Molecular analysis of the bacteriocin-encoding plasmid pDGL1 from *Enterococcus durans* and genetic characterization of the durancin GL locus

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Enterococci constitute a significant component of the lactic acid bacteria normally present in the intestinal microflora and include strains that produce bacteriocins. The genetic determinants for durancin GL in *Enterococcus durans* 41D were identified on the 8347 bp plasmid pDGL1 by plasmid curing experiments. pDGL1 contained nine putative ORFs, with ORF1 and ORF2 encoding plasmid replication proteins, and ORF3 and ORF6 showing high similarity to genes encoding mobilization proteins. The predicted protein encoded by ORF4 showed 74% identity to BacA, a bacteriocin produced by *Enterococcus faecalis*. The deduced DurA protein contained the conserved motif YYGNG, suggesting that durancin GL is a typical subclass IIa bacteriocin. ORF5 was shown to share 85% identity to the immunity protein BacB of *Enterococcus faecalis*. ORF9 displayed 87% sequence identity to a conserved hypothetical protein of unknown function. To further clarify the minimum requirement for durancin GL production, a 547 bp fragment containing the *durAB* gene was fitted with the *Streptococcus thermophilus* P2201 promoter and then subcloned and heterologously expressed in *S. thermophilus* ST128. The result demonstrated that the cloned fragment contained all the genetic components required for durancin GL production.

**INTRODUCTION**

Most lactic acid bacteria (LAB) used in food production have long been ‘generally regarded as safe’ (GRAS) microorganisms. Among LAB, the enterococci found in human or animal digestive systems may be either harmless commensal micro-organisms (Lempia¨inen et al., 2005) or opportunistic pathogens that act as causative agents of a variety of infectious diseases (Kayer, 2003). However, the presence in cheeses of selected strains of enterococci devoid of haemolytic activity and antibiotic resistance factors may be beneficial, since their enzymic activities contribute to the organoleptic qualities of fermented dairy foods (De Vuyst et al., 2003). In addition, several strains of enterococci produce diverse types of bacteriocins that may have potential uses as food preservatives, pharmaceuticals, nutraceuticals and veterinary medicines (Wu et al., 2007; Sánchez et al., 2008; Birri et al., 2010).

Bacteriocins are ribosomally synthesized antimicrobial peptides of bacterial origin that usually inhibit the growth of bacterial species closely related to the producing organism, although broad-spectrum bacteriocins have also been reported (Papagianni, 2003; Nissen-Meyer & Nes, 1997; Cleveland et al., 2001). Extensive research on bacteriocins has revealed that the production of such compounds is widely distributed in both Gram-negative and Gram-positive bacteria (Jack et al., 1995). Bacteriocin-producing elements are frequently associated with a plasmid in the producer strain, but bacteriocin genes can also be encoded on the chromosome or mobile genetic elements such as transposons (Diep & Nes, 2002). In many cases, a conserved N-terminal YYGNG motif is a characteristic feature of subclass IIa (pediocin-like) bacteriocins (Ennahar et al., 2000), which are most commonly found in enterococci displaying antilisterial activity (Nissen-Meyer et al., 2009). Since *Listeria monocytogenes* is a dangerous pathogen responsible for outbreaks of foodborne listeriosis, subclass IIa bacteriocins displaying antilisterial activity have attracted the interest of many research groups.

The production of subclass IIa bacteriocins requires several genetic elements, including a structural gene encoding the bacteriocin precursor, the immunity gene for the immunity

**Abbreviations:** ACO, acridine orange; EB, ethidium bromide; LAB, lactic acid bacteria; RACE, rapid amplification of cDNA ends; RIF, rifampicin; TIS, transcription initiation site.

The GenBank/EMBL/DDBJ accession number for the plasmid pDGL1 sequence of *Enterococcus durans* 41D is HQ696461.
protein that protects the producer organism from its own bacteriocin, and genes for proteins involved in the processing and membrane translocation of the mature bacteriocin peptide. Depending on their transportation and processing pathways, several subclass IIa bacteriocins utilize the sec-dependent translocation system, including enterocin P (Herranz & Driessen, 2005), bacteriocin 31 (Tomita et al., 1996), enterocin T8 (De Kwaadsteniet et al., 2006) and lysteriocin 743A (Kalmokoff et al., 2001), while many of the subclass IIa bacteriocins, especially those containing a double-glycine leader peptide, are secreted by a membrane-associated dedicated ATP-binding cassette (ABC) transporter that concomitantly cleaves off the leader sequence (Nissen-Meyer et al., 2009). The genetic elements for the double-glycine type bacteriocins usually include a processing gene, which encodes a dedicated ABC transporter that helps to process and transport the bacteriocin, and an exporting gene, which encodes an accessory protein required for bacteriocin externalization (Hävarstein et al., 1995; Nes et al., 1996; Dimov et al., 2005; Birri et al., 2010). The genetic elements involved in bacteriocin production and secretion are often organized in one or more gene clusters. For example, the pediocin gene operon is organized in a single gene cluster that may be transferred into new hosts (Coddere & Somkuti, 1999). In the case of the antilisterial bacteriocin in Lactococcus lactis IL-1403, the ABC transporter and accessory genes are located on the chromosome (Venema et al., 1996; Herranz & Driessen, 2005).

Enterococcus durans 41D was previously isolated from a Mexican-style artisanal cheese sample and found to produce a novel bacteriocin (Reny et al., 2009). In this study, the genetic location of the new durancin GL in E. durans 41D was determined by plasmid curing and restriction enzyme analysis. The complete nucleotide sequence of plasmid pDGL1 (8.34 kb) was determined, including the durancin GL production (durA) and immunity (durB) genes. In addition, the genetic requirements for durancin GL production and immunity were further investigated by the heterologous expression of durAB in Streptococcus thermophilus, a food-grade bacterium that is used as a starter culture in yogurt and cheese production.

METHODS

Bacterial strains, plasmids, media and culture conditions. Strains and plasmids used in this study are shown in Table 1. The bacteriocin-producing Ent. durans 41D strain had been previously isolated from Mexican-style artisanal cheese samples (Reny et al., 2009). S. thermophilus and Enterococcus strains were grown in tryptone-yeast extract-lactose (TYL) medium at 37 °C (Somkuti & Steinberg, 1988). Listeria innocua was propagated in brain heart infusion (BHI) medium or tryptone soy broth (TSB) (Difco) under aerobic conditions at 37 °C. Escherichia coli was grown in Luria–Bertani (LB) (Difco) medium at 37 °C. When required, antibiotics (Sigma-Aldrich) including ampicillin (100 μg ml⁻¹ for E. coli) and erythromycin (300 μg ml⁻¹ for E. coli, 15 μg ml⁻¹ for S. thermophilus) were added to the media as selective agents.

Bacteriocin antimicrobial activity assay. Bacteriocin production by the test isolates was examined by the spot-on-the-lawn antimicrobial method (Henderson et al., 1992). Briefly, 5 μl overnight culture was spotted on the surface of BH1 agar plates inoculated with an overnight culture of L. innocua (0.5 %, v/v). The plates were stored at 4 °C for 2 h and then incubated at 37 °C for 10–16 h. The clear zone of inhibition around each spot indicated the presence of active bacteriocin.

Plasmid curing experiments. Curing of plasmids was attempted by growing the cultures in the presence of rifampicin (RIF; Sigma-Aldrich), acridine orange (ACO; Sigma-Aldrich), ethidium bromide (EB; Sigma-Aldrich) and SDS (Sigma-Aldrich) at 37 °C. A 1 % (v/v) inoculum of an Ent. durans 41D overnight culture was added to TLY broth containing 40 μg RIF ml⁻¹, 20 μg ACO ml⁻¹, 20 μg EB ml⁻¹ or 400 μg SDS ml⁻¹. After 20 passages in the same medium, the cultures were serially diluted, plated on TLY agar plates and incubated at 37 °C for 24–48 h. Randomly selected colonies were inoculated in TLY broth for preparation of plasmid DNA by a rapid mini-prep method (O’Sullivan & Kleemhans, 1993), and plasmid profiles of the wild-type strain and its derived variants were analysed by agarose (0.7 %, w/v) gel electrophoresis. The Ent. durans 41D derivatives were identified by using 16S rRNA gene sequencing and BLAST analysis.

DNA sequencing of pDGL1. Plasmid pDGL1 was first purified by CsCl ultracentrifugation (Stougard & Molin, 1981) before digestion with HindIII (New England Biolabs), and purification of the fragments was accomplished by using a QIAquick PCR Purification kit (Qiagen). The DNA fragments obtained were extended by an extra nucleotide (A) by treatment with Taq DNA polymerase (New England Biolabs) to allow ligation into the pGEM-T vector (Invitrogen). The ligation mixture was transformed into E. coli DH5α and the transformants were spread on LB agar plates containing 100 μg ampicillin ml⁻¹, 0.5 mM IPTG (Sigma-Aldrich) and 80 μg X-Gal ml⁻¹ (Sigma-Aldrich). Plasmid DNA from a positive clone was prepared, and both DNA strands of the insert were initially sequenced using the pUC18-derived M13 universal forward and reverse primers. The primer walking strategy was used to complete sequencing with synthetic oligonucleotide primers based on the known sequences using pDGL1 as the template (Table 2).

5’ Rapid amplification of cDNA ends (RACE) for durAB. Ent. durans 41D was grown in TYL to mid-exponential phase, at which time cells were collected and total RNA was isolated using the RiboPure-Bacteria kit (Ambion). Total RNA was treated with TURBO DNase (Ambion) to remove unwanted genomic DNA. To ensure that all DNA was degraded, a PCR was performed using Taq polymerase with durB-specific primers B3 and B4 (Table 2) and the total RNA preparation as template. 5’ RACE analysis was performed using the SMARTer RACE cDNA Amplification kit (Clontech). First-strand cDNA synthesis was performed using a Random Primer Mix (N-15) and the SMARTer TIA oligonucleotide primer, as described by the manufacturer. 5’ RACE PCR was done using Platinum Pfx DNA polymerase (Invitrogen) with the Universal Primer A Mix (Clontech) and the B4 gene-specific primer. Nucleic acid sequence analysis of the 5’ RACE product was performed using an ABI PRISM 3730 DNA analyser (Perkin-Elmer) with ABI PRISM Big Dye Terminator Cycle Sequencing reagent and the B4 gene-specific primer. Sequencer 4.9 (Gene Codes) was used to analyse obtained nucleic acid sequences.

Sequence analysis of pDGL1. The locations of putative ORFs in pDGL1 were identified using the gene prediction programs of GeneMark (Georgia Institute of Technology, Atlanta, GA) (http://exon.gatech.edu/ gmhm2_prok.cgi) and the National Center for Biotechnology Information (NCBI) (US National Library of Medicine) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The BLAST software (NCBI) (http://www.ncbi.nlm.nih.gov) was used to conduct similarity searches with GenBank and EMBL sequence databases. Multiple sequence alignments of related amino acid sequences were constructed using DNAMAN.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>E. coli DH5α</td>
<td>F′ φ80lacZM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK mK φ) phoA supE44 Δ (lacIqZ) galU galK relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>L. innocua</td>
<td>Sensitive to durancin GL; indicator micro-organism</td>
<td>Renye et al. (2009)</td>
</tr>
<tr>
<td><strong>Ent. faecium strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H41B</td>
<td>Isolated from Hispanic-style cheeses, producing enterocin A and enterocin B</td>
<td>Renye et al. (2009)</td>
</tr>
<tr>
<td>H41K</td>
<td>Isolated from Hispanic-style cheeses, possessing the bacteriocin genes entA and entP</td>
<td>Renye et al. (2009)</td>
</tr>
<tr>
<td>H51Ca</td>
<td>Isolated from Queso Fresco sample, producing the bacteriocin gene entA</td>
<td>Renye et al. (2009)</td>
</tr>
<tr>
<td>H51Cb</td>
<td>Isolated from Queso Fresco sample, possessing the bacteriocin gene entP</td>
<td>Renye et al. (2009)</td>
</tr>
<tr>
<td>L50a</td>
<td><em>Ent. faecium</em> L50 cured derivative; containing the big plasmid of the strain</td>
<td>Cintas et al. (1998)</td>
</tr>
<tr>
<td>L50b</td>
<td>Wild-type strain; EntL50, EntP and EntQ producer; containing both plasmids</td>
<td>Cintas et al. (1998)</td>
</tr>
<tr>
<td><strong>Ent. durans strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>H51Cc</td>
<td>Isolated from Queso Fresco sample, antilisterial bacteriocin producer</td>
<td>Renye et al. (2009)</td>
</tr>
<tr>
<td>41D</td>
<td>Wild-type strain; durancin GL producer</td>
<td>This study</td>
</tr>
<tr>
<td>41D-S1</td>
<td><em>Ent. durans</em> 41D cured derivative (pDGL1&lt;sup&gt;+&lt;/sup&gt;, pDGL2&lt;sup&gt;−&lt;/sup&gt;); durancin GL producer</td>
<td>This study</td>
</tr>
<tr>
<td>41D-E1</td>
<td><em>Ent. durans</em> 41D cured derivative (pDGL1&lt;sup&gt;−&lt;/sup&gt;, pDGL2&lt;sup&gt;−&lt;/sup&gt;); durancin GL non-producer</td>
<td>This study</td>
</tr>
<tr>
<td>M29</td>
<td><em>Ent. durans</em> 41D UV-treated derivative (pDGL1&lt;sup&gt;−&lt;/sup&gt;, pDGL2&lt;sup&gt;−&lt;/sup&gt;); durancin GL non-producer</td>
<td>This study</td>
</tr>
<tr>
<td><strong>S. thermophilus strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>ST128</td>
<td>Plasmid-free host strain, non-bacteriocin producer</td>
<td>Somkuti &amp; Steinberg (1988)</td>
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<td>ST128/pMEU5a-1</td>
<td><em>S. thermophilus</em> ST128 derivative carrying pMEU5a-1, durancin GL producer; Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pGEM-T</td>
<td>3.0 kb cloning vector; Amp&lt;sup&gt;+&lt;/sup&gt;; prepared by cutting the pGEM-5Zf&lt;sup&gt;+&lt;/sup&gt; vector with EcoRV and adding a 3′ terminal thymidine to both ends</td>
<td>Promega</td>
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<td>pDGL1</td>
<td>8 kb plasmid from <em>Ent. durans</em> 41D; durancin GL&lt;sup&gt;+&lt;/sup&gt;, immunity&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pPC418</td>
<td>9.1 kb plasmid carrying the P&lt;sub&gt;2201&lt;/sub&gt; promoter; Amp&lt;sup&gt;+&lt;/sup&gt;, Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Coderre &amp; Somkuti (1999)</td>
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<tr>
<td>pMEU5a</td>
<td>5.5 kb shuttle vector; Amp&lt;sup&gt;+&lt;/sup&gt;, Em&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Solaiman &amp; Somkuti (1993)</td>
</tr>
<tr>
<td>pMEU5a-1</td>
<td>pMEU5a derivative with translational fusion of the P&lt;sub&gt;2201&lt;/sub&gt; promoter and durAB gene; durancin GL producer</td>
<td>This study</td>
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Table 2. Primers used in this study

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5′–3′)*</th>
<th>Function or properties</th>
<th>Product (bp)</th>
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<td>U1</td>
<td>CTATCTAGGACCCCAAGGTC</td>
<td>Primer walking</td>
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<tr>
<td>D1</td>
<td>CGGCCAATGATAGTGCTAC</td>
<td>Primer walking</td>
<td></td>
</tr>
<tr>
<td>U2</td>
<td>CTTCATATAATTTTCACT</td>
<td>Primer walking</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>GATTGTAGGAAAAGACG</td>
<td>Primer walking</td>
<td></td>
</tr>
<tr>
<td>U3</td>
<td>TAAGCCAGTGATCCATC</td>
<td>Primer walking</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>TGAGAGGTTGGTTTTGAAGG</td>
<td>Primer walking</td>
<td></td>
</tr>
<tr>
<td>U4</td>
<td>GGGCTCAAACTTTCATC</td>
<td>Primer walking</td>
<td></td>
</tr>
<tr>
<td>D4</td>
<td>CAACTAGGGGAAGATACGC</td>
<td>Primer walking</td>
<td></td>
</tr>
<tr>
<td>U5</td>
<td>GCAGAGATCTCACTT ACTTCA</td>
<td>Primer walking</td>
<td></td>
</tr>
<tr>
<td>D5</td>
<td>CTATTGCAGATCTCACG</td>
<td>Primer walking</td>
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</tr>
<tr>
<td>U6</td>
<td>CCATAAGTTGCTAGTCTCC</td>
<td>Primer walking</td>
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<tr>
<td>D6</td>
<td>AGCGAACTCTTACAAGAAGG</td>
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<tr>
<td>B3</td>
<td>TGTTGCTGGCTGAAACATGAGC</td>
<td>Probe</td>
<td>332</td>
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<tr>
<td>B4</td>
<td>CCATATCGGATGAGCCGACC</td>
<td>Probe</td>
<td></td>
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<tr>
<td>P1</td>
<td>CGGATTTGAGATTCTAGCACTA</td>
<td>P&lt;sub&gt;2201&lt;/sub&gt; promoter forward primer</td>
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<tr>
<td>P2</td>
<td>TACATAAATTTTTTCTCTCTATAATGCATCTTCATTTATT</td>
<td>P&lt;sub&gt;2201&lt;/sub&gt; promoter reverse primer</td>
<td></td>
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<tr>
<td>D1A</td>
<td>CTAAGGGATGATCATTTGAAAGAAAAAATTTTGTATTG</td>
<td>durAB gene forward primer</td>
<td>547</td>
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<tr>
<td>D2A</td>
<td>CTAAGGGATGATCATTTTGTATTG</td>
<td>durAB gene reverse primer</td>
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</table>

*Reverse complement sequences used for fusion PCR are underlined.
software (Lynnon). Analysis of the N-terminal region of the protein was
done with the SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP/)
(Bendtsen et al., 2004).

**MS of durancin GL.** Purified samples of durancin GL were prepared
as previously reported (Du et al., 2012). Analysis of samples by
MALDI-TOF MS was carried out under conditions previously
described (Gilbreth & Somkuti, 2005).

**Construction of recombinant plasmid and DNA transformation.**
To further investigate its biological functions, expression of the *durAB*
gene cluster was attempted in a non-bacteriocin-producing LAB
strain. First, *durAB* was amplified with primers D1A and D2A (Table 2)
using pGEM.L as the template, and the STP-*P2201* promoter was
amplified with primers P1 and P2 (Table 2) using pPC418 (Table 1)
as the template. This was followed by the translational fusion of the
*durAB* and *P2201* fragments using a fusion PCR step with primers P1
and D2A (Table 2). The resulting *P2201-durAB* fragment was ligated
into pMEU5a digested with *KpnI* (New England Biolabs) (Table 1)
using an In-Fusion Advantage PCR Cloning kit (Clontech) according
to the manufacturer’s recommendations, and then transferred to *E.
coli* DH5α to yield pMEU5a-1. DNA sequencing confirmed the
correct construction of this plasmid. The recombinant plasmid
pMEU5a-1 was reisolated and electrotransformed into *S. thermophilus*
ST128 using protocols previously described (Somkuti & Steinberg,
1988). Antimicrobial activity of the recombinant strain *S. thermo-
philus* ST128/pMEU5a-1 (Table 1) was examined by the spot-on-the-
lawn antimicrobial method (Henderson et al., 1992).

**SDS-PAGE and identification of the activity band.** The proteins in
the supernatant from cultures of *S. thermophilus* ST128/pMEU5a-1
were separated by SDS-PAGE using NuPAGE 12 % Bistris gel
(Invitrogen) as previously reported (Renye et al., 2009). The active
band corresponding to recombinant mature DurA protein was
identified by overlaying the washed gel on a BHI agar plate inoculated
with an overnight culture of *L. innocua* (0.5 %, v/v) as the indicator
organism followed by incubation for 8 h at 37 °C (Bhunia et al., 1987).

**RESULTS**

**Plasmid DNA involvement in bacteriocin production.**
The gene cluster required for bacteriocin production may be either
associated with a plasmid or located on chromosomal DNA. Since *Ent. durans* 41D harboured several plasmids
(Fig. 1a), plasmid curing methods were used to study the
possibility of plasmid DNA involvement in bacteriocin
production. The plasmid profiles of colonies isolated after
RIF or ACO treatments remained unchanged (data not
shown). However, plasmid profile analysis of the derivative
designated *Ent. durans* 41D-E1 isolated after EB treatment
showed the loss of several plasmid bands (Fig. 1a). Similar
results were obtained from the derivative designated *Ent. durans*
41D-S1, which was isolated after SDS treatment (Fig.
1b). Supernatants from the *Ent. durans* 41D-S1 culture
retained antilisterial activity, while the *Ent. durans* 41D-E1
culture lost its inhibitory activity against *L. innocua* (Fig. 1c).

Comparison of the plasmid profiles of *Ent. durans* 41D
and the two derivative strains (E1 and S1) indicated the
putative involvement of either an approximately 8 kb or
an approximately 15 kb plasmid in durancin production (Fig. 1d).
When DNA was extracted from the corresponding
bands and digested with *HindIII*, identical fragmentation
patterns were obtained, which implied that the two bands
represented different forms of the same plasmid designated
pDGL1 (Fig. 1e). In addition, the digestion of the 8 kb
plasmid with *HindIII* yielded a 1 kb DNA fragment that
was subcloned into the pGEM-T vector and sequenced.
The nucleotide sequence of a putative ORF detected in this
fragment shared 90 % identity with the known bacteriocin
immunity gene *bacB* on plasmid pYI17 (GenBank accession
no. D78257) of *Enterococcus faecalis* (Tomita et al.,
1996), which tentatively confirmed its role in protecting
*Ent. durans* 41D from the effects of durancin GL.

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**Fig. 1.** (a) Plasmid profiles of 12 *Ent. durans* 41D derivatives
obtained with EB treatment; missing bands in *Ent. durans* strain
41D-E1 are indicated by white arrows in lane 8. (b) Plasmid profiles
of 12 *Ent. durans* 41D derivatives obtained with SDS treatment;
missing bands in *Ent. durans* strain 41D-S1 are indicated by white
arrows in lane 6. (c) Bacteriocin activity of supernatants from
cultures of wild-type *Ent. durans* 41D (W) and the derived mutants
*Ent. durans* 41D-E1 (E1) and *Ent. durans* 41D-S1 (S1). (d) Plasmid
profiles of *Ent. durans* 41D (W) and derivative strains *Ent. durans*
41D-E1 (E1) and *Ent. durans* 41D-S1 (S1). M, supercoiled
molecular size marker. The possible plasmid bands involved in
bacteriocin production are indicated with black lines. (e) *HindIII*
fragments of 8 kb (1) and 15 kb (2) plasmid DNA from *Ent. durans*
41D-S1; M, 1 kb molecular size markers.
DNA sequence of pDGL1

The complete nucleotide sequence of pDGL1 was determined by the primer walking technique. The molecular mass of pDGL1 was 8347 bp and it had a GC content of 34.8%. Sequence analysis of pDGL1 revealed the presence of nine putative ORFs, when the cut-off length for annotated proteins was 50 aa. Each ORF was preceded by a putative Gram-positive ribosome-binding site (RBS), and all but ORF6 were encoded on the same DNA strand.

It appears that the ORFs on pDGL1 can be grouped based on the putative functions of their encoded proteins (Fig. 2a). ORF1 and ORF2 appear to encode proteins required for plasmid replication. ORF1 is preceded by a putative promoter and is predicted to encode a 245 aa protein with 99% identity to the replication protein of a plasmid in *Enterococcus faecium* (Table 3). The region containing the putative promoter of *orf1* includes two sets of direct repeats (nucleotides 3–38, agatatactgtt; and nucleotides 95–182, tgggggataaattgtcacacta), which are also present in the putative replication origin (ori) of the enterocin Q-encoding plasmid pCIZ2 from *Ent. faecium* L50 (Criado et al., 2006).

ORF2, which is located 28 bp downstream of ORF1 and predicted to encode a 211 aa protein, exhibits sequence similarity to a conserved hypothetical protein from *Ent. faecium* (Table 3) and to a putative plasmid replication protein identified in *Ent. durans* 41D. ORF4 and ORF5 are predicted to be an operon that encodes the structural gene *durA* and the immunity gene *durB*, which are responsible for durancin GL production of *Ent. durans* 41D. The putative −10 and −35 sites are in the upstream non-coding region of the operon, while an RBS is located immediately upstream of each of the two ORFs (Fig. 2). 5′ RACE analysis involving the use of a *durB*-specific primer (B4) resulted in the amplification of a 510 bp cDNA fragment. Nucleic acid sequencing of the PCR product confirmed the predicted transcription initiation site (TIS), as shown in Fig. 2. A BLAST search of databases for *durA*-like sequences revealed no significant similarity with other genes, suggesting that *durA* is a bacteriocin gene with novel structural features. The deduced DurA protein is a 71 aa peptide with a molecular mass of 7.9 kDa (Table 3), sharing 74% identity with BacA, a class II bacteriocin produced by *Ent. faecalis* (Tomita et al., 1996). DurA has a potential signal peptidase cleavage site between amino acids 28 and 29 (Fig. 3a), indicating that it may be exported by a sec-dependent transport system. The MALDI-TOF MS spectrum of
Table 3. ORFs in 8347 bp plasmid pDGL1 from *Ent. durans* 41D-S1 and related proteins

<table>
<thead>
<tr>
<th>ORF</th>
<th>Position in nucleotide sequence</th>
<th>Gene</th>
<th>Protein</th>
<th>Closest relative (length, e value, level of amino acid identity, micro-organism)</th>
<th>Accession no.</th>
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<tbody>
<tr>
<td></td>
<td>5'</td>
<td>3'</td>
<td>Designation</td>
<td>G+C (%)</td>
<td>Length (aa)</td>
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<tr>
<td>ORF1</td>
<td>250</td>
<td>987</td>
<td>ORF1</td>
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<td>ORF2</td>
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<td>1650</td>
<td>ORF2</td>
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<td>211</td>
</tr>
<tr>
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<td>IS4</td>
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<td>302</td>
</tr>
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<td>3572</td>
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<td>3871</td>
<td><em>durB</em></td>
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</tr>
<tr>
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<td>4061</td>
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<tr>
<td>ORF7</td>
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<td>5912</td>
<td><em>mobC</em></td>
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</tr>
<tr>
<td>ORF8</td>
<td>5894</td>
<td>6808</td>
<td><em>mobA</em></td>
<td>39.8</td>
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</tr>
<tr>
<td>ORF9</td>
<td>7050</td>
<td>7739</td>
<td>ORF9</td>
<td>34.0</td>
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</table>

The immunity gene (*durB*) is located 17 nt downstream of *durA* and encodes a 94 aa peptide (Table 3), which shares 85% identity with the immunity protein BacB (Tomita *et al.*, 1996). Comparison of the deduced amino acid sequences of DurB with other enterococci immunity proteins showed C-terminal amino acid sequences to be conserved (Fig. 3b). This allowed the deduction that the C-terminal parts of the immunity proteins are involved directly or indirectly in the specific recognition of cognate bacteriocins, as reported previously (Johnsen *et al.*, 2004).

ORF3 encodes a 302 aa protein, which most closely resembles the IS4 transposase of *Ent. faecium* DO (Table 3). ORF6 is located on the complementary strand and encodes a putative 319 aa protein with 99% identity to a transposase from *M. caseolyticus* (Baba *et al.*, 2009). ORF7 encodes a putative 126 aa protein which has 99% identity to a bacterial mobilization protein (MobC) from *Ent. faecium* DO (Table 3). The region containing the putative promoter of orf7 also has the typical features of a transfer origin (oriT) (Francia *et al.*, 2004; Jaworski & Clewell, 1995; Smith & Thomas, 2004), including an inverted repeat sequence (5′−TGT GAC AAG CGG TTA TTT GCA CCA AAT A−3′ and 5′−ATT TGG CTA GCA AAT ATA GAG CTT GCC AAA−3′) and a sequence (GAGCTTGC) with homology to the core sequence of the cis-acting nic site of the IncP/MobP family of Gram-positive plasmids (RYGCTTG) (Smith & Thomas, 2004). These elements are also present in plasmid pCIZ2 (Criado *et al.*, 2006).

ORF8 overlaps the end of orf7 and encodes a 304 aa protein (Table 3) with high similarity to the putative mobilization protein MobA from *Ent. faecium* and to a relaxase/mobilization nuclease domain from *Ent. faecium* (Criado *et al.*, 2006). By aligning the predicted amino acid sequence of ORF8 with the predicted amino acid sequences of several plasmid relaxases and mobilization proteins, it was also found that ORF8 had the typical features of this mobilization protein family, including three highly conserved motifs (I, II and III) at the N-terminal relaxase domain, based on a classification scheme for mobilization regions of bacterial plasmids (Francia *et al.*, 2004).
ORF9 is preceded by a putative promoter and encodes a putative 229 aa protein (Table 3) which exhibits 87% identity to a hypothetical protein from Ent. faecium (van Schaik et al., 2010).

Heterologous durancin GL expression in LAB

To analyse the antimicrobial and immune function of the durAB operon, we attempted its heterologous expression in the thermophilic yogurt and cheese starter culture bacterium S. thermophilus. The approximately 0.7 kb DNA fragment containing the fusion product of the STP2201 promoter and durAB was successfully ligated into pMEU5a, and the resulting recombinant pMEU5a-1 plasmid was electrotansformed into the non-bacteriocin-producing strain S. thermophilus ST128 (Fig. 4a). The cell-free supernatant of S. thermophilus ST128/pMEU5a-1 culture showed antimicrobial activity against L. innocua (Fig. 4b), and was shown by an overlay assay to have a biologically active band with a molecular mass similar to durancin GL of Ent. durans 41D-S1 (Fig. 4c). These results indicated that the durancin locus, consisting of the durA and durB genes, is responsible for bacteriocin production and immunity, and is sufficient for the production of durancin GL in the food-grade S. thermophilus ST128.

Protection effect of the immunity protein DurB to other bacteriocins

To examine whether the enterococcal immunity proteins have a cross-protection effect, we examined the bacteriocin immunity or sensitivity of Ent. faecium H41K and Ent. faecium (accession no. BAA11329) and SE-K4 from Ent. faecium TX1330 (ZP_09980284). The potential signal peptide cleavage site predicted by the SignalP algorithm (Bendtsen et al., 2004) is denoted by the vertical arrow. Identical amino acid residues are indicated by boxes and the conserved amino acid sequence YYGNG of subclass IIa bacteriocins is present in all three sequences. (b) Multiple alignment of DurB with the immunity proteins BacB from Ent. faecalis (accession no. BAA11330) and EntB from Ent. faecium (ABD60752). Cons, conserved residues.

Fig. 3. (a) Multiple alignment of the durancin GL precursor with precursors of closely related subclass IIa enterocins BacA from E. faecalis (accession no. BAA11329) and SE-K4 from Ent. faecium TX1330 (ZP_09980284). The potential signal peptide cleavage site predicted by the SignalP algorithm (Bendtsen et al., 2004) is denoted by the vertical arrow. Identical amino acid residues are indicated by boxes and the conserved amino acid sequence YYGNG of subclass IIa bacteriocins is present in all three sequences. (b) Multiple alignment of DurB with the immunity proteins BacB from Ent. faecalis (accession no. BAA11330) and EntB from Ent. faecium (ABD60752). Cons, conserved residues.

Fig. 4. Heterologous expression of the durancin GL gene in S. thermophilus. (a) Fusion PCR result for the P2201 promoter and durAB gene. Lanes: 1, 1 kb molecular marker; 2, fusion product of the P2201 promoter and durAB gene; 3, PCR amplification of the durAB gene from pMEU5a-1 using primers D1A and D2A; 4, PCR amplification of the P2201 promoter from pMEU5a-1 using primers P1 and P2; 5, 100 bp molecular marker. (b) Bacteriocin activity in supernatants from cultures of S. thermophilus ST128/pMEU5a and S. thermophilus ST128/pMEU5a-1 grown in non-erythromycin-containing TYL broth as determined by an agar diffusion assay using L. innocua as the target micro-organism. (c) Antimicrobial activity of durancin GL heterologously produced by S. thermophilus ST128/pMEU5a-1 after overlay with the indicator strain L. innocua. Lanes: 1, SeeBlue Plus2 Pre-Stained Standard (Invitrogen) – the positions of protein molecular mass markers are indicated by the horizontal lines.
durans 41D-S1 to the bacteriocins from different Enterococcus strains. The results showed that Ent. faecium H41K was sensitive to the bacteriocins from H41B, H51Ca, H51Cb, H51Cc, L50a and L50b, but immune to the bacteriocin from 41D-S1 (Fig. 5a), while 41D-S1 was sensitive to the bacteriocins from H41B, H51Ca, H51Cb, H51Cc, L50a and L50b, but immune to H41K (Fig. 5b). These findings suggested that cross-protection existed between H41K and 41D-S1, but the immunity protein DurB cannot provide protection against the bacteriocins produced by most other strains of enterococci that were included in the study.

**DISCUSSION**

The results of the genetic characterization of durancin GL permitted its classification as a novel subclass Ia bacteriocin produced by Ent. durans 41D, a strain isolated from Mexican-style artisanal cheese samples. The location of the genetic determinants responsible for durancin GL production on the 8 kb plasmid pDGL1 was confirmed by using plasmid curing agents. The smaller plasmid (approximately 4 kb) of Ent. durans 41D was cured by treatment with SDS, while the larger plasmid (pDGL1, 8 kb) was eliminated by treatment with EB.

DNA sequencing and analysis of pDGL1 revealed the presence of durancin GL structural (durA) and immunity (durB) genes, as well as seven ORFs involved in plasmid replication and mobilization based on homology searches by blast software (Fig. 2, Table 3). Since the MALDI-TOF MS spectrum confirmed the calculated molecular mass (4.97 kDa) of the mature DurA peptide based on nucleotide sequence analysis of durA, it was reasonable to conclude that durancin GL contains a hydrophilic N-terminal region, a conserved YYGNG motif and a relatively hydrophobic C-terminal region, indicating that it is a subclass Ia bacteriocin (Nissen-Meyer et al., 2009; Birri et al., 2010), with activity against Listeria and other enterococci as previously reported (Du et al., 2012). Subclass Ia bacteriocins have been divided into four subgroups based on sequence similarities and differences in the C-terminal region (Nissen-Meyer et al., 2009). Durancin GL belongs to subgroup 4, which includes bacteriocin 31 (mature peptide of BacA), bacteriocin RC714, bacteriocin T8, penocin A and enterocin SE-K4. Based on the deduced amino acid sequence of the mature peptides, bacteriocin 31, which is produced by Ent. faecalis Y1717 (Tomita et al., 1996), is the closest structural relative of durancin GL (Fig. 3a). Similar to durancin GL, the genetic determinants for bacteriocin 31 are also located on an operon composed of bacA and bacB on plasmid pY1717 (Tomita et al., 1996). Both durancin GL and bacteriocin 31 pre-peptides include leader sequences that are recognized by a sec-dependent translocation system and do not require bacteriocin-specific translocation proteins. This may explain why the gene locus involved in durancin GL production is characterized by a simpler structure than that of most of the other subclass Ia bacteriocins with a double-glycine leader sequence, including mundticin KS produced by Enterococcus mundtii NFRI 7393 and enterocin Q produced by Ent. faecium L50, which are secreted by a dedicated membrane-associated ABC transporter (Nissen-Meyer et al., 2009; Kawamoto et al., 2002; Criado et al., 2006).

Bacteria that produce subclass Ia bacteriocins also produce a cognate immunity protein that protects the producing organism from being killed by its own bacteriocin (Axelsson & Holck, 1995; Dayem et al., 1996; Hühne et al., 1996; Quadri et al., 1995, 1997; Venema et al., 1995; Johnsen et al., 2004; Criado et al., 2006). The immunity proteins of pediocin-like bacteriocins have recently been divided into subgroups A, B and C based on sequence similarities (Finland et al., 2005). The alignment results between DurB and bacteriocin31-im (BacB) (Fig. 3b) suggest that the durancin GL immunity protein (DurB) may be classified as a member of subgroup C. Although immunity proteins show a high degree of specificity to the bacteriocins they recognize, cross-immunity may be observed in some cases, where either the bacteriocins or the immunity proteins belong to the same sequence-based subgroup (Finland et al., 2002). The immunity protein DurB apparently did not provide protection against the bacteriocins produced by
most *Enterococcus* strains, suggesting that DurB has a high degree of specificity in recognizing its cognate bacteriocin, since cross-protection was found only between H41K and 41D-S1 (Fig. 5).

In addition to the genes determined for durancin GL production and plasmid replication, four ORFs encoding two transposases (ORF3 and ORF6), a putative mobilization protein (ORF7) and a relaxase/mobilization nuclease domain protein (ORF8) were found in pDGL1 (Table 3). Sequences similar to orf8 have also been reported in another plasmid responsible for bacteriocin production, the 7 kb plasmid pCIZ2 from *Ent. faecium* L50, and also in the 2 kb DNA contiguous fragment from the *Enterococcus hirae* DCH5 genome (Criado et al., 2006; Sánchez et al., 2007). Although the presence of these elements suggests that the mobilization proteins may contribute to the transfer of bacteriocin gene clusters in enterococci, additional work is required to demonstrate their involvement in such events.

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REFERENCES


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