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Occurrence of Strains Producing Specific Antibacterial Inhibitory Agents in Five Genera of Enterobacteriaceae

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Abstract. Striking differences in the production of specific inhibitory agents affecting other strains of the same (or of related) species were found between genera of the family Enterobacteriaceae. We tested 50–163 strains each of the potentially pathogenic genera: *Escherichia, Citrobacter, Enterobacter, Kluyvera*, and *Leclercia* for their ability to produce bacteriophages, high-molecular-weight (HMW) and low-molecular-weight (LMW) bacteriocins and siderophores against the same sets of strains, using the cross-test method. The genus *Escherichia* differs substantially from all other Enterobacteriaceae, harboring a notable proportion of lysogenic (36.6%) and colicinogenic (13.9%) strains. Only 18.2% of the *Citrobacter* strains are lysogenic and only rarely are they colicinogenic, although in 7.3%, they produce phage tail-like bacteriocins. On the other hand, *Kluyvera* strains were only in 1.8% lysogenic, no colicinogenic strains were found, but in 7.3%, they produced siderophores causing zones of growth inhibition in agar cultures of strains of the same genus. In *Leclercia*, 10.0% of the strains were lysogenic, 2.0% produced HMW bacteriocins, no colicinogenic strains were found and 2.0% produced siderophores. *Enterobacter* has shown 23.1% of strains producing siderophores, whereas merely 7.7% were lysogenic, 1.9% colicinogenic and 3.8% formed phage tail-like bacteriocins. HMW bacteriocins of *Enterobacter* strains disposed of an unusually wide spectrum of activity. The siderophore activity spectrum was rather wide in any genus, but the siderophores were usually not produced by strains producing phages or colicins.

Bacteria frequently produce agents inhibiting other strains of the same and of more or less closely related species. This action—and the strain specificity of it—is mostly determined by specific receptors on the cell surface (cell wall or cell membrane). The production of such agents and the susceptibility to them might be experimentally proven by the interaction of a producer strain with the susceptible one in a common culture.

Technically, such an interaction can be revealed most easily by inhibition zones around colonies of producer strains in agar cultures. From various physical and physico-chemical parameters of the inhibition zones, the production of at least four types of inhibition factors can be deduced: of bacteriophages (in lysogenic strains), of protein bacteriocins or polypeptide microcins [low-molecular-weight (LMW) bacteriocins], of corpuscular [high-molecular-weight (HMW)] bacteriocins (in bacteriocinogenic strains) or of siderophores. At least several of them participate in the determination of a selective growth advantage of the producer strains compared to nonproducer bacteria. Nonpathogenic strains with a selective growth advantage over pathogenic or potentially pathogenic strains might be applied as probiotics, if further experimental tests will justify such a design [2].

We undertook several series of producer strains screening among conditionally pathogenic species in five genera of the family Enterobacteriaceae. We were stimulated by our previous finding that the natural occurrence of lysogenic and bacteriocinogenic strains

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strikingly differ in three species of *Escherichia*: *E. fergusonii, E. vulneris*, and *E. hermanii* [18].

Materials and Methods

Bacterial Strains Tested. Fifty strains of *Leclercia adecarboxylata*, 55 strains of the genus *Kluyvera* (34 strains of *K. ascorbata* and 21 strains of *K. cryocrescens*), 55 strains of *Citrobacter youngae*, 52 strains of the genus *Enterobacter* (25 strains of *E. cloacae*, 19 strains of *E. agglomerans*, and 8 strains of *E. aerogenes*) were supplied by the National Reference Laboratory for Salmonellae, National Health Institute in Prague. The bacterial genera and species of used strains were identified using standard classification methods for the family Enterobacteriaceae [3]. The results of commercially available biochemical tests were evaluated by the TNW Pro Auto 6.0 Win program (Czech Collection of Microorganisms, Masaryk University, Czech Republic). More detailed information on strains used in this study is available at one of the authors (Dr. Dědičová).

Most strains were isolated from nonviable environmental materials; some were isolated from biological (human or veterinary) sources. None was isolated from any pathological material. The experiments on 53 *E. coli* strains were published separately [17].

Nutrient Media. The media used were as follows: TY broth: 8 g caseine enzymic hydrolysate type I, 5 g yeast extract powder (both components: HI Media Laboratories, India), 5 g NaCl per 1000 mL of water. Nutrient agar No 2 Imuna, Slovakia: 5 g meat extract, 5 g peptone, 6.25 g nutrient base No 1, 6.25 g nutrient base No 2, 2.5 g NaCl, 1.5% (w/v) agar in water for base layers and 1.0% (w/v) agar for soft upper layers, pH 7.2.

For the detection of siderophore production, restricting the growth of indicator strains, the nutrient agar was supplemented with 200 μM 2,2′ dipyridyl and 100 μM nitrilotriacetate. The spontaneous production of bacteriophages was enhanced by adding 5 μg of mitomycin C per 1 mL of upper layer soft agar [= 0.0005% (w/v) mitomycin C]. For confirmation of narrow inhibiton zones of HMW bacteriocins, 0.1% (w/v) trypsin was added to the soft agar upper layers.

Experimental Procedures. The basic protocol was founded on the classical Fredericq's [4] agar stab test for colicinogeny, applied in a cross-test. Fresh broth cultures were inoculated with a needle stab into base agar plates and the plates were incubated at 37°C for 48 h. The grown-up bacterial accruals were killed by chloroform vapors and each plate was covered with a soft agar upper layer containing 10^7 cells/mL of a fresh broth culture of strain tested for susceptibility. After solidification, the plates were incubated at 37°C overnight. All 50–55 strains of each genus were cross-tested as possible producers and as possible indicators. In addition, four ''universal'' indicators of *E. coli* (i.e., strains K12-Row, C6-ϕ, B1, and P400) and one indicator of *Shigella sonnei* (strain 17) were used. Each pair of strains was tested at least twice.

In lysogenic producers, phage plaques (usually tiny ones) were reproducibly formed, scattered around their grown accruals. The phages were autoreproducible in series; that is, a small amount of sample from the middle of each plaque was resuspended in 100 μL of distilled water that was placed on 0.5 mL of chloroform to ensure sterility. Ten microliters of such sterile samples were used to inoculate the fresh broth culture of susceptible bacteria. After overnight incubation, bacteria were removed by centrifugation and the clear supernatant was placed in the tube containing 0.5 mL of chloroform. Ten microliters of serial supernatant dilutions were added to the soft agar upper layer containing 10^7 cells/mL of a fresh broth culture of a susceptible strain. After solidification, the plates were incubated at 37°C overnight. Phage particles formed plaques on the plates, whereas similar samples from colicin inhibition zones did not result in any growth inhibition. Three different zones of growth inhibition were distinguished based on the diameter of the zone (and thus the molecular weight of the inhibitory compound), sensitivity to trypsin, and the degree of growth inhibition around the producer. HMW bacteriocins are particles composed of many protein subunits forming conspicuously narrow zones of complete growth inhibitions that are resistant to trypsin [14, 15]. LMW bacteriocins are proteins sensitive to trypsin treatment [4] that form zones of variable diameter showing complete growth inhibition;, no LMW bacteriocin zone was formed on agar with 0.1% (w/v) trypsin. Additionally, a siderophore-caused inhibition zone was usually very wide (due to its low molecular weight), only partially hampering the growth, not delineated by a distinct edge, and not sensitive to trypsin. In comparison to HMW and LMW bacteriocins, siderophores formed much more distinct zones of growth inhibition on iron-limited agar (i.e., on nutrient agar with the addition of iron-chelating compounds; see the subsection Nutrient Media). The inability to utilize ferric siderophores from the medium (especially under iron-restricted conditions) is a growth disadvantage that is likely to reflect the absence of a functional siderophore transporter system in inhibited strains. In general, the siderophore strain specificity was very low in most cases. Using the described experimental approach, it cannot be ruled out that other growth-inhibiting substances could meet the criteria used for the identification of bacteriocins and siderophores.

Three control producers were included in the study comprising the HMW bacteriocin producer (*Citrobacter youngae* 29 [16]), the LMW bacteriocin producer (strain *E. coli* 5K pDS1 producing colicin U [13]), and the aerobactin producer (strain *E. coli* K311 [10]). Control *E. coli* indicator strains were K12-Row, C6-ϕ, B1, and P400 [18]. Colicin Js control indicator was *Shigella sonnei* (strain 17) [18]. Each pair of strains was tested at least twice.

All of the results were noted as those of plus/minus assays. The simple ways of application thereof were based on experience with thousands of producing, nonproducing, susceptible, and unsusceptible strains. Thus, all of the bacterial growth-inhibiting agents could be proved and differentiated unequivocally from each other.

Aerobactin was detected as described previously [1] on iron-limited nutrient agar plates containing 0.2 mM 2,2′ dipyridyl and 0.1 mM nitrilotriacetate as the iron-chelating compounds. *E. coli* strain H1887 was used as an aerobactin indicator, strain H1886 as a negative control, and strain K311 as a positive control in the aerobactin assay [10]. Each experiment was repeated at least three times.

Results

***Escherichia* spp.** Here, we combine the three species producer incidence values published previously (110 tested strains [18]) with that for 53 strains of *E. coli* to ascertain the average incidence for the genus *Escherichia.* In *E. coli*, 39.6% of strains produced bacteriophages, being lysogenic, 43.4% produced colicins (LMW bacteriocins), 5.7% of strains produced corpuscular (HMW) bacteriocins, and 15.1% released siderophores (mostly aerobactin [17]). Out of 163 strains tested in the genus *Escherichia*, we calculated the following results: 36.6% of strains lysogenic, 13.9% of strains producing LMW colicins, 1.4% of strains forming HMW bacteriocins, and 5.3% of strains releasing siderophores.

Table 1. Reciprocal inhibitory effects of the strains *Citrobacter youngae*

Producer strain	Sensitive strains for the agent produced			
	Bacteriophage	HMW bacteriocin	LMW bacteriocin	Siderophore
6	12			
11	12			
12	6, 20, 21, 29, 39, 46			
18	26			
19	26			
20	29, 39			
21	3, 12, 37			
26		18, 19		
29		12		
31	3, 6, 11, 12, 13, 21, 25, 29 39, 46, 47			
35	26			
39		54		
47		31		
52				4, 17, 40, Row, ϕ, B1, P400, Shs17
54	39			
55				4, 17

***Citrobacter youngae*.** From 55 strains tested, 10 (18.2%) produced bacteriophages; these were different from each other and were more closely characterized in a special study [16]. Four *Citrobacter* strains (i.e., 7.3%) produced HMW bacteriocins, none of them produced any standard LMW bacteriocin (similar to colicins), and two (3.6%) strains produced specific siderophores different from aerobactin. Four strains produced bacteriophages and HMW bacteriocins together. All of the positive results are summarized in Table 1.

***Enterobacter* spp.**

Fifty-two strains of 3 species belonging to the genus *Enterobacter* were examined: 25 strains of *E. cloacae*, 19 strains of *E. agglomerans*, and 8 strains of *E. aerogenes*. All 52 strains were tested in a cross-test, as stated in the Materials and Methods section.

In *E. cloacae*, three (12%) strains produced bacteriophages, no strains formed bacteriocins (HMW or LMW), and six (24%) strains produced siderophores. In *E. agglomerans*, one (5.3%) strain was lysogenic, none formed a HMW bacteriocin, one (5.3%) formed a LMW bacteriocin, and four (21%) strains produced siderophores. In *E. aerogenes*, no strain produced a bacteriophage or an LMW bacteriocin, two (25%) formed HMW bacteriocins, and one (12.5%) produced siderophores. All of the positive results are summarized in Table 2. Altogether, in the genus *Enterobacter*, we found 4 (7.7%) strains producing bacteriophages, 2 (3.8%) strains forming HMW bacteriocins, 1 (1.9%) strain producing a LMW bacteriocin, and 12 (23.1%) strains producing siderophores. Five strains produced aerobactin (2, 6, 11, 19, and 20; Table 2). Two strains (43 and 44) stimulated growth of *E. coli ent*A but not of *E. coli fep*A mutant strain (data not shown), suggesting that the synthesized siderophore was enterochelin. Additional five strains synthesized untyped siderophores. Out of these producers, siderophores of strains 10 and 45 inhibited growth of both *Escherichia* and *Enterobacter* indicators, whereas growth inhibition of strains 31, 32, and 51 caused by siderophore production was limited to the *Enterobacter* indicators (Table 2). In Table 2, strains 1–25 belong to *E. cloacae*, strains 26–44 to *E. agglomerans*, and strains 45–52 to *E. aerogenes*.

***Kluyvera* spp.** Fifty-five strains of 2 species of the genus *Kluyvera* were tested: 34 strains of the species *K. ascorbata* and 21 strains of *K. cryocrescens*. All 55 strains were tested as one set in a standard cross-test.

In *K. ascorbata*, one (2.9%) strain was lysogenic and produced a phage and two (5.9%) strains produced a siderophore; none produced HMW or LMW bacteriocins. In *K. cryocrescens*, only two (9.5%) strains formed siderophores; again, none produced bacteriocins and none produced bacteriophage. Hence, in the genus *Kluyvera*, altogether, only one strain (1.8%) produced bacteriophage and four (7.3%) strains formed siderophores different from aerobactin. No production of other agents was noted. All of the results are summarized in Table 3; in this table, strains 1–34 belong to *K. ascorbata* and strains 35–55 belong to *K. cryocrescens*.

***Leclercia adecarboxylata*.** From 50 strains of *Leclercia adecarboxylata*, 5 (i.e., 10%) were lysogenic, 1 (2%) produced an HMW bacteriocin, none formed an LMW bacteriocin, and 1 (2%) produced a siderophore. For the summary and strain specificity survey of these results, see Table 4.

Discussion

Genera in the family Enterobacteriaceae are generally distinguished on the basis of morphological, ecological, immunological, biochemical, sequential, and other parameters, in accordance with other families of bacteria. The same criteria direct delimitation of species within the genera. Less attention has been devoted to physiological parameters, as well as to the capability to synthesize and release exocellular products inhibiting

Table 2. Reciprocal inhibitory effects of the strains *Enterobacter* spp.

	Sensitive strains for the agent produced			
Producer strain	Bacteriophage	HMW bacteriocin	LMW bacteriocin	Siderophore
1	4, 14, 52			
2				16
6				9
7	2			
10				7, 9, 12–16, 20, 44, 52, Row, ϕ, B1, P400, Shs17
11				1, 4, 5, 10, 16, ϕ
19				16, 37, 44
20				7, 40, 48
25	12, 20			
26	12			
31				7, 34, 39–42
32				2, 9, 7, 12, 16–18, 20, 34, 39–42
43			29, 33, 48, 50	26
44				36
45				7, 12–14, 17, 19, 20, 25, 26, 44, 52, Row, ϕ, B1, P400, Shs17
50		2–8, 16, 17, 20, 22, 24, 31–34, 38, 39, 42, 43, 45		
51		2–7, 16, 17, 20, 22, 24, 31–34, 38, 39, 42, 43, 45		4, 17

Table 3. Reciprocal inhibitory effects of the strains *Kluyvera* spp. (strains 1–34 belong to *K. ascorbata* and strains 35–55 belong to *K. cryocrescens*)

	Sensitive strains for the agent produced			
Producer strain	Bacteriophage	HMW bacteriocin	LMW bacteriocin	Siderophore
9				11, 35, 51, 52
22				11, 17, 35, 45, 48, 49, 51, 52, 54
28	53			
39				2, 3, 15, 17, 35, 49, 52
46				2–6, 8, 9, 11, 15–17, 20–22, 24–27, 29, 31–35, 38, 39, 44, 47–49, 51–53, Row, ϕ, B1, P400, Shs17

Table 4. Reciprocal inhibitory effects of the strains *Leclercia adecarboxylata*

	Sensitive strains for the agent produced			
Producer strain	Bacteriophage	HMW bacteriocin	LMW bacteriocin	Siderophore
10		44		
12	3, 10			
16				Row, ϕ, B1, P400, Shs17
21	30			
44	10, 14, 21, 30, 32, 34, 42			
45	2			
50	2			

growth of other strains, of the same or of related species (genera).

In this article, we show the existence of profound differences in this respect among five genera of Enterobacteriaceae. Recently, we have shown analogous differences among three novel species of *Escherichia* [18]. Taking the genus *Escherichia* as a whole, we can again state profound differences between it and the four genera examined here.

Whereas about 40% of naturally occurring human strains are lysogenic in *E. coli* and 57% in *E. hermanii*, only 2% (*Kluyvera*) to 18% (*Citrobacter*) lysogenic strains can be found (applying the same screening method) in the four genera investigated in the same family. Whereas 43% strains produce molecular (protein) colicins in *E. coli* (although none in *E. vulneris* or *E. hermanii*), 0% (three genera) to 2% (*Enterobacter*) only are colicinogenic in the four genera in question. Although 6% of strains were found producing corpus-

cular (HMW) bacteriocins in our *E. coli* set of strains, 0% (*Kluyvera*) to 7% (*Citrobacter*) of strains produced them in these four genera, documenting the known low incidence of such particles in Enterobacteriaceae in general.

On the other hand, the genus *Enterobacter* is conspicuously rich in siderophore-producing strains; 23% of strains release siderophores into their surroundings, whereas the occurrence of siderophore production in the other four genera investigated keeps within the limits of 2% (*Leclercia*) to 7% (*Kluyvera*), reaching 15% in *E. coli.* Inhibition of indicator strains caused by the siderophore release around the producer strains likely represents differences in the siderophore synthesis and uptake systems between producer and indicator strains.

Of course, conspicuous differences might not be overlooked in individual species of a single genus, occurring in some production capabilities. Thus, the incidence of lysogeny fluctuates from 0% (*E. aerogenes*) to 12% (*E. cloacae*) within the genus *Enterobacter* or the incidence of HMW bacteriocins production from 0% (*E. cloacae, E. agglomerans*) to 25% (*E. aerogenes*) in the same genus. Surprisingly, no species differences like that were found for siderophore production. Nevertheless, our strain collections of the *Enterobacter* species (*E. cloacae, E. agglomerans*, *E. aerogenes*) and of *Kluyvera* (*K. cryocrescens*, *K. ascorbata*) were not extensive enough to ensure a reliable statistical significance. Also, vice versa, the selection of strains for the sets representing the two genera monospecific in our collections (*Citrobacter* and *Leclercia*) might not be representative enough for a particular genus. It appears probable that the same or similar inhibitive effects are to be supposed and expected in the natural medium of the enterobacteria tested (i.e., in the gut flora) [2].

It is hardly possible to compare the incidence data stated with those published previously with respect to the steadily developing system of bacterial genera, including the emergence of both newly defined and acknowledged species and genera. Thus, no data concerning the incidence of lysogeny, bacteriocinogeny or siderophore production in the species *Citrobacter youngae* are available, although four temperate bacteriophages of *Citrobacter freundii* have been reported [6], Similarly, production of colicin A was described for *C.* (former *Escherichia*) *freundii* as early as in 1948 [5] and the incidence of colicinogenic strains in the genus *Citrobacter* of 5% [11] to 13% [9]. In the genus *Enterobacter*, the previously published data on the bacteriocinogeny incidence among strains are inconsistent: 5% for the genus as a whole [8] to 27% for *E. cloacae* [7]. No data on *Kluyvera* or *Leclercia* have been published so far. Interestingly enough, we found only 1 from 19 strains to be colicinogenic in *E. agglomerans*, whereas none from 25 strains was bacteriocinogenic in *E. cloacae.*

Taken together, we showed the marked differences in the capability of strains of five enterobacterial genera to synthesize and release exocellular products inhibiting the growth of other strains. Hence, it is tempting to suggest these frequencies of inhibitive factor production to be acknowledged—at least in certain cases—as an auxiliary, rather specific criterion for genus/species delimitation within Enterobacteriaceae. According to our experience, the genus *Escherichia* (four species) is strikingly rich in strains producing bacteriophages (37%) as well as strains producing colicins (14%; among the *E. coli* species, the colicin-producing strains represent 43%). More often, a genus or species harboring lysogenic strains with a relatively high frequency harbors colicinogenic strains usually with a lower frequency; for example, *C. youngae* with 18% of lysogenic strains has hardly any colicinogenic ones (although 7% of strains produce HMW bacteriocins) or *L. adecarboxylata* with 10% of lysogenic strains has hardly any colicinogenic ones. Of course, all of the proportion numbers stated must be considered as purely statistical ones, subject to all kinds of statistical error.

A similar study mapping the incidence of inhibitory factors in the genera *Salmonella* and *Yersinia* are underway and the preliminary data show marked differences when compared to each other or to *E. coli.*

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