

METAL ION RESISTANCE OF THE BACTERIOCIN PRODUCING ENTEROCOCCI

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Summary

Ten bacteriocin-producing *Enterococcus faecium* strains with urease activity (1.10-6.2 nkat.mL⁻¹) were isolated from the rumen of 2-8 weeks old calves. All strains were resistant against disodium arsenate at a minimal inhibition concentration - MIC 5g.L⁻¹ and mercury chloride (MIC = 10-20 mg.L⁻¹). Eight strains were resistant against silver nitrate (MIC = 40-50 mg.L⁻¹) and three against cadmium sulphate (MIC = 70 mg.L⁻¹). All enterococci tested were resistant at least of one of seven antibiotics used. The resistance against six antibiotics was found in A23 strain. Values of adherence index ranged from 5.02 to 20.4 enterococci adhered per one epithelial cell of rumen wall. All isolates produced bacteriocins which inhibited the growth at least of one of five indicator organisms. The EF1 strain with a good affinity to the epithelial cell (15.2 ± 1.2) produced bacteriocin substance with antimicrobial activity against grampositive and gramnegative indicator bacteria.

(Key Words: Enterococci, Metal Ion Resistance, Bacteriocin, Rumen, Calves)

Introduction

In general, the ecological problems includes an important research area in the all world, involving also agriculture mainly animal production. In ruminants is interesting to study the interactions among rumen microorganisms or microflora of digestive tract with heavy metals. Source of heavy metals in digestive tract is the most frequently from nutrition. The interaction of rumen microorganisms with heavy metals, as a bioindicator of animal metal contamination, has been a priority environmental concern. There is a lack of informations about the occurrence of the metal ion resistance in normal physiological strains isolated from the rumen. Adherent rumen enterococci are present in relatively high counts in the rumen wall of young ruminants (Kmet' et al., 1984) and they may play and important role in the rumen nitrogen metabolism by urease activity (Cook, 1976). As a model strain we have chosen bacteriocin producing *Enterococcus faecium* strain (isolated from the rumen of calves) as a bacteria with selective colonizing advantage. The

other importance to chose rumen enterococci for study of their metal ions resistance was information on plasmid detection in these species (Laukova et al., 1991). Many bacterial strains contain genetic determinants of resistances to heavy metals (Silver and Misra, 1984). The topic of plasmid-mediated heavy metal resistance has been frequently reviewed over the last decade (Foster, 1987). Therefore, the idea for the experiment was to give information on metal ion resistance of enterococci, which are also bacteriocin producers. Bacteriocins are proteins or polypeptides affected against closely related and also against food - borne pathogenic bacteria (Toba et al., 1991; Okereke and Montville, 1991).

Materials and Methods

Bacterial strains

Ten *Enterococcus faecium* strains were isolated from the rumen of 2-8 weeks old calves. Calves were fed on milk replacer containing (dry matter) 70.5% of dried skimmed milk, 18% of fat, 8.5% of enzymatically treated starch, 1% of glucose, 1% of starch syrup and 1% of vitamin supplement. Apart from this, they were fed a ration "TKS". This mixture contains (dry matter) 30% of wheat, 2% of maize, 4% of barley, 12% of

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wheat feed meal, 22.5% of extracted crushed groundnut, 8% of dehydrated protein, 0.5% of fodder salt, 2% of mineral supplements, 1% of biofactor supplement. The samples of the rumen contents were obtained by the stomach tube, 4 hours after morning feeding. They were serially diluted and cultured on Azide Blood Agar Base Medium (Oxoid, UK) with addition of the 60 g sodium chloride per l. To identify the isolates, the Strepto Test identification system for streptococci and enterococci (Lachema Brno, Czech Republic) was used according to the manufacturers instructions. On the basis of the results of tests and according to Ruoff (1990) were isolates allotted to the species *Enterococcus faecium*.

Determination of resistance against heavy metal ions

The heavy metal ion resistance of the rumen enterococci was determined using semiquantitative agar dilution method. Heavy metal salt solutions, such as disodium arsenate-Asa, cadmium sulphate-Cd, mercury chloride-Hg and silver nitrate-Ag were used in this test. Each strain was examined on the Nutrient Agar No. 2 (Oxoid) plates with the supplementation of the metal salt solutions in the relevant concentration. After overnight incubation at 37°C the lowest concentration of the metal ions preventing growth of tested strains was taken as the minimal inhibition concentration (MIC). In MIC tests, isolates were graded as resistant or sensitive respectively as follows: CD > 64 and < 4. mg.L⁻¹, inorganic Hg > 1.25-5.0 and < 0.63 mg.L⁻¹, Asa > 2.0-4.0 and < 0.25-1.0 g.L⁻¹ (Poston and Saw Hee, 1991), Ag > 40-70 mg.L⁻¹ and < 20 mg.L⁻¹ (Grewal and Tiwari, 1990).

Determination of resistance against antibiotics

Antibiotic resistance of enterococci against seven antibiotics was examined by disk method using a commercial Sensi-La-disks (Lachema, Brno, Czech Republic). It was used erythromycin (10 µg/disk) streptomycin, tetracyclin, chloramphenicol and kanamycin (30 µg/disk), penicillin (10 IU per disk) and ampicillin (20 µg/disk).

Determination of bacteriocin production

Bacteriocin production was determined according to (Skalka et al., 1983). *Staphylococcus aureus* Oxford 209 P. *Corynebacterium renale*

CCM 5740 (Veterinary Faculty Brno, Czech Republic), *Streptococcus bovis* AO 24/85 (Institute of Experimental Veterinary Medicine, Kosice, Slovakia), *Enterococcus faecium* EF 26/42 and wild isolate of siderophore producing *Escherichia coli* EC 5 (isolate of this laboratory) were used as indicator strains.

Determination of adherence

Adherence of enterococci to the rumen wall epithelial cells was examined microscopically according to (Kmet' et al., 1984). Adherence index was expressed as an average counts of bacteria adhered per one epithelial cell of the rumen wall.

Determination of urease activity (E.C. 3.5.1.5.)

Urease activity was measured by spectrophotometric method according to (Cook, 1976) and expressed in nkat. ml⁻¹.

The results of adherence and urease activity are expressed as the arithmetical average ± S.E.M.

Results

Bacteriocin production, resistance against heavy metal ions and antibiotics of tested *Enterococcus faecium* strains can be seen in table 1. All strains were resistant against disodium arsenate (MIC > 5 g.L⁻¹) and mercury chloride (MIC = 10-20 mg.L⁻¹). Eight strains of ten tested *Enterococcus faecium* strains were resistant against silver nitrate (MIC = 40-50 mg.L⁻¹) and three against cadmium sulphate (MIC = 70 mg.L⁻¹) - table 2. *E. faecium* A23 was resistant against six antibiotics (ICT, PNC, AMP, KAN, STM, ERY) and three metal ions (Hg, Asa, Ag). This strain produced bacteriocin, which was active against three indicator strains. Enterococci EF2, MID, EF1, EF3 were mono respectively biresistant using antibiotics with a combination of the biresistance or polyresistance (EF3) against metal ions. EF1 strain was with a super strong bacteriocin production against all grampositive and gramnegative indicator strains. Five bacteria of ten bacteriocin producing strains were also active against pathogenic *E. coli* EC5.

Values of adherent index ranged from 5.02 ± 0.50 to 20.4 ± 1.02 enterococci adhered per one epithelial cell of rumen wall. MID strain showed the highest adherence index-20.4 ± 1.0 bacteria adhered to one epithelial cell of rumen wall.

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Urease activity ranged of 1.10 ± 0.55 to 6.2 ± 0.36 nkat.ml⁻¹ (table 3). It means that these *Enterococcus faecium* belong to the strains with

medium or relatively high affinity to epithelial cells of the rumen wall (except strains A22 and R2A) and low urease activity.

TABLE 1. RESISTANCE AGAINST HEAVY METAL IONS AND ANTIBIOTICS AND ANTIMICROBIAL ACTIVITY OF TESTED STRAINS

Strains	Metal ion and drug resistance	Zone of inhibition				
		1	2	3	4	5
A 22	Hg, Asa, TCT, KAN, PNC	+	+	+	-	+
A 26	Hg, Asa, TCT, KAN, PNC, ERY	+	-	+	+	+
A 23	Hg, Asa, Ag, TCT, AMP, KAN, STM, ERY, PNC	+	-	-	+	+
EF 1	Hg, Asa, ERY, PNC	+	+	+	+	+
EF 2	Hg, Asa, PNC	+	+	+	-	-
EF 3	Hg, Asa, Cd, AMP, PNC	-	+	-	-	-
M1 D	Hg, Asa, TCT	+	+	-	-	-
M4 A	Hg, Asa, STM, ERY, PNC	+	-	+	+	-
R2 A	Hg, Asa, Cd, KAN, STM, ERY, PNC	-	+	-	-	+
R2 C	Hg, Asa, Cd, ERY, PNC, CHL	+	+	+	+	

TCT-tetracyclin, AMP-ampicillin, KAN-kanamycin, STM-streptomycin, ERY-erythromycin, PNC-penicillin, CHL-chloramphenicol, Hg-mercury, Asa-arsenate, Ag-silver, Cd-cadmium.

Indicators strains: 1-*Staphylococcus aureus* OXFORD 209P, 2-*Corynebacterium renale* CCM 5740, 3-*Streptococcus bovis* AO 24/85, 4-*Enterococcus faecium* 26/42, 5-*Escherichia coli* EC 5.

Zone of inhibition +, No zone of inhibition -.

TABLE 2. MINIMAL INHIBITION CONCENTRATIONS mg. l⁻¹ OF METAL IONS

Strain	Cadmium (Cd)	Mercury (Hg)	Silver (Ag)
A 22	60	20	50
A 26	60	20	50
A 23	60	10	50
EF 1	50	20	50
EF 2	50	10	50
EF 3	70	10	30
M1D	30	10	20
M4A	30	10	40
R2A	70	10	50
R2C	70	10	50

All strains were resistant against disodium arsenate (Asa)-MIC > 5g.l⁻¹.

TABLE 3. ADHERENCE AND UREASE ACTIVITY OF THE RUMEN *Enterococcus faecium* STRAINS

Strain No.	Adherence index*	Urease (nkat.ml ⁻¹)*
A 22	5.0 ± 0.5	6.2 ± 0.3
A 26	14.7 ± 1.5	6.7 ± 0.4
A 23	13.7 ± 1.9	1.6 ± 0.1
EF 1	15.2 ± 1.2	2.2 ± 0.1
EF 3	14.7 ± 1.8	1.6 ± 0.1
M1D	20.4 ± 1.0	4.1 ± 0.2
M4A	16.3 ± 0.5	1.1 ± 0.55
R2A	5.5 ± 1.7	2.5 ± 0.1
R2C	11.0 ± 1.7	1.2 ± 0.5

* Arithmetical average \pm S.E.M

Discussion

The frequency of resistance against heavy metal ions and antibiotics in rumen enterococci probably reflects the degree of environmental contamination with these agents. The antibiotic treatment of animal infections, especially a diarrhoeal and respiratory syndrome of calves is a source of selection pressure for the development of the resistance in normal physiological microflora. The correlation between resistance against metal ions and antibiotics has been reported in bacterial species of different origins (Cenci et al., 1982) and resistance can be plasmid encoded (Foster, 1983., Kaur and Rosen, 1992). The finding of coresistance against metal ions and antibiotics was also seen in this study. However, this study provides evidence for the occurrence of the resistance and properties involved in the colonization of the rumen epithelial wall. Urease activity of the adherent strains was lower than in rumen fluid as was reported by Wallace et al. (1979) but enterococci are the first lactic acid bacteria colonizing gastrointestinal tract of young ruminants. Enterococcal strains tested in this study produced bacteriocin substances with the inhibitory activity not only against gram-positive bacteria but also against gram-negative pathogenic *E. coli*. The protective effect of *Enterococcus faecium* on the pathogenic strain *E. coli* O 55 in the gnotobiotic pigs was described by Talafantová and Mandel (1985) but its effect was based on the lactic acid production as in a majority currently used probiotics. Positive bacteriocin effect of *E. faecium* against *Clostridium* sp. was described by Koniárová et al. (1992). The common importance of bacteriocin production, urease activity, adherence and resistance against metal ions or antibiotics resulted in knowledge that these properties can be plasmid encoded (Cook, 1976, Kmet, 1980, Bondi et al., 1984, Foster, 1987, Parrot, 1990). Practical relevance of this information is in possibility to use these strains for other study, especially for molecular genetic analysis because as was reported by Lauková et al. (1990) in some adherent, bacteriocin and urease producing, antibiotics resistant rumen bacteria plasmids have been determined. The predominant results showed the presence of plasmids also in presented *Enterococcus faecium* strains (Lauková and Mareková, unpublished

data). Generally, gram-negative bacteria are considered to be more resistant than gram-positive bacteria against antimicrobial agents and it seems that rumen lactic acid bacteria e.g. enterococci can be a good bioindicator model for animal environmental studies.

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