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Bacteriocin from Honeybee Beebread *Enterococcus avium*, Active against *Listeria monocytogenes*

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Enterococcus avium **isolated from** ***Apis mellifera*** **beebread produces a thermoresistant bacteriocin with a strain-dependent inhibitory effect on** ***Listeria*** **and without effect on gram-negative bacteria. The bacteriocin appeared to be a polypeptide of about 6 kDa. Genetic analyses revealed no extrachromosomal material in** ***E. avium*****.**

Our general objective is to characterize and select lactic acid bacteria (LAB) that may be of probiotic relevance (1–3). Beebread is processed pollen stored with the addition of various enzymes and honey, which is subjected to lactic acid fermentation (11) by LAB present in flowers, silage, and the environment. Enterococci have been isolated from vegetable matter, reptiles, and insects, but there are no references to these microorganisms associated with honeybees (9, 17). Since no previous studies of LAB associated with the common honeybee were found, we screened the *Apis mellifera* intestinal tract and beebread samples for these microorganisms.

Enterococcus avium PA1 was isolated from *Streptococcus* selective medium (1) incubated at 37°C for 24 to 48 h and characterized by biochemical tests (8), by carbohydrate fermentation pattern (APICH50), and on the basis of its 16S rRNA sequences.

Inhibition assays performed with *E. avium* PA1 cell-free supernatant (CFS) from brain heart infusion (BHI) broth were studied with the well diffusion assay (18). Twenty-three microliters of CFS was placed in wells cut in BHI agar plates previously seeded with the indicator strains (final concentration, ca. 1×10^9 CFU ml^{-1}). The plates were incubated at 25 to 30°C for 12 to 24 h and examined for inhibition halos. The inhibitory substance suspension titer was determined by serial twofold dilution and expressed in arbitrary units (AU) per milliliter (7). Indicator strains and their sensitivities to *E. avium* PA1 CFS at pH 5.5 are indicated in Table 1.

The physicochemical nature of the antagonistic substance was determined by studying the anti-*Listeria* activity of the CFS at pH 5.5 heated to 121°C for 15 min in an autoclave and treated with proteolytic enzymes (trypsin, papain, α-chymotrypsin, and pepsin), catalase, and lysozyme. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was performed with 20 μl CFS mixed with 7 μl of running buffer and heated at 100°C for 5 min (16). After 3 h electrophoresis at 65 V, gel was removed and assayed for molecular weight estimation and biological assay (4). Extrachromosomal material was also determined in *E. avium* PA1 cells (5).

The mode of action of bacteriocin on nonproliferating *L. monocytogenes* cells was studied. An overnight culture of *L. monocytogenes* 01/198 in BHI broth was harvested by centrifugation, and cells were resuspended in phosphate buffer (0.05 M, pH 7.00) to a final concentration of ca. 10^9 CFU ml^{-1}. A bacteriocin solution was mixed in equal amounts with the cell suspension and incubated for 2 h at 37°C. Counts of listeriae were determined on BHI agar (1.5%, wt/vol) incubated at 30°C for 24 h.

The assays were performed in triplicate. Data were analyzed by Tukey's test, and differences were considered significant at the $P < 0.05$ level.

The metabolite synthesized by *E. avium* PA1 did not inhibit *Lactobacillus* or gram-negative pathogens like *Salmonella* and *Klebsiella* spp. However, all *Listeria* sp. strains tested were inhibited. The effect was strain dependent, as shown in Table 1. The antimicrobial activity of *E. avium* PA1 CFS at pH 5.5 completely disappeared with the proteolytic enzyme treatment, but its action was unaffected by catalase or lysozyme. It was highly resistant to heat since its anti-*Listeria* activity persisted after 15 min at 121°C. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of different CFS revealed only one band with biological activity against *L. monocytogenes* Scott A. Its molecular mass was around 6 kDa (data not shown). These results demonstrate the presence of a bacteriocin-like molecule (6, 10, 12, 13).

Listeria strains 01/01, 00-3/364, 01/155, and 99/267 were more resistant to the bacteriocin, while 01/2000, 99/625, and 99/128 were the most sensitive. Strains 00/270, 01/155, 01/01, 99/267, and 01/198 showed a double inhibition halo with well-defined colonies growing between both limits. The remaining *Listeria* strains presented lesser growth halos without detection of individual colonies, suggesting a bacteriostatic effect of the bacteriocin. This bacteriocin was bactericidal against *L. monocytogenes* 01/198 since the log number of viable cells fell from 9.08 ± 0.03 to 6.60 ± 0.04 after 2 h of contact with 11,130 AU

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TABLE 1. Indicator strains and their sensitivities to *E. avium* PA1 CFS at pH 5.5

Indicator strain	Source[a]	Titer (AU ml^{-1})[b]
Enterococcus hirae 8043	ATCC	5,120
Enterococcus avium 31/96	Instituto Carlos Malbrán	5,560
Enterococcus faecium A4[c]	CA	5,560
Enterococcus faecium Y1b[c]	CA	5,560
Enterococcus faecium G2d[c]	CA	5,560
Enterococcus faecium C4[a]	CA	5,560
Enterococcus faecium 1385	CRL	Negative
Lactobacillus sp. strain MCA18	CA	Negative
Lactobacillus sp.[d] strain 16	CA	Negative
Lactobacillus sp.[d] strain 20	CA	Negative
Salmonella enterica serovar Gallinarum	INTA	Negative
Salmonella enterica serovar Pullorum	INTA	Negative
Salmonella enterica serovar Enteritidis	INTA	Negative
Salmonella enterica serovar Typhimurium	INTA	Negative
Listeria monocytogenes Scott A	IHT	22,260
Listeria monocytogenes 00/270	Instituto Carlos Malbrán	22,260
Listeria monocytogenes 01/2000	Instituto Carlos Malbrán	11,130
Listeria sp. strain 99/316	Instituto Carlos Malbrán	22,260
Listeria sp. strain 99/625	Instituto Carlos Malbrán	22,260
Listeria sp. strain 99/128	Instituto Carlos Malbrán	22,260
Listeria monocytogenes 01/198	Instituto Carlos Malbrán	20,480
Listeria monocytogenes 01/01	Instituto Carlos Malbrán	5,565
Listeria sp. strain 00–3/364	Instituto Carlos Malbrán	1,391
Listeria sp. strain 01/155	Instituto Carlos Malbrán	2,782
Listeria sp. strain 99/267	Instituto Carlos Malbrán	5,565
Listeria monocytogenes 99/287	Instituto Carlos Malbrán	22,260
Klebsiella sp.	CA	Negative

[a] ATCC, American Type Culture Collection; CRL, Centro de Referencia para Lactobacilos; IHT, Institute for Hygiene and Toxicology, Karlsruhe, Germany; Instituto Carlos Malbrán, Instituto de Microbiologúa, Buenos Aires, Argentina; INTA, Instituto Nacional de Tecnología Agropecuaria, Balcarce, Buenos Aires, Argentina; CA, Carina Audisio, INIQUI, Universidad Nacional de Salta, Salta, Argentina.
[b] Determined by well difussion assay.
[c] Strain isolated from different honey samples in our laboratory.
[d] Strain isolated from honeybee intestinal tract in our laboratory.

ml^{-1} at 25°C. In contrast, the bacteriocin showed a bacteriostatic effect against those strains (*L. monocytogenes* 99/287; *Listeria* spp. 00-3/364 and 99/316) with a halo of lesser growth and no individual colonies. Therefore, caution should be exercised when generalizing about the bactericidal effect of bacteriocins synthesized by enterococci. We do not know yet whether the colonies growing in the halo are spontaneous bacteriocin-resistant cells or recovered cells after sublethal injury. Almost all enterococci tested were inhibited by bacteriocin, but *E. faecium* CRL1385 was totally resistant.

No bacteriocin synthesis was found at 4 or 10°C, and its production was not dependent on the growth medium. It was generated even in the presence of 1% (wt/vol) honey as the sole carbon source. In all cases, bacteriocin production started after 3 h of incubation at 37°C, as observed in *E. faecium* CRL1385 (15). The titer (22,260 AU ml^{-1}) remained unchanged after 6 h of incubation and 24 h of culture. Besides, *E. avium* PA1 produced its bacteriocin in a high-ionic-strength medium (4.5% [wt/vol] NaCl) and after 24 h at 45°C (11,130 and 20,480 AU ml^{-1}, respectively). The substance's stability in storage at diverse temperatures (−20°C, 4°C, and 25°C) and for several months was remarkable. It could thus be used in high-temperature industrial processes or as a promising natural alternative to control food-borne infection.

No plasmids were shown in *E. avium* PA1 with the techniques employed. Further experiments are being performed to confirm where the bacteriocin production information is encoded and thus determine the stability of this property. In previous assays with *E. avium* whole cells as the template, several PCRs were done using known enterocin primers such as *ent* A, *ent* B, *ent* P, *ent* L50 AB, *ent* AS-48, and *bac* 31 (10) but no products were obtained. Other experiments are being performed to determine if this is a new bacteriocin molecule.

Until now there has been no evidence of any bacteriocin produced by an *E. avium* strain (14). This bacterium's ability to inhibit honeybee pathogens would be used for honey preservation.

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