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EFFECT OF LONG-TERM MERCURY TREATMENT ON COMPOSITION OF RUMEN PROTOZOAN *ENTODINIUM CAUDATUM* ASSOCIATED BACTERIAL POPULATION

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ABSTRACT

Upon long term *in vitro* culture of *Entodinium caudatum* with increased dose of mercury, the mercury resistance of *E. caudatum* culture increased from initial level of less than 0.5 microgram per ml to more than 10 micrograms per ml. No changes of mercury chemical status were detected in protozoal culture, indicating the lack of detoxification activities of mercury reductase. Denaturing gradient gel electrophoresis (DGGE) analysis of associated bacterial populations revealed clear shift in eubacterial population structure with hydrogen sulphide producing bacteria overgrowing but very limited changes in archaebacterial population structure. The data obtained indicated that free living bacteria protect protozoan cells and their archaebacterial endosymbionts by eliminating mercury into its insoluble form.

Key words: rumen; *Entodinium*; mercury; bacteria; DGGE

INTRODUCTION

Ruminants can be exposed to toxic concentration of heavy metals by consumption of contaminated feed and water. The microorganisms present in the reticulorumen initially come into contact with materials consumed by the animal and a number of interactions may occur. The ingested toxic elements can be inhibitory to both the fermentation activity and growth of the microorganisms present in the rumen. Microorganisms can decrease productivity of the animals by transformation of the element to a more toxic form, e.g. methylation of mercury (Barkay and Wagner-Dobler, 2005). On the other hand, microbes may also decrease the toxicity of the elements to the animal by their conversion into less toxic forms (Faix et al., 2005).

Ciliate protozoa represent 18 - 32 % of the total microbial biomass in the rumen, therefore they are believed to contribute to the interactions of rumen microbes with heavy metals. However, few studies have been carried out to elucidate the interactions of heavy metals with rumen ciliates (Kisidayova et al., 2001).

The aim of the present study was to elucidate the role of bacteria-protozoa interactions in response to stress evoked by high doses of long-term mercury treatment.

MATERIAL AND METHODS

The ciliate *Entodinium caudatum* was isolated from sheep rumen mixed ciliate population and grown from single cell in a basic mineral culture medium described by Williams and Coleman (1992). The long-term *E. caudatum* culture was daily supplemented with mercury, the concentration of which was gradually increased from 1.5 mg l^{-1} up to 30 mg $l^{-1}$of Hg^{2+} in the form of $HgCl_2$ solution. The Hg-doses were doubled in monthly intervals (1.5-3-6-12-24-30 mg l^{-1} of Hg^{2+}).

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Simultaneously, the control culture was maintained in the absence of mercury.

The mercury content and species were determined in total culture, bacterial, ciliate (cathode) and anode fractions adapting the fractionation method used by Kisidayova et al. (2001). The total content of mercury was determined (Houserova et al., 2005) with an atomic absorption spectrophotometry using thermo-oxidative decomposition of a sample (AMA 254 Advanced Mercury Analyzer). A high performance liquid chromatography in combination with atomic fluorescence spectrophotometric detection using cold vapour technique (HPLC/CV-AFS) to produce elemental mercury was applied for separation and determination of mercury species (methyl mercury, ethylmercury, phenylmercury, and divalent inorganic mercury). Acid hydrolysis in microwave extractor was used for separation and isolation of the mercury species from samples (Houserova et al., 2006).

Total DNA from single *E. caudatum* cells were isolated using CHELEX-100 method as described by Regensbogenova et al. (2004). Total DNA from total culture, bacterial, ciliate (cathode) and anode fractions were purified acording to Pospiech and Neumann (1995). Purified DNA was directly used in PCR amplifications carried out in Personal Thermal Cycler MJ Mini (Bio-Rad Laboratories, Richmond, USA). For the presence of *mer*A sequences in analysed DNA, primers and PCR conditions according to (Schaefer et al., 2004) were used. Samples for DGGE analysis were amplified using eubacterial (Muyzer et al., 1993) or archaebacterial primers (van Hoek et al., 2000) and conditions specified elsewhere.

PCR samples were applied directly onto 8% (wt/vol) polyacrylamide gel in 1× TAE, with denaturant gradient from 35 to 60% (where 100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 50 V and a temperature of 60°C for 17 h. Selected DGGE bands were excised from gel, re-amplified and sequenced at Macrogen sequencing facility (Macrogen, South Korea).

RESULTS AND DISCUSSION

Upon long term *in vitro* culture of *Entodinium caudatum* with increased doses of mercury, the mercury resistance of *E. caudatum* culture increased from initial level of less than 0.5 microgram per ml to more than 10 micrograms per ml. No changes in chemical status of mercury were detected in protozoal culture, indicating the lack of detoxification by mercury reductase enzyme (Table 1).

Table 1: Analysis chemical status of mercury in protozoal and bacterial fractions of *Entodinium caudatum* culture *in vitro*

Content	Bacterial fraction	Protozoal fraction
Total Hg [mg/ml]	4.65 ± 0.12	1.18 ± 0.07
Hg^{2+} [mg/ml]	3.86 ± 0.15	1.18 ± 0.08
Ethyl-mercury [mg/ml]	0	0
Methyl-mercury [mg/ml]	0	0

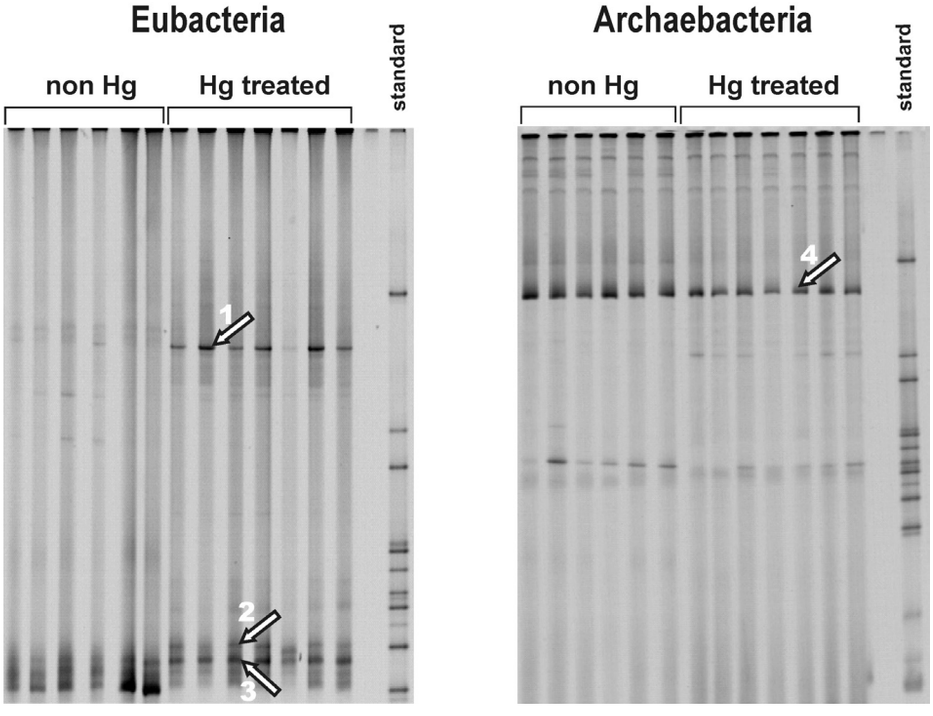

Fig. 1:

DGGE analysis of eubacterial (left panel) and archaebacterial (right panel) populations associated with Entodinium caudatum cell. White arrows indicate the DGGE bands which were excised from gel, re-amplified and sequenced

In protozoal fraction all mercury was present in bi-valent form indicating the lack of mercury reductase activity. In correlation with this observation no *mer*A homologous sequences were detected in this fraction (data not shown). Bacterial fraction was positive for *mer*A sequences and total mercury content was significantly higher than the content of chemically bound mercury, indicating the presence of insoluble zero-valent mercury in this fraction (Table 1).

To evaluate the molecular basis of increased *E. caudatum* mercury resistance associated bacteria were analysed by means of Denaturing Gradient Gel Electrophoresis – DGGE (Muyzer et al., 1993), as it is known that many bacteria, especially methanogens exist in close association with the rumen ciliates.

Total eu- and archaebacterial 16S-rRNA genes were amplified from picked single *E. caudatum* cells. For DGGE analysis variable V3 regions were reamplified and analysed with a DCode Universal Mutation Detection System (Figure 1).

The data indicate clear shift in eubacterial population but only limited effect was observed for archaebacteria associated with *E. caudatum*. To identify bacterial species which were affected by mercury treatment selected DGGE bands were excised form gel and sequenced. The data are summarized in Table 2.

Table 2: Blastn analysis of selected DGGE bands

Band No.	Blastn best hit	Similarity (%)
1	*Prevotella spp.*	98.60
2	*Lactobacillus ovatus*	99.10
3	*Enterococcus durans*	98.30
4	*Methanomicrobium mobile*	99.20

A predominant archaeal symbiont was identified as *Methanomicrobium mobile* in both mercury treated and control samples. This archaeon is a typical representative of rumen methanogenes (Yanagita et al., 2000). Observed shift in eubacterial population was due to increased numbers of prevotellas and *Lactobacillus ovatus* and *Enterococcus durans* species in mercury treated culture as indicated by sequence analysis of predominant DGGE bands. All these bacteria are normal inhabitants of rumen ecosystems and especially species of *Prevotella* genus are known as high hydrogen sulphide producers (Avgustin et al., 1997).

The formation of insoluble mercury salts as a result of sulfide production in the rumen is considered one of the primary mechanisms for detoxification of mercury and other heavy metals in the ruminant digestive system (Suttle, 1975)

The data obtained indicated that free living bacteria protect protozoal cells and their archaebacterial endosymbionts by eliminating mercury into its insoluble form.

CONCLUSIONS

Mercury resistance of *Entodinium caudatum* culture increased from initial level less than 0.5 microgram per ml to more than 10 microgramgs per ml during two years of mercury treatment. No changes in chemical status of mercury were detected in protozoal culture, indicating the lack of detoxification by mercury reductase enzyme and DGGE analysis of associated bacterial population revealed clear shift in eubacterial population structure with hydrogen sulphide producing bacteria overgrowing, while very limited changes were observed in archaebacterial population structure. Based on this data it can be concluded that free living bacteria protect protozoal cells and their archaebacterial endosymbionts by eliminating mercury into its insoluble form.

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