

## Isolation of human pathogen *Escherichia albertii* from faeces of seals (*Leptonychotes weddelli*) in James Ross Island, Antarctica

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### Abstract

A set of nine gram-negative fermenting rods biochemically identified as *Escherichia coli* was isolated from faeces of seals. These bacteria were characterized by phenotypic classification, 16S rDNA sequence analyses, automated ribotyping, study of whole-cell protein profiles by SDS-PAGE and finally by bacteriocin production. The results of our polyphasic taxonomic study supported the recognition of P4652, P4653 and P4740 isolates as true members of *Escherichia albertii* species – probably a major enteric human pathogen. To our best knowledge, this is the first evidence showing that *E. albertii* produces bacteriocin, colicin D. Obtained data unambiguously showed inconvenience of commercial identification systems to distinguish both *Escherichia* species due to missing data of *E. albertii* in the commercial databases. The results of *Escherichia* isolates taxonomy suggest seals as a novel source of human and animal pathogen, *E. albertii* in the Antarctic region.

**Key words:** identification, bacteriocin, biotyping, ribotyping, SDS-PAGE

The GenBank accession numbers for the 16S rRNA gene sequences of *Escherichia albertii* P4652 (CCM 8505) and P4653 are KF891381 and KF891382, respectively.

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## Introduction

Antarctica is the specific cold environment area far from human activities that exhibit totally natural ecosystem. Analyses of microbiota from animals inhabiting such cold environments reveal their biodiversity and specific relationships. Enteric bacteria in the gut of animals may entail positive (*e.g.* probiotics such as lactobacilli) but also detrimental (*e.g.* *Salmonella* infections) consequences. Regulation of the intestinal flora depends on complex interactions between many factors determined by the host environment, *e.g.* secretion of gastric acid, intestinal motility, local immunity, structure of the inner gut and mucous layers, as well as diet (Potti *et al.* 2002). The genus *Escherichia*, one of the most important enteric bacteria, is composed of five valid species, including the type species *Escherichia coli* and four less frequently encountered members: *Escherichia hermannii*, *Escherichia vulneris*, *Escherichia fergusonii* and *Escherichia albertii* (Euzéby 1997). However, the current taxonomic position of *Escherichia* spp. is little complicated. Comparative sequence analysis between the genes for 16S rRNA of *E. coli*, *E. vulneris*, and *E. hermannii* placed *E. coli* and *E. vulneris* together in a tightly related cluster with shigellae, and *E. hermannii* between *Salmonella* spp. and *Citrobacter freundii* (Cilia *et al.* 1996). Recently, based on phylogenetic data, Ezaki (2010) suggested that only two species of genus *Escherichia* should be valid, *E. coli* and *E. albertii*.

Species *E. albertii* was described ten years ago by Huys and co-workers (2003) as the pathogen associated with diarrheal disease in Bangladesh children. Afterwards, *E. albertii* was found as probable emerging major human enteric pathogen because many strains might have been misidentified as enterohemorrhagic (EHEC) or

enteropathogenic (EPEC) *E. coli* (Ooka *et al.* 2012). This species is not only a human pathogen but was found in wild and domestic birds worldwide, both healthy and death birds (Oaks *et al.* 2010, Oh *et al.* 2011). The bird and human *E. albertii* isolates are heterogeneous and do not segregate on the basis of host, geographic origin, or disease status. Strains of *E. albertii* possessed intimin (*eae*) and cytolethal distending toxin (*cdtB*) genes as putative virulence factors (Oaks *et al.* 2010). Recently, the *E. albertii* was found as the causative agent of human gastroenteritis outbreak (Konno *et al.* 2012, Ooka *et al.* 2013).

*Escherichia* spp., as well as many bacterial species, produce antimicrobial agents called bacteriocins (comprise colicins, microcins). Production of bacteriocins is considered to be an important feature of interactions between microbial populations (Cascales *et al.* 2007). Although there is an evidence of the role of some bacteriocins in bacterial virulence (Šmajš *et al.* 2010), in probiotic phenotype of *E. coli* strains (Šmajš *et al.* 2008), and in *E. coli* colonization of gastrointestinal tract (Gillor *et al.* 2009), the exact role of bacteriocin production on bacterial population level is unclear. New information about the frequency of bacteriocins and their molecular types help understand a bacteriocin role in bacterial populations (Šmajš *et al.* 2012).

In this study, we have classified nine strains of the genus *Escherichia* isolated from faeces of seals (*Leptonychotes weddelli*) in James Ross Island, Antarctica, in the austral summer of 2012-13. These isolates were analysed on the basis of phenotypic, genetic and phylogenetic data to the species level and both *E. coli* and *E. albertii* were proved.

**Material and Methods**

*Bacterial strains*

The study was carried out in austral summer of 2012-13 in James Ross Island, Antarctica (63.778338°S, 57.780993°W) and the culture-dependent technique was used to characterize the diversity of enteric bacteria in faeces of seals (*L. weddelli*). A group of nine *Escherichia* strains was isolated from faecal samples of randomly discovered seals in the Lachman cape in the frame of cultivable faecal bacteria communities study project. The samples were taken from seal faeces and/or rectum with sterile swab/transport tube system E-Swabs (Dispolab, Czech Republic), kept at 4°C and brought to the Czech Collection of Microorganisms, Masaryk University, Czech Republic where they were plated and analysed. The time from sampling to initial culture in the laboratory was from

21 to 49 days. The strains, originating from different seals (Table 1), were isolated on Columbia blood agar (Bio-Rad, Czech Republic) by primo-cultivation of samples maintained in E-Swabs (Dispolab, Czech Republic). Subcultures, based on distinct colony differences, were cultivated again on Columbia agar (Bio-Rad, Czech Republic) supplemented with 7% sheep blood and after incubation at 30°C for 24 hours were used for further experiments. A selected representative strain P4652 was deposited in the Czech Collection of Microorganisms (<http://www.sci.muni.cz/ccm>) as *Escherichia albertii* CCM 8505. Reference strains *E. albertii* CCM 7160<sup>T</sup> and *E. coli* CCM 4225 and CCM 5172<sup>T</sup> were used for ribotyping and SDS-PAGE experiments.

Isolate No:	Source	Isolate No:	Source
<i>Escherichia albertii</i>		<i>Escherichia coli</i>	
P4652	fresh seal faeces ( <i>L. weddelli</i> )	P4656	seal faeces on iceberg (unknown species)
P4653	fresh seal faeces ( <i>L. weddelli</i> )	P4657	seal faeces on iceberg (unknown species)
P4740	fresh seal faeces ( <i>L. weddelli</i> )	P4664	fresh seal faeces ( <i>L. weddelli</i> )
		P4665	fresh seal faeces ( <i>L. weddelli</i> )
		P4722	smear from seal rectum ( <i>L. weddelli</i> )
		P4733	fresh seal faeces ( <i>L. weddelli</i> )

**Table 1.** Source of isolation (Lachman Cape, James Ross Island, Antarctica).

*Phenotyping*

The phenotypic characteristics of the isolates were initially performed using two commercial identification kits ENTERO-test 24 (Erba Lachema, Czech Republic) and Biolog Identification System, GP2 Micro Plate (Biolog, USA) according to the manufacturer’s instructions. Furthermore, some physiological and biochemical tube or plate tests (e.g. catalase, oxidase,

gas from glucose, motility, growth at 5°C and 37°C, beta glucuronidase, hydrolysis of gelatine etc.) were additionally carried out according to Abbott et al. (2003) to confirm assignment of isolates into the genus *Escherichia* and to supplement test results with a few key tests missing in identification kits.

### *Bacteriocins*

Bacteriocin production was tested in seven strains identified biochemically as *E. coli* (P4652, P4653, P4656, P4657, P4664, P4665 and P4733) in parallel using four different agar plates containing TY agar, nutrient agar, TY agar supplemented with mitomycin C, and TY agar supplemented with trypsin (Šmajš *et al.* 2010). The rich TY medium consisted of yeast extract (Hi-Media) 5 g l<sup>-1</sup>, tryptone (Hi-Media) 8 g l<sup>-1</sup>, and sodium chloride 5 g l<sup>-1</sup>; the TY agar consisted of a base layer

(1.5%, w/v, solid agar) and a top layer (0.7%, w/v, soft agar). As a relatively unenriched medium, a Difco™ nutrient broth (Difco Laboratories, USA) 8 g l<sup>-1</sup>, NaCl 5 g l<sup>-1</sup>, was used for 1.5% (w/v) agar plates. For induction of colicin production, the base agar layer was supplemented with 0.01% (w/v) mitomycin C. To test protease sensitivity of the inhibitive agents, 0.1% (w/v) trypsin was added to the base layer of agar.

### *Detection of bacteriocin producers, identification of bacteriocin types and E. coli phylogenetic group*

Detection of bacteriocin production was performed as described previously (Šmarda *et al.* 2006). Briefly, the agar plates were inoculated using stab of fresh broth cultures and the plates were incubated at 37°C for 48 hours. The obtained macrocolonies were killed using chloroform vapours and each plate was then overlaid with a soft agar (3 ml) containing 10<sup>7</sup> cells ml<sup>-1</sup> of an indicator strains (*E. coli* K12 - Row, *E. coli* C6 - φ, *Shigella sonnei* 17, *E. coli* P400, *E. coli* S40, *E. coli* 5K). The plates were incubated at 37°C overnight. The formation of inhibition zones on agar plates indicated a

production of inhibition agents. All the seven strains were tested on four various parallel agar plates against six various indicators, *i.e.* 24 individual tests for each tested seal isolate. For identification of individual bacteriocin types, PCR amplifications of bacteriocin coding determinants were performed according to Gordon *et al.* (2006) and Šmajš *et al.* (2010). Phylogenetic group of strains was performed using colony triplex PCR, based on amplification of two genes (*chuA*, *yjaA*) and DNA fragment TSPE4.C2, according to Clermont *et al.* (2000).

### *16S rDNA sequencing and construction of phylogenetic tree*

Sequencing of 16S rRNA gene was performed as described previously (Pontes *et al.*, 2009). Sequencing was performed by MicroSynth, Austria. Sequence analyses were performed using Lasergene soft-

ware (DNASTAR v. 7.1.0., USA). The phylogenetic tree was generated using software MEGA v. 5.2 (Tamura *et al.*, 2011).

### *Ribotyping*

Well-isolated colonies on Columbia agar plates supplemented with sheep blood (incubated at 30°C for 24 h) were used as a template for the RiboPrinter® Microbial

Characterisation System (DuPont Qualicon, USA), according to the standard protocol provided by the manufacturer. Obtained ribotype patterns were processed

and automatically identified by a DuPont Qualicon database DUP 2008 by using RiboExplorer v. 2.1.4216.0 operating software (DuPont Qualicon, USA). Cluster

analysis of ribotype profiles was performed as described previously (Sedláček et al., 2013) using the BioNumerics software v. 6.6 (Applied-Maths, Belgium).

#### *Whole cell protein analysis*

The whole cell protein profile was analyzed by sodium dodecyl sulphate polyacrylamid gel electrophoresis (SDS-PAGE) according to Pot et al. (1994). Cell proteins were extracted from cultures cultivated under standard conditions (24h at 37°C, nutrient agar CM3, Oxoid). Wide range marker (Sigma) ranging from 6.5 to 205 kDa was used as a molecular weight

marker. Protein bands were visualised using Coomassie Blue R-250 staining. Cluster analysis of protein profiles was performed using BioNumerics software v. 6.6 (Applied-Maths, Belgium). The dendrogram was constructed using the UPGMA algorithm based on Pearson correlation coefficient.



**Fig. 1.** Bacteriological smear from seal rectum (Photo: Pavel Jurajda).

## **Results and Discussion**

All nine isolates were gram-negative fermenting rods, catalase positive but oxidase negative. The biochemical properties of isolates were determined primarily by using the ENTERotest 24 and additional phenotypic test were conducted on Biolog system. Both commercial identification

kits, often used by clinical laboratories to identify gram-negative rods, clearly distinguished seals isolates from others enteric bacteria and identified them to the species level mainly as *E. coli* or *E. coli* atypical and inactive (Table 2).

No. of isolate	Identification according to:		
	ENTEROtest 24	Biolog GN2 plate	RiboPrinter
P4652	<i>E. coli</i> atypical	<i>E. coli</i> inactive	unidentified
P4653	<i>E. coli</i> atypical	<i>E. coli</i> inactive	unidentified
P4656	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
P4657	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
P4664	<i>E. coli</i>	<i>Citrobacter</i> sp.	<i>E. coli</i>
P4665	<i>E. coli</i>	<i>Citrobacter freundii</i>	<i>E. coli</i>
P4722	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
P4733	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>
P4740	<i>E. coli</i> atypical	<i>E. coli</i> inactive	unidentified

**Table 2.** Identification of escherichiae isolates by selected commercial systems.

The biochemical test results of the nine isolates were very similar. Isolates P4652, P4653 and P4740 identified as atypical *E. coli* or *E. coli* inactive were distinct from other isolates in one physiological and three biochemical tests. They were non motile, beta-glucuronidase negative and unable to ferment sorbitol and melibiose, *cf. E. coli* identification test results and reference data (Abbott *et al.* 2003).

We used PCR based methods for identification of 33 bacteriocin types (26 colicin types and 7 microcin types) in seven isolates. For two isolates (P4656, P4657), the production of bacteriocin was not observed. Remaining five isolates produced one or more bacteriocin types. Two isolates were monoproducers of colicin D,

two isolates produced two microcins, H47 and M, and one isolate was polyproducer of two microcins (H47 and M) and colicin E1 (Table 3). In addition, phylogenetic groups were tested. Four isolates belonged into group D, one isolate into group B2 and two isolates (P4652, P4653) failed in amplification of any sequence determinant (*chuA*, *yjaA*, TSPE4.C2). According to this result, isolates cannot be classified in any group or can belong in group A (Table 3). Virulent strains of *E. coli* mainly belong to the group B2 (Clermont *et al.* 2000). Considering, that the isolate P4733 belongs to B2 group and produce microcin H47 and colicin E1, this isolate could be another potentially pathogenic strain isolated from seals.

Isolate No.	Bacteriocin type	<i>chuA</i> detection	<i>yjaA</i> detection	TSPE4.C2	<i>E. coli</i> phylogenetic group
P4652	D	-	-	-	A or not classified
P4653	D	-	-	-	A or not classified
P4656	-	+	-	-	D
P4657	-	+	-	-	D
P4664	H47, M	+	-	-	D
P4665	H47, M	+	-	-	D
P4733	H47, M, E1	+	+	-	B2

**Table 3.** Bacteriocin types and *E. coli* phylogenetic groups.

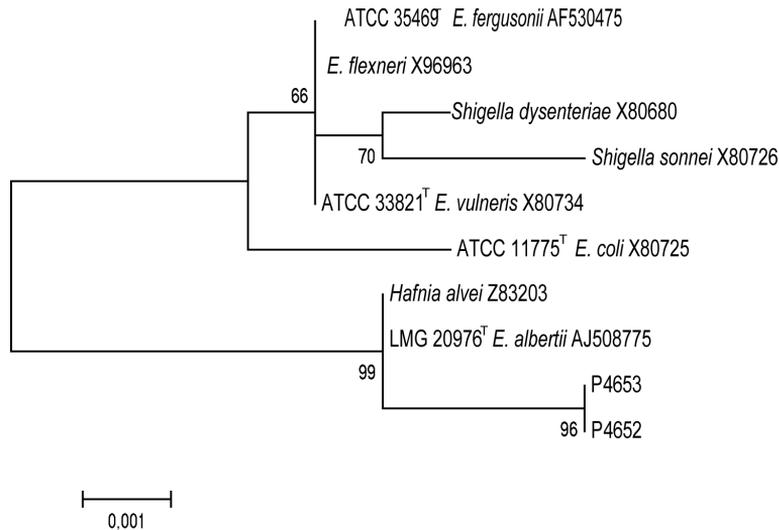
We have identified an incidence of bacteriocin D, H47, M and E1. Microcin H47 and colicin E1 were described as potential virulence factor (Šmajš *et al.* 2010).

Colicin D is rarely isolated type of bacteriocin. In the study published by Gordon *et al.* O'Brien (2006), 102 producer strains of *E. coli* from Australia were

analysed and colicin D was not detected at all.

The 1350 bp of 16S rDNA (out of 1350 bp) sequences were analyzed for isolates P4652, P4653 and references strains, download from the database RDP (Cole et al. 2009), tested between coordinates 67-1417 (16S rDNA, 1494 bp). Both se-

quence of strains P4652 and P4653 were identical and showed 99.7% homology to species *E. albertii* (LMG 20976<sup>T</sup>). The variability in 1350 bp long fragment was found only in three positions - G1107A, C1109T, C1113T. The position of the two seal isolates in phylogenetic tree is shown in Fig. 2.



**Fig. 2.** The unrooted tree of locus 16S rDNA. The tree was generated by MEGA 5, using bootstrapping maximum likelihood algorithm and Tamura Nei model. The bar scale represents the number of nucleotide changes per 1 nt site. Bootstrap probability values greater than 66 (percentage of 1000 tree replication) are indicated at branch-points. Used sequences were download from the database RDP.

Sequencing of 16S rDNA showed, that strains P4652 and P4653, producing bacteriocin D, belonged to the species *E. albertii*, a recently described member of the *Enterobacteriaceae* and associated with diarrheal illness in humans and birds. To our knowledge, this is the first evidence showing that *E. albertii* produces some bacteriocin, colicin D.

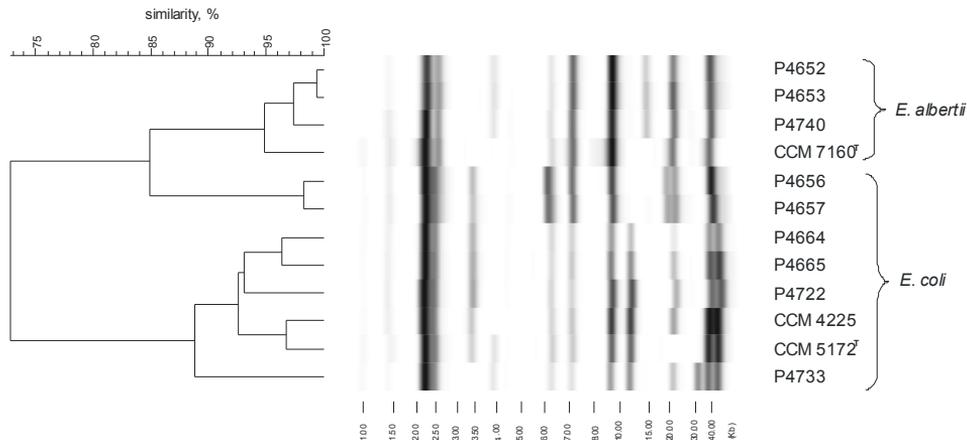
Based on distinct phenotypic identification and 16S rDNA sequencing results, additional analysis (ribotyping, whole-cell protein analysis) were suggested and performed to clarify taxonomic position of isolated *Escherichia* spp. members from

seal faeces. The automated ribotyping by a RiboPrinter has been shown to provide rapid, accurate and reproducible genetic information for the identification of many species (Brisse et al. 2002, Hartel et al. 2007). In our study, six isolates were identified as *E. coli* while three remaining isolates (P4652, P4653 and P4740) stayed unidentified (Table 2).

Cluster analysis of ribotype patterns divided the studied *Escherichia* isolates into two unequivocally separate groups (Fig. 3) clearly corresponding with 16S rDNA sequencing data for P4652 and P4653 isolates. Three isolates (P4652, P4653 and

P4740) have almost identical ribotypes which were very similar to a restriction profile of *E. albertii* type strain CCM 7160<sup>T</sup>. This group with coincident restriction profiles (similarity > 95%) for-

med separate ribotypes clearly distinct from the rest of analysed *Escherichia* strains. The fragment of 15 kb was determined for all three isolates of seals.

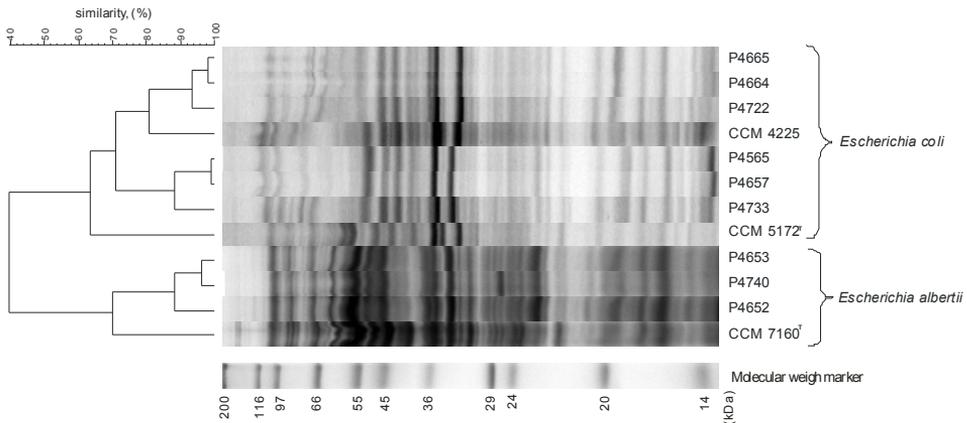


**Fig. 3.** Dendrogram based on the cluster analysis of ribotype patterns (*EcoRI*) obtained by the RiboPrinter Identification System from faecal *Escherichia* isolates and from the type and reference strains of escherichiae. The dendrogram was calculated with Pearson's correlation coefficients with UPGMA clustering method.

These data strongly support previous finding that P4652, P4653 and P4740 isolates were highly divergent from *E. coli* strains and represent members of *E. albertii* species. Current commercial biochemical identification systems misidentified these isolates as *E. coli* due to absence of *E. albertii* species data in databases. It is difficult to discriminate *E. albertii* from *Escherichia* spp. by using routine bacterial identification systems based on biochemical properties (Abbott *et al.* 2003, Hyma *et al.* 2005, Oaks *et al.* 2010). A large number of *E. albertii* strains might have been misidentified as EPEC or EHEC because they possess the *eae* gene which encodes intimin (Konno *et al.* 2012, Ooka *et al.* 2012). For validation of presumption, that we had isolated *E. albertii* strains from faeces of seals, the whole-cell protein

analysis was performed with representative isolates and reference strains.

The cluster analysis of protein profiles clearly separated the strains into two sub-clusters at similarity level of 39% (Fig. 4). The three isolates (P4653, P4740 and P4652) showed a close similarity (86%) and were grouped together with the type strain of *E. albertii* CCM 7160<sup>T</sup> (at 70% similarity level). The second cluster comprised two CCM reference strains of *E. coli* (CCM 5176<sup>T</sup> and CCM 4225) and six isolates from seals identified by phenotyping as *E. coli*. Results of the whole-cell protein analysis revealed that the three isolates of biochemically atypical or inactive *E. coli* (P4653, P4740 and P4652) are more related to *E. albertii* than to *E. coli*.



**Fig. 4.** Dendrogram based on the whole-cell protein profiles of faecal *Escherichia* isolates and from the type and reference strains of escherichiae. The dendrogram was constructed using Pearson's correlation coefficients with UPGMA clustering method.

Obtained 16S rDNA sequencing data, ribotyping and SDS-PAGE results confirmed placement of P4652, P4653 and P4740 isolates as true members of *E. albertii* species. The *E. albertii* is a newly emerging enteric pathogen that has been associated with sporadic infections among humans and birds (Nimri, 2013). However, the role of *E. albertii* among seal population is unclear and as well as whether all faecal strains from seals are associated with diarrheal cases. As detected in our

study, the faeces from seals could be the source of such potential pathogens. Whether *E. albertii* can be transmitted from animals to humans is unknown, although MLST and PFGE data by Oaks et al. (2010) suggest that zoonoses or anthroponoses are possible. Further studies are needed to assess virulence and the occurrence of virulence genes among these strains from seals or to discover a distribution of *E. albertii* strains among other Antarctic avian and mammal populations.

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