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EFFECT OF ENTEROCINS CCM4231 AND V24 ON THE CELLS OF ENVIRONMENTAL ISOLATES *Acinetobacter* spp.

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Abstract

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The inhibitory effect of bacteriocins (enterocins) produced by different enterococcal isolates against *Acinetobacter* spp. strains AL1 and AL115 and/or on the resting cells of AL1 strain was tested under *in vitro* conditions. These poly-resistant strains (resistant to 6 from 12 antibiotics) were isolated from the cattle dung water and feces from chamois. Their growth was inhibited by treatment of crude extracts of enterocins CCM4231, V24, EC24, EK13 with activity ranged from 100 to 1600 Arbitrary units per ml. When log phase cells of *Acinetobacter* spp. AL1 strain were treated by crude extracts (CE) CCM4231 and V24 at temperatures 4 °C and 33 °C, the decrease of surviving cells in comparison to the control, one order of magnitude was found. In contrary, when exponential phase cells were harvested and treated by CE CCM4231 and V24, at 4 °C no reduction was noted. While at 33 °C the decrease of cells 2 orders and one order of magnitude was measured. Here, the effect of some enterocins against Gram-negative bacteria was confirmed as well as the way for further experiments to apply enterocins for the control and/or for maintaining the microbial balance in waste ecosystem.

Bacteriocins, excrements, Acinetobacter spp., anti-microbial effect

Livestock management is associated with liquid and solid manure production. The manure possesses a fertilizing value that should be used as much as possible to replace expensive chemical fertilizers. However, the excessive utilization of animal manures in agriculture has resulted, in some areas of Europe with dense animal populations, in serious environmental pollution (Strauch and Ballarini 1994). That is, the manure is a source of different Gram-positive and Gram-negative microorganisms, the pathogenic species including (Lauková et al. 2000a). Animal as well as public health hazard is faced in front of possibilities of diseases transmission. Genus *Acinetobacter* involves Gram-negative, oxidase-negative, non-motile rods taxonomically allotted to the Family *Moraxellaceae* which are ubiquitously distributed in the environment (Bifulco et al. 1989; Rossau et al. 1991). They were detected even in the activated sludge (Rossetti et al. 1997). Bergogne-Berezin (1995) presented the increasing significance of outbreaks of *Acinetobacter* spp. Therefore, there are the impacts leading to search for methods that would be more effective against surviving contaminants. In this paper, the effect of bacteriocins (enterocins) produced by ruminal and environmental enterococci is reported. Moreover, the effect of enterocins CCM4231 and V24 produced by *Enterococcus faecium* CCM4231 and *Ent. faecalis* V24 strains on resting cells of *Acinetobacter* spp. AL1 strain is included. The aim of this study resulted from our previous studies using CCM4231 and V24 bacteriocins to reduce contaminant bacteria in cattle dung water or slurry (Lauková et al. 1998; Lauková et al. 2000b).

Materials and Methods

Strains, media and growth conditions

The strains of *Acinetobacter* spp. were isolated from cattle dung water as well as from feces of chamois by standard microbiological dilution method. Selected isolates were maintained on Trypticase soy broth and agar

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enriched with 0. 6 % of yeast extract (TSB, TSA and with 5 % of defibrinated sheep blood, Becton & Dickinson, Cockeysville, USA) at 30 °C and 37 °C for 24 h and 48 h. Phenotypic identification of species was done by BBL Crystal ID System - Enteric/Nonfermenter Gram -negative ID kit (Becton & Dickinson).

Antibiotic sensitivity and/or resistance testing

Resistance and/or sensitivity to antibiotics was checked by agar diffusion method (Bauer et al. 1966) according to the guidelines of the National Committee for Clinical Laboratory standards (1988) on Trypticase soy agar enriched with 5 % of defibrinated sheep blood using antibiotic discs. Discs were purchased from Becton & Dickinson and the following concentrations were used: gentamicin, ampicillin (GEN, AMP - 10 μg), erythromycin (ERY - 15 μg), kanamycin, tetracycline, rifampicine, vancomycin, streptomycin, chloramphenicol, cefalotine, penicillin (KAN, TCT, RIF, VAN, STR, CHC, CEF, PNC - 30 μg) and bacitracin (BAC – 2 units). Inhibition zone diameters were measured after 18 h - 24 h of incubation at 37 °C of the strains tested. The standard strain, *Staphylococcus aureus* ATCC 6538, was incubated simultaneously as a control.

Preparing of the crude bacteriocin extracts (enterocins)

To test *in vitro* activity of bacteriocins (enterocins) against *Acinetobacter* spp., their crude extracts (CE) were prepared. However, for this test CE of several bacteriocin - producing enterococci (such as *Enterococcus faecium* CCM4231, EK13, *Enterococcus casseliflavus* EC24, *Enterococcus faecalis* V24) were used. CE were prepared as follows: enterococci were grown for 18 h at 37 °C in MRS broth (Becton & Dickinson). Cell-free supernatant fluids were collected by centrifugation (30 min at 10 000 *g*), treated by EDTA, heated for 30 min at 80 °C, cooled and concentrated in a rotary evaporator. Before testing of these CE against *Acinetobacter* spp., their efficacy against the most sensitive indicator organism (*Listeria innocua* Li1, Hans Blom, Matforsk, Norway) was checked by the critical dilution method using agar spot test (De Vuyst et al. 1996) on Trypticase soy agar with 0.6 % of yeast extract (1.5 % and 0.7 %, w/v). And, an inhibitory activity was defined as the reciprocal of the highest dilution which demonstrated complete inhibition of the indicator and expressed in Arbitrary units (AU) per millilitre of culture medium.

Effect of the crude bacteriocin extracts CCM 4231 and V24 on resting cells of *Acinetobacter* spp. AL1

Cells of a middle logarithmic (mid-log) phase and/or exponential phase culture of *Acinetobacter* spp. AL1, grown in Trypticase soy broth with 0. 6 % of yeast extract at 37 °C, were harvested, washed with PBS-buffer pH 7.0 and resuspended to an O.D. of 1.0 in this buffer. 800 AU ml^{-1} of CE CCM4231 and V24 were added to the cell suspensions. After 30 min at 33 °C as well as 4 °C the number of surviving cells was determined. The cell number was also checked from the control samples (without CE addition). The cells survival was checked on TSYA with 0.5 % of defibrinated sheep blood and with trypsine (1mg ml^{-1}) and expressed in colony forming units per ml (cfu ml^{-1}). *Acinetobacter* spp. can grow also under low temperatures. That is, the incubation at low and optimal temperatures was tested to detect an inhibitory activity of CE from the point of their further possible application.

AL1 strain was selected for this experiment because of its better ability to grow in media.

Results and Discussion

On the basis of phenotypic studies the isolates were allotted to *Acinetobacter* spp. They were found to be oxidase-negative, catalase-positive with positive p-nitro-DL-phenylalanine reaction which is typical for *Acinetobacter* spp. However, developed from these results, they cannot be detaily specified to the species level. The defined isolates were marked as *Acinetobacter* spp. AL1 and AL115. Exact specification of *Acinetobacter* spp., e. g. by the method such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis or colony hybridization with a genus-specific 16S rRNA-targeted alkaline phosphatase-labeled oligonucleotide probe by Guardabassi et al. (1998) or by Ellis et al. (1998) confirmed the frequent occurrence of this genus in the wastes. However, the strains of *Acinetobacter* spp. are not only contaminant bacteria. They can play an important role in horizontal gene transfer in soil microcosms and so to be a mechanism for bacterial adaptation to changing environments (Nielsen et al. 2000). Some of *Acinetobacter* spp. can even participate in degradation of phenol in the environment.

Both strains of *Acinetobacter* spp. presented in this study were poly-resistant with their resistance to following antibiotics: ampicillin, erythromycin, vancomycin, penicillin, chloramphenicol and bacitracin. It means, they showed resistance to 6 from 12 antibiotics used. Their remarkable ability to be and/or to develop resistance to antimicrobial agents,

makes these microorganisms particularly suitable for monitoring antibiotic resistance in the environment (Towner 1997; Guardabassi et al. 1998). The presence of antibiotic-resistant bacteria e. g. in cattle dung or in the waste system derived from use of antibiotics in animal husbandry or in agriculture. It assesses the ecological impact from the view to maintain an ecological balance concerning to bacterial flora in nature.

However, concerning our aspect (the occurrence of *Acinetobacter* spp. in animal waste which can be used for land application), the method how to reduce their frequency in the environment mentioned is searched. That is why the treatment by bacteriocins was also applied against the isolates under *in vitro* conditions in this study. CE produced by EC24 strain inhibited both *Acinetobacter* isolates reaching activity 800 and 100 AU ml^{-1} (Table 1). Using CE EK13, the isolates were inhibited by activity 400 and 1600 AU ml^{-1}. When CE V24 and CCM4231 was used to treat AL115 strain, the activity 100 AU ml^{-1} was measured. In the case of AL1 strain it was 100 and 400 AU ml^{-1}. That is, the inhibitory activity of bacteriocins (enterocins) produced by enterococci against Gram-negative bacteria was found as previously reported by Lauková et al. (1993, 1998a, 2000c).

Table 1
Treatment of two strains of *Acinetobacter* spp. by enterocins

Acinetobacter spp.	EK131	V242	EC243	CCM42314
AL1	400*	100	800	400
AL115	1600	100	100	100

[1]Crude extract/CE (enterocin) of *Enterococcus faecium* EK13; [2]CE of *Ent. faecalis* V24; [4]CE of *Ent. faecium* CCM4231. *Inhibitory (bacteriocin) activity is expressed in Arbitrary units per ml (AU ml^{-1}).

Table 2
Effect of crude extracts (enterocins) CCM4231 and V24 on resting cells of *Acinetobacter* sp. AL1 harvested in log growth phase

Control at 4 °C[1]	Sample + CE CCM4231 at 4 °C[2]	Sample + CE CCM4231 at 33 °C[3]	Control at 33 °C[4]
2.4×10^8	1.0×10^7	1.2×10^7	1.8×10^8
Control at 4 °C	Sample +CE CCM4231 at 4 °C[6]	Sample + CE CCM4231 at 33 °C[7]	Control at 33 °C[8]
2.4×10^8	5.0×10^7	2.8×10^7	1.8×10^8

The number of surviving cells is expressed in cfu ml^{-1}. [1]Harvested cells of AL1 strain resuspended in PBS-buffer cultivated at 4 °C for 30 min. [2]Cells of AL1 strain treated by crude extract (enterocin) CCM4231; cultivation at 4 °C. [3]Cells of AL1 strain without enterocin; cultivation at 33 °C. [4]Cells of AL1 strain treated by enterocin CCM4231; cultivation at 33 °C. [5, 7]Cells of AL1 strain without enterocin V24; cultivation at 4 °C and 33 °C. [6,8]Cells of AL1 strain treated by enterocin V24; cultivation at 4 °C and 33 °C.

Effect of CE CCM4231 and V24 on resting cells of *Acinetobacter* spp. AL1 is given in Table 2 and Table 3. When AL1 strain was harvested in a mid-log phase, resuspended in PBS-buffer and treated by CE CCM4231 and V24 individually, a decrease in the viable cell number of one order of magnitude was noted (from 10^8 cfu ml^{-1} to 10^7 cfu ml^{-1}) under both cultivation temperatures (4 °C and °33 C). On the other hand, when AL1 strain was harvested in an exponential phase, treatment by CE CCM4231 as well as V24 at concentrations mentioned above showed the reduction in the viable cell number of 2 orders as well as one order of magnitude at a temperature of 33 °C (from 10^9 to 10^7 cfu ml^{-1} and/or from 10^8 to 10^7 cfu ml^{-1}). In contrary, at 4 °C, no inhibition in cells number from exponential phase growth was counted. The best inhibitory effect of enterocins against indicator bacteria affected in a log phase of their growth during batch cultivation was also reported in our previous studies (Lauková et al. 1999, Lauková and

Table 3
Effect of crude extracts (enterocins) CCM4231 and V24 on resting cells of *Acinetobacter* sp. AL1 harvested in exponential growth phase

Control at 4 °C[1]	Sample +CE CCM4231 at 4 °C[2]	Sample + CE CCM4231 at 33 °C[3]	Control at 33 °C[4]
3.7×10^8	1.1×10^8	6.5×10^7	1.2×10^9
Control at 4 °C[5]	Sample +CE V24 at 4 °C[6]	Sample + CE V24 at 33 °C[7]	Control at 33 °C[8]
3.7×10^8	1.2×10^8	7.8×10^7	1.2×10^9

The number of surviving cells is expressed in cfu ml^{-1}. [1, 3]Cells of AL1 strain harvested in exponential growth phase and reusupended in PBS-buffer; cultivation at 4 °C and 33 °C. [3,4]Cells of AL1 strain harvested in exponential growht phase, resuspended in PBS-buffer and treated by enterocin (CE) CCM4231; cultivation at both temperatures. [5,7]Cells of AL1 strain without enterocin V24; cultivation at both temperatures. [1,8]Cells of AL1 strain treated by enterocin V24; cultivation at both temperatures.

Czikková 1999). However, the aspect of temperature and sensitivity of the indicator organism (structure of cell wall, etc.) must be also taken into account.

In conclusion, the cell number of *Acinetobacter* spp. AL1 strain in a mid - log phase was inhibited by CE of enterocins tested under both low and optimal temperatures (4 °C and 33 °C). When AL1 strain was harvested in an exponential phase of growth, enterocins reduced its cells count at the temperature of 33 °C only. All CE inhibited both *Acinetobacter* spp. by activity ranged from 100 to 1600 AU ml^{-1}. It indicates the correct direction and/or way to continue in the experiments with enterocins applying in wide range of treatment associated with environmental and/or agricultural microbiology or biotechnology.

Účinok enterocínov CCM4231 a V24 na bunky environmentálnych kmeňov *Acinetobacter* spp.

Bol testovaný inhibičný účinok bakteriocínov (enterocínov) produkovaných rôznymi enterokokmi na bunky (z tzv. strednej logaritmickej a exponenciálnej fázy rastu) environmentálnych izolátov *Acinetobacter* spp. AL1 a AL115 za podmienok *in vitro*. Tieto polyrezistentné kmene (s rezistenciou na 6 z 12 testovaných antibiotík) boli izolované z hnojovice hovädzieho dobytka a z faeces kamzíkov. Ich rast bol inhibovaný tzv. „hrubými extraktmi“ (HE) bakteriocínov – enterocínov (CCM4231, V24, EC24 a EK13) s aktivitou v rozsahu 100 až 1600 Arbitrárnych jednotiek (AU ml^{-1}). Pri ošetrení buniek kmeňa *Acinetobacter* spp. AL1 (zo strednej logaritmickej fázy rastu) enterocínmi CCM4231 a V24 pri teplotách 4 °C a 33 °C, bol zistený pokles prežívajúcich buniek v porovnaní s kontrolou o jeden matematický rád. Naproti tomu, bunky z exponenciálnej fázy rastu kmeňa AL1 boli inhibované resp. redukované len pri teplote 33 °C (o 2 a jeden matematické rády). Pri teplote 4 °C nebola zaznamenaná redukcia buniek kmeňa AL1. Uvedené výsledky potvrdili inhibíciu Gram-negatívnych baktérií enterocínmi a naznačili správnosť aplikačného testovania enterocínov pre ich využitie na kontrolu mikrobiálnej rovnováhy v ekosystéme exkrementov.

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