A NEW FAMILY OF LOW MOLECULAR WEIGHT ANTIBIOTICS FROM ENTEROBACTERIA

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SUMMARY

An apparently new family of antibiotics has been detected in the culture media of bacteria isolated from human feces. In a preliminary investigation ten different compounds have been partially purified and characterized. All of them are thermostable, soluble in methanol-water (5:1) and have a molecular weight of about 1000 or less. They show a different degree of specificity as growth inhibitors of a series of enterobacteria as well as other non-enteric microorganisms. Current work strongly suggests the existence of other similar compounds among Enterobacteriaceae and other bacterial families.

INTRODUCTION

Present knowledge on the chemical communication between the cells of microbial ecosystems is scarce. It is even the case for the bacterial population of the human intestinal tract, where there is a rather well defined community of microorganisms that show, however, a dynamic succesion in both species composition and relative abundance of each type, under either normal or pathological conditions. Succesions in these systems are not infrequently rapid and very specific in regard to the main invaders, which are able somehow to efiectively displace other closely related bacterial species by mechanisms poorly understood. Colicins, for instance, have been invoked to explain these displacements among intestinal enterobacteria, but the avail-

able evidence does not seem to account for such phenomenon (1). There might be other chemical agents endowed with the required information to act competitively in a specific manner.

On this basis, we have undertaken a systematic search for compounds produced by enteric bacteria isolated from the intestinal content of infants, which could exert antibiotic effects on related microorganisms. The design of the screening was made to look for compounds of relatively low molecular weight, and, at the same time, to exclude interference by conventional colicins. Preliminary results indicate the occurence of such compounds. Thus, several types of substances have been detected and partially characterized, which show a different degree of specificity as growth inhibitors of other enterobacteria. Likewise, the results obtained suggest the existance of more types of these compounds among Enterobacteriaceae and other bacterial families.

MATERIALS AND METHODS. Escherichia coli strains K 12 (3,300) B, W and 405 (Mc Leod strain) were routinely used as markers for the screening procedure. For the specificity study of purified preparation of the different antibiotics, the collection of microorganisms isolated and identified in our Service of Bacteriology was employed. Most of the strains selected for the exploration of antibiotic production were facultative aerobic enterobacteria, and were obtained from the feces of hospitalized infants.

Chemicals. Methanol of analytical quality was obtained from either Merck or Carlo Erba; methionine analogs, pronase and subtilysin type VII from Sigma; amino acids, vitamins, and purine and pyrimidine bases from Calbiochem; Sephadex G-10 and G-15 from Pharmacia; Amicon filters are made by Amicon, Lexington, Mass.; and Pellicon filters by Millipore, Bedford, Mass.

Procedure. The antibiosis assays were performed as follows: agar-minimal medium plates (see below) were inoculated with the marker strains of E. coli by the soft agar double-layer technique; upon hardening of the medium, a film (6 x 5 cm) of Cellophane was placed on the agar surface of each plate. Smears of potential producing strains were seeded in different spots on the Cellophane surface, usually 6 per plate. After being incubated for 24 hours at 37ºC, the plates were checked for antibiosis (see Figure 1). The rationale of this procedure was to exclude interference by conventional colicins, since these agents have a molecular weight of 40,000 upwards and are thus retained by the Cellophane membrane, which allows otherwise the passing of substances with a M.W. of about 15,000 downwards.

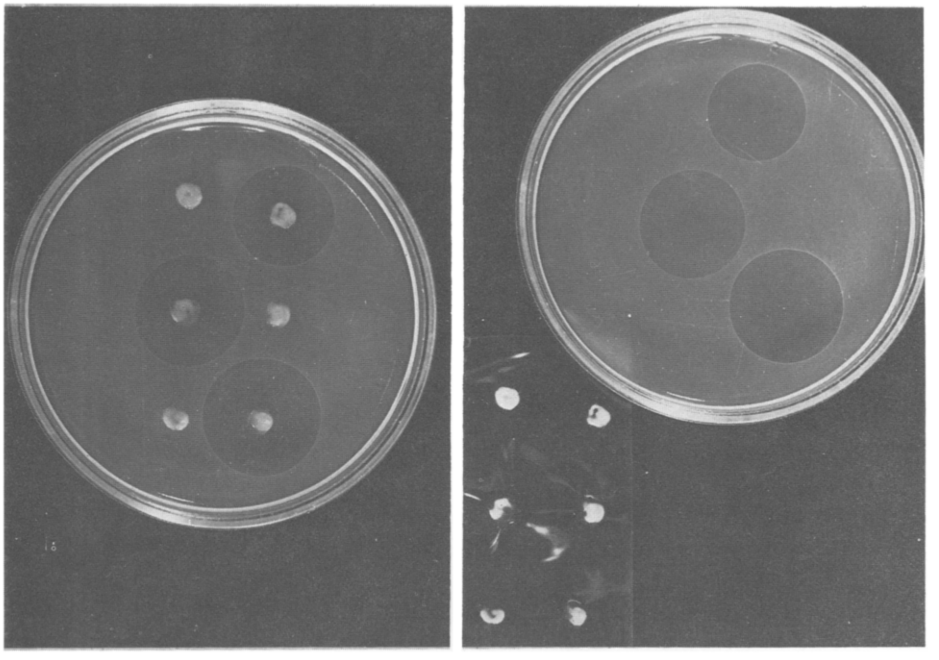

Figure 1. Antibiotic assay for 6 enterobacteria isolated from infantile feces on the growth of E. coli K 12. The picture shows the same plate, before and after laying aside the Cellophane film (lower part) on which had been grown the isolates. It can be seen the inhibitory effect of 3 of them.

Microorganisms that showed clear inhibition halos on any of the markers growth were subsequently cultured in minimal medium "63" (2) with 0.2% glucose, pH 7, the same used to prepare the agar plates (in which traces of thiamine were also added). 2 l flasks with 1 l of medium in each were inoculated and incubated overnight at 37ºC in a rotary shaker. Cells were removed by centrifugation and the supernatants were used for further studies. Antibiotic activities were approximately estimated by measuring directly, or after serial dilution of the samples, the diameters of the inhibition halos produced by aliquots (0.01 ml) of the active preparations.

RESULTS AND DISCUSSION

About 15% of the nearly four hundred isolates that were assayed produced inhibition zones of a diameter greater than the corresponding colonies, on one or more of the markers lawns. The most active cells were selected for further investigation. Most of the supernatants obtained from the cultures of these microorganisms showed antibiotic activity on the same markers. These liquids were concen-

Table I: Some properties of the enterobacterial antibiotics

Properties / antibiotics	15	17 w	17 e	76	136	138	140	146	152	290
Molecular weight [a]	< 500	>1000	<1000	< 500	<500	~1000	<500	<500	< 500	>500 <1000
Thermostability (100ºC-30 min)	+	+	+	+	+	+	+	+	+	+
Adsorption on activated charcoal	-	+	+	-	-	-	-	-	-	+
Resistant to pH 1 [b]	+	-	+	+	+	-	-	+	-	-
" to pH 12 [b]	+	-	+	+	+	-	+	-	+	+
" to pronase [c]	+	-	+	+	+	+	+	+	+	+
" to subtilysin [c]	+	-	+	+	+	+	+	+	+	+
Antagonism by amino acids, vitamins or nitrogenous bases [d]	Met	No	No	No	Met	Met	No	No	No	No

a) According to filtration through Amicon PM-10 and UM-05 (M.W. less than 10,000 and 500, respectively) and Pellicon PSAC 02510 (less than 1,000) membranes. b) By adding 5 N HCl (pH 1) and 5 N NH_4OH (pH 12); the samples were exposed to these pHs for 3h, then brought to neutral pH. c) Assayed at a final concentration of 10 and 20 µg/ml for 3h at 37ºC. d) All used at a final concentration of 10 µg/ml; vitamin B_1, B_6, PABA, nicotinic acid and inositol were assayed; the amino acids, and purine and pyrimidine bases tried were the usual constituents of proteins and nucleic acids, respectively.

trated at vacuum at 40ºC, down to 1:20 of the original volume, and then precipitated with 5 volumes of methanol. Most of the activities were found to be preserved in the supernatants after this treatment, which otherwise allowed the elimination of the bulk of salts and macromolecules of the concentrates. Each supernatant was again concentrated in the same way down to 1:500 of the original volume, and then heated at 100ºC for 30 min, cooled, and thoroughly mixed with 5% of activated charcoal during 10 min; the charcoal was afterwards discarded by centrifugation. None of the antibiotic activities of these preparations was apparently affected by the heating; some of them, however, were lost by the charcoal treatment.

The "x 500" concentrates, treated with charcoal when appropriate, were the standard preparations used to obtain the data summarized in Table I, which includes some properties of 10 of the antibiotic activities found.

Antibiotic activities 15, 17 (w and e) 76, 136, 138, 140 and 146 are produced by 7 different strains of E. coli ; 152 and 290 by 2 strains identified as Pseudomonas aeruginosa.

All the antibiotic activities shared three common features, namely, solubility in methanol-water (5:1), low molecular weight and thermostability. They are however distinguishable in regard to the other properties listed in Table I as well as to the antibiotic specificity, which is shown in Table II. It is at present doubtful whether activities 140 (E. coli) and 152 (P. aeruginosa) are identical or not; the morphology of their growth inhibition halos is clearly distinct, and there are some differences in their specificity spectra (see Table II).

Three of the antibiotic activities (15, 136 and 138) are fully antagonized by l-methionine at a final concentration of 10 μg/ml. None of the activities except one (17 w) is inactivated by the proteases pronase and subtilysin. Inactivation by these enzymes of 17 w indicates an oligopeptide structure for it, since its molecular weight is higher than 1000 but lower than 5000, according to its behaviour in Sephadex G-15 and G-25 chromatography and its filtration through Amicon PM-10 but not through Pellicon PSAC 02510 membranes.

Table II: Antibiotic specificity on enterobacteria and other microorganisms

Organisms / Antibiotics	15	17w	17e	76	136	138	140	146	152	290
Escherichia (20)	20	4	16	4	20	12	5	17	8	14
Proteus (11)	11	0	11	6	0	2	0	11	0	10
Enterobacter (7)	5	1	7	1	4	4	0	4	0	4
Klebsiella (3)	3	0	3	0	3	1	0	0	0	3
Serratia (3)	3	0	3	0	3	0	0	0	0	3
Salmonella (3)	2	0	2	2	2	3	1	3	0	2
Pseudomonas (3)	3	3	3	3	0	3	3	3	2	1
Levinea (2)	2	0	2	2	2	2	0	2	0	2
Shigella (2)	2	1	2	1	0	0	0	2	0	0
Citrobacter (2)	2	0	2	0	2	2	0	2	0	1
Acinetobacter (2)	2	0	2	0	2	2	0	2	0	1
Streptococcus (2)	2	0	0	0	0	0	0	1	0	2
Staphylococcus (5)	0	0	0	5	0	0	0	0	5	5

All the Escherichia species were of the coli type. Figures in parenthesis in the first column indicate the number of different strains assayed for each genus. The figures in the other columns correspond to the number of strains that were inhibited by the antibiotic, in each case. The antibiosis assays were carried out with 0.01 ml aliquots of the "x 500" preparations; the antibiotic-producing strains were also sensitive to their own "x 500" preparations, but were insensitive to the growing of their own colonies by the Cellophane technique (see Procedure).

It is possible that 17 e is a derivative of 17 w. Both are produced by the same strain of E. coli and have other common features (see Table I). They differ, however, besides in their sensitivity to proteases, in their M.W., solubility in ethanol (17 e being soluble) and antibiotic spectra (see Table II). Further information on the chemical

nature of the antibiotic entities here reported would eventually require their physical isolation, a task in which we are presently involved.

On the whole, it can be presumed the occurrence of more types of antibiotic entities of a similar character to the ones reported here, at least among enteric bacteria. Because of their characteristics, they could be tentatively grouped into a family, for which the generic name of microcins is proposed, which seems to be distinguished from the main known classes of conventional antibiotics, which might be clustered into two general groups: (a) those produced by Actinomycetes, mostly by Streptomyces species. Their chemical structures are very heterogenous and, in general, they show wide spectra of antibiotic action; (b) those produced by sporogenic bacteria of the genus Bacillus, like bacitracins, tyrocidins and polymyxins, which are of a cyclic peptide character and are not found free in the culture media (3). They, too, are usually active on many types of bacteria. On the contrary, the antibiotics identified in this investigation: (a) are produced by non-sporogenic bacteria isolated from the human intestinal tract, for which no antibiogenic capacity has been described (except the synthesis of colicins, which in fact are not considered as conventional antibiotics); (b) are excreted into the culture media, and (c) they act on closely related microorganisms.

It is to be expected that the occurrence of "microcins" is not restricted to the mammalian intestine ecosystems. In any case, their presence poses a major question, regarding their physiological role in the habitats where they occur. On a teleonomic premise, they might be related to the ecological competition that gives place to the dynamic microbial succesions mentioned above as a typical feature of the bacterial community of the intestine. If so, the study of "microcins" and their inhibitory mechanisms would be a relevant subject to achieve a better understanding of this ecosystem, including its common alterations by the enteropathogenic microbial flora. It seems also predictable that the availability of different types of purified "microcins" might lead eventually to some instances of therapeutic utility. In this

regard, it is to be noted that none of the E. coli antibiotic producing strains described in this report belongs to the enteropathogenic class, according to the standard techniques of serotypification.

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REFERENCES

1. Ikari, N.S., Kenton, D.M., and Young, V.M. (1969) Proc. Soc. Exp. Biol. Med., 130, 1280-1285.
2. Sistrom, W.R. (1958) Biochem. Biophys. Acta., 29, 579-587.
3. Bodanszky, M., and Perlman, D. (1969) Science., 163, 352-358.