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Inhibition of *Listeria monocytogenes* by *Enterococcus mundtii* isolated from soil

T. Bigwood [a,b], J.A. Hudson [a,*], J. Cooney [a,c], L. McIntyre [a,1], C. Billington [a], J.A. Heinemann [b], F. Wall [c]

[a] Food Safety Programme, Institute of Environmental Science and Research Ltd (ESR), Christchurch Science Centre, PO Box 29-181, Ilam, Christchurch, New Zealand
[b] School of Biological Sciences, University of Canterbury, Christchurch, New Zealand
[c] CPIT, 130 Madras Street, Christchurch, New Zealand

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ABSTRACT

Two bacterial isolates with inhibitory activity against *Listeria monocytogenes* and *Enterococcus faecalis* were obtained from soil. Genotypic and phenotypic characterization identified them as *Enterococcus mundtii*, a species whose ability to compete with *L. monocytogenes* is relatively unexplored compared to other members of the genus. The thermal stability of the inhibitory factor and its sensitivity to proteolytic enzymes indicate that it is most likely a bacteriocin. Both isolates grew at comparable rates to *L. monocytogenes* at 5 °C and 10 °C *in vitro*. One isolate killed *L. monocytogenes* when it reached concentrations of 10^6–10^8 CFU ml^{-1}. Minimum inocula of 10^6 and 10^5 CFU ml^{-1} of *E. mundtii* were required to reduce and maintain *L. monocytogenes* concentrations beneath the level of detection at 5 °C and 10 °C, respectively. *In situ* experiments at 5 °C showed that *E. mundtii* inhibited the growth of *L. monocytogenes* on vacuum-packed cold smoked salmon during its four week shelf life. *E. mundtii* could, therefore, control the growth of *L. monocytogenes* at low temperatures, indicating a potential application in controlling this pathogen in chilled foods. To control growth of *Listeria*, the concentration of *E. mundtii* needs to be high, but it is possible that a purified bacteriocin could be used to achieve the same effect.

1. Introduction

Bacteriocinogenic enterococci have been shown to control *Listeria monocytogenes* in a number of foods including dairy (Achemchem et al., 2006) and meat products (Hugas et al., 2003). Activity against other foodborne pathogens, such as *Bacillus cereus*, has also been recorded (Muñoz et al., 2004). Bacteriocinogenic enterococci are also important in food fermentations. For example the addition of *Enterococcus faecium* as an adjunct starter during the manufacture of feta cheese enhanced its organoleptic qualities (Sarantinopoulos et al., 2002).

The first report of a bacteriocin produced by *Enterococcus mundtii* was by Bennik et al. (1998), who showed that the bacteriocin, mundticin, inhibited *L. monocytogenes*, *Clostridium botulinum* and a number of lactic acid bacteria. The characteristics of mundticin suggested potential for its use as a biocontrol agent in foods. Subsequently, many reports have described the properties of bacteriocins from *E. mundtii* (Campos et al., 2006; De Kwaadsteniet et al., 2005; Ferreira et al., 2007; Kawamoto et al., 2002; Solichová et al., 2012; Todorov et al., 2005).

The control of *L. monocytogenes* in foods by bacteriocins from *E. faecium* and *Enterococcus faecalis* has been well described (Achemchem et al., 2006; Ananou et al., 2005) but, by comparison, data on the control of pathogens on foods by *E. mundtii* are scarce. We are aware of only two reports of *E. mundtii* being used in the biocontrol of *L. monocytogenes* in a food, namely in mung bean sprouts (Bennik et al., 1999) and fresh Minas cheese (Vera Pingitore et al., 2012). In the study on bean sprouts, biocontrol did not occur when the bacterium itself was used, but some biocontrol was apparent when the bacteriocin alone was applied to the food. However, in the cheese, *E. mundtii* CRL 35 prevented growth of *L. monocytogenes* for 12 days when incubated at 8–12 °C.

The bacteriocin from *E. mundtii* ST46A has been shown to limit the growth of *L. monocytogenes* in a model human gastrointestinal tract system with infant formula as the growth substrate (Botes et al., 2008).

Here, we report on the isolation and characterization of *E. mundtii* from soil and describe the anti-listerial properties of the cell-free supernatant and the organism itself when grown in liquid

* Corresponding author. Tel.: +64 3 351 0099; fax: +64 3 351 0010.
E-mail addresses: bigwoodteresa@hotmail.com (T. Bigwood), andrew.hudson@esr.cri.nz (J.A. Hudson), j_cooney@xtra.co.nz (J. Cooney), lmcintyre@harper-adams.ac.uk (L. McIntyre), craig.billington@esr.cri.nz (C. Billington), jack.heinemann@canterbury.ac.nz (J.A. Heinemann), Frances.Wall@cpit.ac.nz (F. Wall).
[1] Present address: Department of Food Science and Agri-Food Supply Management, Harper Adams University College, Edgmond, Newport, Shropshire, United Kingdom.

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culture and on cold smoked salmon in co-culture with the pathogen.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

In work screening for the presence of bacteriophages in soil which could infect *L. monocytogenes*, two inhibitory bacterial colonies were noted through the production of zones of inhibition in a lawn of *L. monocytogenes*. These isolates (designated SUB and SUC) were isolated into pure culture and subsequently identified as *E. mundtii* as described below. Inocula were grown at 37 °C overnight in Trypticase Soy Broth (TSB; Oxoid, Basingstoke, UK) with shaking.

Cultures used to determine the antimicrobial spectrum of the isolates were obtained from the New Zealand Reference Culture Collection, Medical Section (NZRM) (www.esr.cri.nz/competencies/communicabledisease/Pages/nzrcc.aspx), as follows: *Aeromonas hydrophila* (NCTC 8049), *B. cereus* (NCTC 8035), *Enterobacter aerogenes* (NCTC 10006), *E. faecalis* (NCTC 775), *Escherichia coli* (ATCC 25922), *E. coli* O157:H7 (NZRM 3647 and NCTC 12900), *Klebsiella pneumoniae* (NCTC 9633), *L. monocytogenes* (NCTC 7973 and 2000/47), *L. innocua* (NCTC 11288), *L. ivanovii* (NZRM 797), *Pseudomonas aeruginosa* (NCTC 10662), *Salmonella* Menston (NZRM 383), *S.* Typhimurium PT160 (NZRM 4214), *Shigella sonnei* (ATCC 9290), *Staphylococcus aureus* (NZRM 917), *Staphylococcus epidermidis* (ATCC 12228) and *Yersinia enterocolitica* (ATCC 9610). *E. coli* RR1 (Bolivar et al., 1977) was also used. The *L. monocytogenes* isolate used in growth inhibition experiments was 2000/47, a strain associated with clinical cases of listeriosis in New Zealand (Sim et al., 2002).

2.2. Identification of isolates SUB and SUC

Genomic DNA was extracted from the bacteria using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA). The 16S rRNA gene was amplified from the genomic DNA by PCR, generating a ~1500 bp amplicon bounded by the outer primer pair 16SF27 and 16SR1541 (Weisburg et al., 1991). Amplification of the 16S rRNA gene was carried out in a GeneAmp 9700 thermocycler (Applied Biosystems, Carlsbad, CA, USA) using the following reaction conditions: 35 denaturation cycles for 30 s at 94 °C, annealing for 30 s at 55 °C and elongation for 45 s at 72 °C. Amplicon detection was by agarose gel electrophoresis of 5 μl of PCR product through a horizontal 2% agarose gel in 0.5 × Tris-borate-EDTA buffer. After electrophoresis, amplicons were stained with ethidium bromide and visualised by UV light irradiation prior to image capture.

The amplicon was purified using the High Pure 96 UF Cleanup Kit (Roche, Indianapolis, IN, USA) before carrying out the sequencing reactions. Primers (Lane, 1991) internal to the outer primers (F357, F530, F, R518, R1087 and R1492) were used for amplification and sequencing of the initial PCR product to obtain overlapping products in both directions. All PCR products were sequenced using an ABI PRISM BigDye terminator DNA sequencing kit (Applied Biosystems, Foster City, CA, USA) and analysed on a model 3730XL ABI DNA sequencer (Applied Biosystems), following the manufacturer's instructions.

Sequence analysis was performed using BioNumerics version 5 software (Applied Maths, Sint-Martens-Latem, Belgium). The data obtained with the six primers were combined and aligned to produce a consensus sequence for each isolate, which was then compared to the sequences in GenBank® using the Basic Local Alignment Search Tool (Altschul et al., 1990).

Both isolates were tested using API 50CH test strips (BioMerieux Clinical Diagnostics, Hazelwood, MO, USA) incubated at 37 °C, and examined after 24 h and 48 h incubation. Haemolysis was assessed by growing the isolates on Columbia Blood Agar containing 5% defibrinated sheep blood (Fort Richard Laboratories, Auckland, New Zealand) at 37 °C or 30 °C for 24 h. Aesculin hydrolysis was assessed by growing the organisms on bile aesculin agar (Oxoid, Basingstoke, UK) and incubating them at 37 °C overnight. Motility was examined using a wet mount under phase contrast microscopy.

2.3. Determination of inhibitory range

To determine the inhibitory spectrum of the *E. mundtii* isolates they were grown as streaks across the centre of a pre-dried TSA plate (TSB containing 1.5% (w/v) agar) with incubation for 16 h at 37 °C. Simultaneously, indicator organisms were grown overnight and 0.1 ml of this culture inoculated into 10 ml TSB and incubated at 37 °C for 3–4 h. A 0.1 ml volume was subsequently added to 3 ml TSA containing 0.7% agar equilibrated at 46.5 °C, the tube briefly mixed, and then poured as an overlay onto the plate with growth of *E. mundtii*. Plates were incubated overnight at 37 °C before being examined for zones for clearance of the bacterial lawn in the agar overlay.

2.4. Characterisation of the anti-listerial agent

Overnight broth cultures of the *E. mundtii* isolates, SUB and SUC were centrifuged at 3000 *g* for 10 min to remove the cells. A subsample of the cell-free supernatant (CFS) was taken and boiled for 30 min. Volumes (100 μl) of the boiled and unboiled CFS were then dispensed into wells aseptically cut in TSA plates which had been inoculated with 100 μl of a *L. monocytogenes* 2000/47 culture with an optical density (600 nm) of 0.1 spread over the entire surface. The plates were incubated at 30 °C overnight before measuring the zone of inhibition in the *L. monocytogenes* lawn, if formed. The pH of the CFS samples was measured to exclude acid production as the inhibitory mechanism.

To determine the effect of proteases on the anti-listerial activity, cultures were grown overnight at 37 °C and centrifuged as described above. The CFS was incubated at 25 °C or 37 °C for 2–4 h individually in the presence of trypsin, pepsin, α-chymotrypsin and proteinase K (Sigma, St. Louis, MO, USA). Each protease was made up at an initial concentration of 10 mg ml^{-1} in phosphate buffered saline (Oxoid, Basingstoke, UK) and then used at a final concentration of 1 mg ml^{-1} (900 μl of CFS and 100 μl of enzyme solution). CFS without enzyme (but with buffer), enzyme solutions alone and buffer alone acted as controls. Anti-bacterial activity was determined by the agar well diffusion method described above. The absence of a zone of inhibition around the well indicated the hydrolysis of the antibacterial factor.

To determine the effect of growth conditions on the production of the antibacterial compound, an overnight culture of *E. mundtii* SUC at 37 °C in TSB was centrifuged at 3000 *g* for 10 min and the supernatant discarded. The cells were resuspended in SM buffer (0.05 M TRIS, 0.1 M NaCl, 0.008 M $MgSO_4$, 0.01% (w/v) gelatin pH 7.5), the suspension re-centrifuged, and the supernatant removed. This washing step was then repeated. Cells were resuspended in SM buffer to achieve an OD (600 nm) of ~0.1. A loopful of this suspension was streaked onto a pre-poured TSA plate and incubated at 37 °C to assess its purity. The cell suspension (0.1 ml) was added to growth media (1.3 ml) in four replicate tubes and they were incubated overnight under different conditions (Table 1). Antibacterial activity was measured by adding the CFS samples (0.1 ml) to wells cut into overlay agar plates prepared as described above and incubating them overnight at 37 °C. The sizes of