Microbiology, Vol. 73, No. 2, 2004, pp. 150–155. Translated from Mikrobiologiya, Vol. 73, No. 2, 2004, pp. 188–194.

EXPERIMENTAL ARTICLES

Characterization of Enterobacteria Producing the Low-Molecular-Weight Antibiotics Microcins

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Received August 13, 2002; in final form, April 28, 2003

Abstract—A comparative study of the morphological, cultural, physiological, and biochemical properties of the microcinogenic strains EcS 5/98, EcS 6/98, and EcB 214/99 and the known microcin C51 producer *Escherichia coli* M17(p74) showed that these strains belong to the species *E. coli.* The strains produced microcins with molecular masses lower than 10 kDa. Microcin biosynthesis was stimulated by a deficiency of nutrients in the cultivation media. The microcins were found to be resistant to thermolysin but were degraded by pronase, protolichetrem, and the *Bacillus mesentericus* metalloproteinase. This indicated that the microcins are peptides or contain peptides in their molecules. The study of cross immunity to the microcins and the sequencing of their genetic determinants showed that the microcins of strains EcS 5/98 and EcS 6/98 are of B type, whereas the microcin of strain EcB 214/99 presumably belongs to another type, since it suppresses the growth of the producers of C and B-type microcins. The new microcin producers possess antibacterial activity against natural isolates belonging to the genera *Escherichia* and *Salmonella*, against a wide range of colicinogenic *Escherichia* strains, and against collection *Salmonella* cultures.

Key words: *Escherichia*, biology, microcinogeny, antagonism.

It has long been known that enterobacteria can synthesize not only bacteriocins but also low-molecular-weight antibiotics, called microcins [1]. The microcins are a heterogeneous group of plasmid-encoded compounds that are secreted into the medium and suppress the growth of many gram-negative enterobacteria of the genera *Escherichia, Salmonella, Shigella, Proteus, Klebsiella, Serratia, Enterobacter, Citrobacter, Pseudomonas*, and others.

The currently characterized microcins belong to seven types, A, B, C, D, E, H, and J [2–6], and efforts go on in study of their structure, mechanism of action, genetic control, and role in the ecology of enteric bacteria. The antagonistic activity of microcins implies that they can be used to control the population of gram-negative bacteria in the human and animal intestines.

The aim of this work was to study the physiology, biochemistry, and genetics of microcinogenic escherichia strains isolated from the gastrointestinal tracts of pigs and cattle.

MATERIALS AND METHODS

Experiments were carried out with strains EcS 5/98 and EcS 6/98, isolated from pigs, and strain EcB 214/99, isolated from cattle.

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The antagonistic activity of the strains was studied with *Escherichia coli* K-12, *Escherichia coli* 113-3, and other freshly isolated and collection strains of *E. coli* as the test cultures. The nutrient media used were the minimal medium of Khmel *et al.* [7] and its modified version enriched in yeast extract and glucose (0.2%); Luria–Bertani (LB) agar; minimal Davis agar; tryptose agar (TA); fish meal (FM) broth and agar in various versions; nutrient broth; and agar. Tryptose, LB, and FM agars were either full or quater-rich (in the nutrient content). The media contained 1.5% agar.

The synthesis of microcins was studied as follows: Bacteria were plated onto a cellophane film laid on the surface of agar plates. After one day of incubation, the film was removed together with the grown microcin-producing colonies. The agar plates were sterilized with chloroform and overlaid with soft agar (0.7%) containing the exponential-phase cells of a test culture. After one day of incubation, the plates were examined for the size of growth inhibition zones. The absence of such zones was an indication that the test culture was resistant to microcins. In these experiments, the *E. coli* strains M17(p74) and BZB 2283, which produce microcins C51 and B17, respectively, were used as the positive controls.

Strains EcS 5/98, EcS 6/98, and EcB 214/99 were studied for their morphology; gram staining; ability to

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grow at 15 and 45°C; survival at 60°C; tolerance to 0.2, 0.4, and 0.6% phenol; tolerance to 2, 4, and 6% ethanol; and tolerance to 2 and 4 dry wt % bile. The strains were tested for growth on Kligler iron agar, Simmons citrate agar, acetate and malonate agars; for hydrolysis of phenylalanine and urea; and for the ability to ferment carbohydrates and higher alcohols. These tests, as well as the methyl red and Voges–Proskauer tests, were performed as described by Golubeva *et al.* [8].

The antibiotic resistance of the microcinogenic strains was studied by using paper disks containing (μg/disk) oxacillin, 10; chloramphenicol, 30; streptomycin, 30; gentamicin, 120; rifampicin, 5; neomycin, 30; erythromycin, 15; tetracycline, 30; kanamycin, 30; vancomycin, 30; klindamycin, 2; claritromycin, 15; and amikacin, 30. The sensitivity of the strain to nalidixic and fusidic acids was studied at their concentrations of 20 and 150 μg/ml, respectively.

The sensitivity of the microcins to proteolysis was studied with thermolysin (a neutral protease of *Bacillus thermoproteolyticus*) purchased from Serva; bovine trypsin (Serva); subtilisin E (a serine proteinase isolated from *Bacillus subtilis* in the Laboratory of Protein Chemistry at the Institute for Genetics and Selection of Industrial Microorganisms); metalloproteinase from *Bacillus mesentericus* [9]; protolichetrem (a commercial preparation from the culture liquid of *Bacillus licheniformis that* contains an alkaline protease of the subtilisin Karlsberg–type, a metalloproteinase, and glutamyl endopeptidase); and pronase from *Streptomyces griseus* (Fluka). The concentration of the proteolytic enzymes was 2 mg/ml.

The genetic determinants of the microcins were cloned as described by Groisman and Casadaban [10]. Strain EcS 5/98 was lysogenized with bacteriophage Mu cts and plasmid Mud5005 [10]. The phage library obtained by the thermoinduction of the double lysogen was screened by means of transduction into *E. coli* HB101::Mu cts, followed by selection on agar plates containing the EcS 5/98 microcin. The transductants were grown at the permissive temperature (30°C). Quater-rich tryptose agar plates were inoculated with strain EcS 5/98 and incubated for 1 day. The plates with a lawn of EcS 5/98 cells were treated with chloroform to lyse the cells. After desiccation, the plates were overlaid with a thin agar layer. Such plates possessed antagonistic activity against *E. coli* HB101 over a period of 3–4 days. The microcin-resistant transductants were tested for the genetic markers of *E. coli* HB101 [11] and the ability to suppress the growth of the recipient strain *E. coli* HB101::Mu cts. The location of genetic determinants on phagemids was confirmed by observing the transfer of the respective phenotypic traits during the transformation of the phagemid DNA of the Mu cts–lysogenic strains. In this case, we used selection for the phagemid marker of kanamycin resistance.

Plasmid and phagemid DNAs were isolated by the rapid alkaline extraction procedure [12] and analyzed with the aid of restriction endonucleases. For nucleotide sequencing, the DNA extracts were purified by mixing a cleared lysate with an equal volume of 8 M ammonium acetate. Phagemid fragments were subcloned with vector pUC19 [13] as recommended in a laboratory manual [11]. Plasmid DNA was sequenced with an *fmol* DNA Sequencing System kit (Promega) by using oligonucleotide primers complementary to the vector parts of plasmids pUC19 or Mud5005. The nucleotide sequences obtained were compared with nucleotide sequences available in GenBank by using the BLAST program [14].

RESULTS AND DISCUSSION

The microcinogenic strains EcS 5/98, EcS 6/98, and EcB 214/99, as well as the microcin C51-producing strain *E. coli* M17(p74), are gram-negative rods with rounded ends. When cultivated on Endo medium, the strains produced dark red colonies with a brilliance like that of metals. The strains could grow at temperatures between 15 and 45°C but could not survive 30-min exposure to 60°C. The strains were found to be tolerant to 0.6% phenol, 2% ethanol, and 20% bile (strain EcB 214/99, even to 40% bile). They did not form H_2S, did not produce gases from glucose, showed a positive reaction in the methyl red test and a negative reaction in the Voges–Proskauer test, were unable to utilize acetate and malate as carbon sources, and did not hydrolyze phenylalanine and urea. Strains EcS 5/98 and EcB 214/99 were able to grow on Simmons citrate agar.

All the strains produced acids from lactose, maltose, mannitol, glucose, xylose, trehalose, and sorbitol but were unable to ferment inositol, dulcitol, and salicin. Arabinose was fermented by strains *E. coli* M17(p74), EcS 5/98, and EcS 6/98 but not EcB 214/99. Sucrose was fermented by *E. coli* M17(p74) but not by EcS 5/98 and EcS 6/98. Strain EcB 214/99 showed variable ability to ferment sucrose.

All these data allowed the strains under study to be assigned to the species *E. coli*.

The production of microcins was tested on tryptose, LB, and FM agars, as well as on the minimal agar of Khmel *et al.* [7]. When tested on full-strength tryptose agar, only the strains *E. coli* M17(p74) and EcB 214/99 produced zones of inhibited growth. On the quater-rich tryptose agar, the growth inhibition zones induced by these strains increased in size from 20 to 30 mm and from 12 to 16 mm, respectively. Strains EcS 5/98 and EcS 6/98, which did not produce growth inhibition zones on the full-strength tryptose agar, did produce them on the quater-rich tryptose agar (Table 1).

Similarly, the microcinogenic strains produced growth inhibition zones on the quater-rich LB agar but not on the full-strength LB agar. On the full-strength FM agar, microcins were produced by strains EcS 5/98 and EcB 214/99. On the quater-rich FM agar, microcin was produced by strain EcS 6/98 as well. The growth

Table 1. The effect of cultivation conditions on microcin production

Strain	Diameter of growth inhibition zone, mm						
	Tryptose agar		LB agar		FM agar		Ref. [6]
	full	quater-rich	full	quater-rich	full	quater-rich	
E. coli M17(p74)	20	30	–	25	ND	ND	22
E. coli S 5/98	–	19	–	14	15	17	–
E. coli S 6/98	–	17	–	15	–	17	–
E. coli B 214/99	12	16	–	18	15	15	15

Note: ND stands for "not determined"; the sign "–" indicates the absence of a growth inhibition zone.

Table 2. The sensitivity of microcins to proteolytic enzymes

Proteolytic enzyme	Microcins produced by			
	E. coli M17(p74)	EcS 5/98	EcS 6/98	EcB 214/99
Thermolysin	–	–	–	–
Trypsin	+	–	–	–
Subtilisin E	+	+	–	+
Metalloproteinase from *B. mesentericus*	+	+	+	+
Protolichetrem	+	+	+	+
Pronase	+	+	+	+

Note: The signs "+" and "–" indicate, respectively, the sensitivity and the resistance of microcins to attack by the proteolytic enzymes.

Table 3. The cross sensitivity of microcin-producing strains

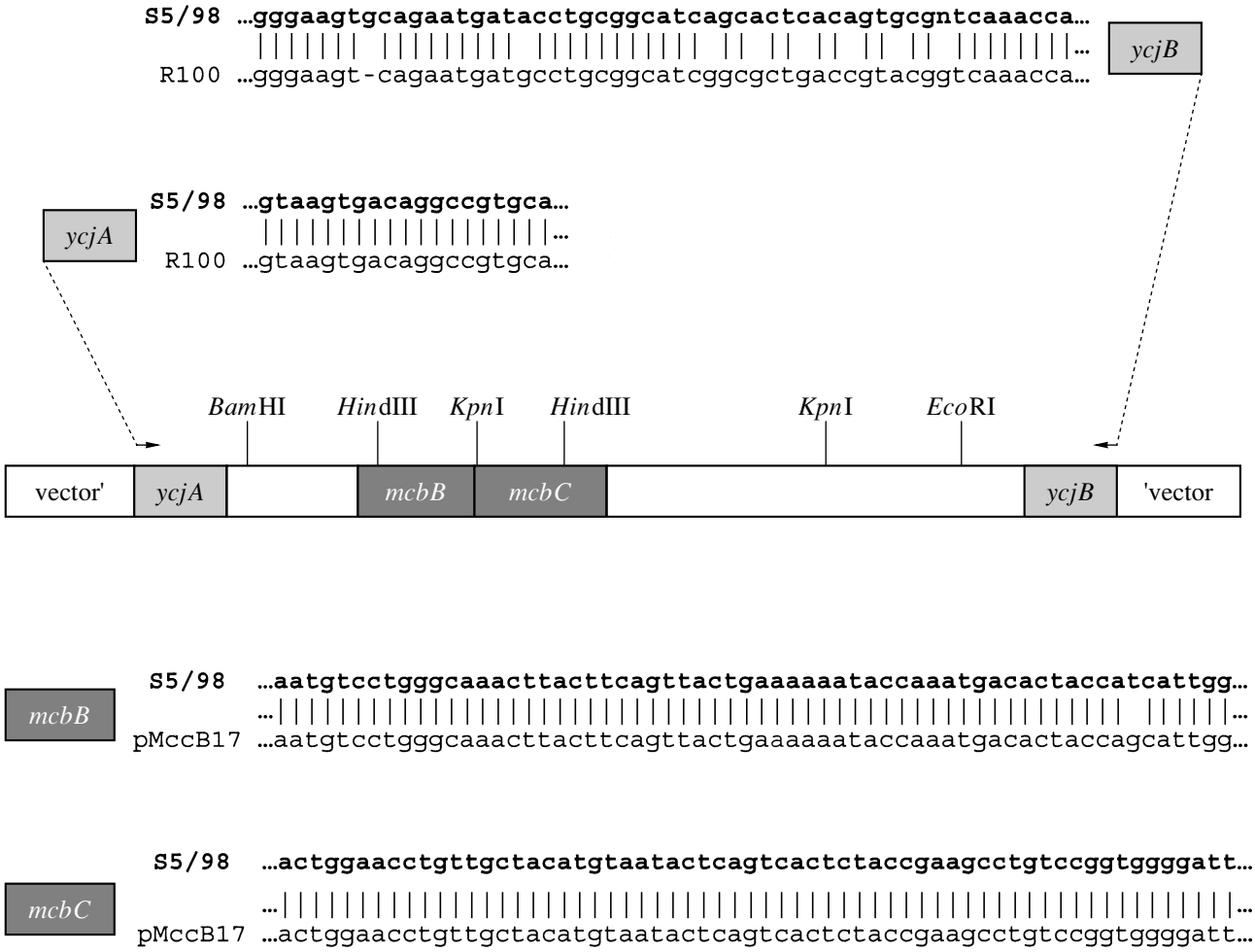
Test strain	Diameter of growth inhibition zone, mm				
	E. coli M17(p74)	EcS 5/98	EcS 6/98	EcB 214/99	*E. coli* BZB 2283
E. coli M17(p74)	–	25	23	26	10
EcS 5/98	17	–	–	8	–
EcS 6/98	20	–	–	7	–
EcB 214/99	11	–	–	–	–
E. coli BZB 2283	40	–	–	9	–

Note: The sign "–" indicates the absence of a growth inhibition zone.

Table 4. The microcin sensitivity of the wild-type *Escherichia* and *Salmonella* strains isolated from various domestic animals

Animal	Strains of the genus	Number of tested strains	Percent microcin-sensitive strains		
			C 51	EcS 5/98	EcS 6/98
Cattle:					
cows	*Escherichia*	200	ND	84	ND
	Salmonella	47	ND	42.6	ND
bull calves	*Escherichia*	84	ND	69	69
Pigs	*Escherichia*	50	52	90	90
	Salmonella	30	50	80	80
Broiler chickens	*Escherichia*	17	88.2	82.3	82.3
	Salmonella	23	56.5	56.5	56.5
Cats	*Escherichia*	100	100	99	99

Note: ND stands for "not determined."



The structure of a DNA fragment of strain EcS 5/98 in the phagemid Mud5005 responsible for type-B microcin production. Nucleotide sequences are aligned with the homologous regions of plasmids R100 and pMccB17.

inhibition zone induced by EcS 5/98 on this agar was greater than on the full-strength FM agar. On the minimal agar of Khmel *et al.* [7] with yeast extract and glucose, microcins were produced only by *E. coli* M17(p74) and EcB 214/99.

These data confirm the earlier observations that nutrient deficiency promotes microcin synthesis in bacteria of the genus *Escherichia.* The absence of microcin production in strains EcS 5/98 and EcS 6/98 cultured on the minimal medium of Khmel *et al.* suggests that not only nutrient deficiency but also other factors may influence microcin synthesis.

The fact that the microcins were able to pass through cellophane film indicates that their molecular mass is less than 10 kDa.

All the microcinogenic strains were sensitive to almost all of the antibiotics tested, except that strain EcB 214/99 was resistant to 150 μg/ml fusidic acid. This contradicts the data available in the literature that natural, nonantagonistic *Escherichia* strains are resistant to many antibiotics. We believe that the low antibiotic resistance of the microcinogenic escherichia may be related to their production of microcins, which provide for the competitiveness of such escherichia in natural microbial communities, thereby compensating for the absence of antibiotic synthesis.

To gain some information on the chemical nature of the microcins, they were treated with different proteolytic enzymes directly on the surface of the agar plates. All the microcins were found to be inactivated by pronase, protolichetrem, and the *B. mesentericus* metalloproteinase but not by thermolysin (Table 2). The microcin produced by strain EcS 6/98 was resistant to subtilisin E and trypsin. The microcins of strains EcS 5/98 and EcB 214/99 were resistant to trypsin. These data suggest that the microcins under study are of a peptide nature and may differ from each other. The latter suggestion needs further studies with the use of purified microcin preparations.

Table 5. The antibacterial activity of microcin-producing strains

Test culture	Diameter of growth inhibition zone, mm			
	E. coli M17(p74)	EcS 5/98	EcS 6/98	EcB 214/99
Salmonella heidelberg 287	16	–	–	–
Sal. stanley 5716	22	–	–	–
Sal. kirkee	21	–	–	–
Sal. give	30	12	10	10
Sal. bovis morbipicans 988	29	8	8	8
Sal. dublin 42	28		–	–
Sal. london 1446	36	17	17	16
Sal. gaminare	30	11	13	11
Sal. utrecht	22	–	–	–
Sal. derby	21	7	9	8
Sal. amager 2399	18	8	8	10
Sal. sendai	–	–	–	–
Sal. rostock	–	12	12	10
Sal. readiry	33	20	18	21
Sal. typhimurium (colicins E1 and J)	15	–	–	–
Sal. enteritidis 41997	18	11	10	10
Klebsiella sp. 4140 (K-7)	–	16	12	12
Escherichia coli 113-3	28	22	18	20
Proteus sp. 14	–	–	–	–
Proteus sp. 2091	–	–	–	–
Providencia stuartii	–	–	–	–
Providencia alcalifaciens	–	–	–	–
Staphylococcus aureus	–	–	–	–
Staphylococcus saprophiticus	–	–	–	–
Staphylococcus epidermidis	–	–	–	–

Note: The sign "–" indicates the absence of a growth inhibition zone.

The seven presently known microcin types [2–6] are mainly classified according to the cross immunity of the producing strains, i.e., their resistance to microcins of the same type and sensitivity to microcins of the other types. In addition, microcins are classified according to their antagonistic properties, structure, and mechanism of action [2].

Our experiments showed that the microcinogenic strains EcS 5/98, EcS 6/98, and EcB 214/99 are inhibitory to the microcin C51–producing strain *E. coli* M17(p74) and, vice versa, are sensitive to this microcin (Table 3). Consequently, the EcS 5/98, EcS 6/98, and EcB 214/99 microcins are not of C type. Strains EcS 5/98 and EcS 6/98 did not inhibit the microcin B17–producing strain *E. coli* BZB 2283, suggesting that the microcins of these two strains may belong to B type. As for strain EcB 214/99, it suppressed the growth of the C- and B-type microcin producers and, hence, probably synthesizes a microcin of a different type. This inference requires further studies.

To verify the supposition that strain EcS 5/98 produces a B-type microcin, we cloned the genetic determinants responsible for the production of the microcin and immunity to it. Recombinant strains were obtained by screening the phage library of strain EcS 5/98 created on vector Mud5005 [10] as described in the Materials and Methods section. The partial sequencing of the cloned fragments showed that they are homologous to the *mcbB* and *mcbC* genes involved in the production of microcin B17 (figure) [15]. A similarity search with the use of the BLAST program showed that the adjacent sequences are similar to the plasmid genes *IncFII*, particularly to the ORFs with an unidentified function of the neighboring *yciB* and *yciA* genes of plasmid R100 (GenBank accession number AP000342). The homologous sequences of EcS 5/98 were separated by an insertion containing the genes that control the production of the microcin and immunity to it. The alkaline extraction of EcS 5/98 cells did not yield a plasmid (data not shown). This can be due either to a large size of the res-

ident plasmid or to its integration with the chromosomal DNA of strain EcS 5/98. It should be noted that this strain is not resistant to the antibiotics encoded by plasmid R100. In turn, this plasmid does not contain genes responsible for microcin biosynthesis.

The antibacterial activity profile is an important characteristic of microcin-producing strains. Relevant experiments showed that the growth of 69–84% of *Escherichia* strains and 42.6% of *Salmonella* strains of wild type isolated from cattle was suppressed by the microcins of strains EcS 5/98 and EcS 6/98 (Table 4). These microcins also inhibited the growth of 90% of escherichia and 80% of salmonella isolated from pig feces, whereas microcin C51 inhibited the growth of 52% of escherichia and 50% of salmonella. Microcin C51 also inhibited more than half of the *E. coli* strains isolated from the chicken intestines. All the microcins studied suppressed the growth of the *E. coli* strains isolated from cat feces, some colicinogenic escherichia and salmonella, and one klebsiella strain, whereas bacteria of the genera *Proteus, Providencia*, and *Staphylococcus* turned out to be resistant to the microcins (Table 5). These data show that the antagonistic activity profiles of strains EcS 5/98 and EcS 6/98 are very similar. The electropherograms of the restriction enzyme digests of the total nucleic acids of these strains were also nearly identical (data not shown). All this, together with the very similar physiological and biochemical properties of EcS 5/98 and EcS 6/98, testifies that these strains are identical.

ACKNOWLEDGMENTS

We are grateful to I.A. Khmel for providing microcinogenic strains and test cultures and to A.B. Shevelev for providing the proteolytic enzymes.

This work was supported by the Russian Foundation for Basic Research, project no. 01-04-96027, and by grant no. NSh-1712.2003.4 from the Russian Ministry of Industry, Science, and Technology.

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