonicus ACA-DC 198 did not produce macedocin when cultured in MRS and M17 broth, macedocin produced only when the strain was grown in skim milk (Georgalaki et al., 2010). In this study, we successfully obtained a Bac phenotype by 200-fold or more diluting of a 10-h-old culture of L. pentosus 31-1 in skim milk medium, and it was used to detect the inducing peptide. Since quite a lot of LAB could grow in skim milk medium, this medium may be a promising candidate for investigating QS in class II bacteriocin producing LAB. Induction is an all-or-nothing process in skim milk medium, but this was not seen in MRS broth, the
interference may attribute to the undefined peptides in the complex nitrogen sources of MRS broth. Therefore, these results led to the establishment of a reliable model for detecting QS in class II bacteriocinogenic LAB strains.

Our results showed that there was an optimal amount of FII-22 added to the cultures to exhibit its induction activity. 0.5 µg/ml of FII-22 was enough for inducing bacteriocin production in L. pentosus 31-1, while high concentration was unfavorable for bacteriocin induction. It might also be cautiously speculated that bacteriocin production has been suppressed by the excess inducing peptide. According to Leroy and De Vuyst (2002), once all receptors were saturated with induction factor, bacteriocin production would be switched off.

Induced by FII-22 led to an earlier bacteriocin production in L. pentosus 31-1 grown in skim milk medium compared with that of the Bac" culture control, similar result was also found in QS controlled antimicrobial peptide production of Bacillus sp. strain HILY-85,54728 (Schmitz et al., 2006). The earlier the addition of FII-22 to the culture, the earlier it switch on bacteriocin production, but the amount of bacteriocin production is relatively low; the later the addition of FII-22, the more bacteriocin production at 60 h, which could ascribe to the differences of the amount of bacteriocin-producing cells being onset.

In this study, based on a hypothesis that low-molecular peptide was related with the QS system of class II bacteriocin producing LAB (Quadri, 2003), we selected FII-22 with the molecular size between 1 and 3 kDa as signal molecule to induce bacteriocin production. Further investigations are required to identify the inducing molecules using completely purified ones. And more studies are needed for further confirmation of the presence of quorum sensing mechanism in L. pentosus 31-1.

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