Two New E Colicins, E8 and E9, Produced by a Strain of *Escherichia coli*

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We have isolated a strain of *Escherichia coli* from chicken caeca which produces two E colicins and colicin M. This strain has seven plasmids, five of which have been transferred to *E. coli* K12. Two *E. coli* K12 derivatives which produce the two E colicins separately have been tested against seven standard E colicin producing strains which define seven different immunity groups. Our results indicate that these new E colicins define two further immunity groups, E8 and E9.

INTRODUCTION

Colicins are plasmid-determined antibiotic proteins which are produced by some strains of enterobacteria and are active on strains of *Escherichia coli*. We screened strains of enterobacteria isolated from chickens for colicin production and found one, *E. coli* strain J, which had colicin E and colicin M activities. E colicins are those which require the *btuB* gene product for adsorption (this gene product is also used for adsorption of phage BF-23 and uptake of vitamin B12) and are inactive on *btuB* mutants. Colicin M uses the *tonA* gene product, also used by phage ϕ 80, and is inactive on *tonA* mutants.

E colicins are further classified into immunity groups on the basis of their activity on other colicin producing, *btuB*⁺ strains. If inactive on an E-colicin producer it is placed in the same immunity group. If active on a producer, it is placed in a separate immunity group. Thus far seven different E colicin immunity groups have been reported (see Males & Stocker, 1982). Genetic analysis of strain J showed that it produces two E colicins. Immunity testing between derivatives producing each of these E colicins separately and strains representing the colicin E immunity groups E1 to E7 indicate that the E colicins of strain J define two further immunity groups, E8 and E9.

METHODS

Bacterial strains and bacteriophages. These are shown in Table 1.

Media. Oxoid Nutrient Agar and Nutrient Broth were used for routine culturing and for colicin testing. Minimal medium was M9 (Miller, 1972) supplemented with L-amino acids (20 μ g ml⁻¹) as required, glucose (0.2%, w/v), raffinose (filter-sterilized 0.5%, w/v). Tetracycline was used at 10 μ g ml⁻¹, nalidixic acid at 25 μ g ml⁻¹, and streptomycin at 100 μ g ml⁻¹.

Mating conditions. Equal volumes of donor and recipient strains (at $2-5 \times 10^8$ cells ml⁻¹) were mixed, shaken slowly at 37 °C for 2–4 h and then plated directly on selective media. Trypsin, when used to inactivate colicin in the mating mixture, was added to 300 μ g ml⁻¹.

Colicin testing. The stab/overlay method was used. Presumptive colicinogenic strains were stabbed into nutrient plates and incubated overnight at 37 °C. The plates were exposed to chloroform vapour, and overlaid with 2.5 ml Davis agar (in distilled water) containing approximately 10^7 bacteria. After overnight incubation plates were scored for zones of inhibition.

Plasmid preparation and electrophoresis. For agarose gel electrophoresis, plasmid DNA was prepared from 1 ml of culture grown overnight in nutrient broth, using the alkaline lysis procedure of Birnboim & Doly (1979), and electrophoresed overnight in 0.7% (w/v) agarose in 0.04 M-Tris/acetate buffer pH 8.0 at 25 V. For transformation, plasmid DNA was prepared by the 'small-scale' cleared lysate method of Hirsch *et al.* (1980) from 1 litre of cells grown at 37 °C.

Table 1. *Bacterial strains and phages*

Strain designation	Relevant properties	Source
J	Natural isolate producing colicins E and M; Tc ^r ; Raf ⁺	Chicken caeca, Hall Farm, Hingham, UK
JE5506	Multiply auxotrophic; colicin E-s; colicin M-s	M. Inouye, SUNY, Stonybrook, NY, USA
RJ179	Spontaneous NaI ^r mutant of JE5506	This laboratory
PC264	Spontaneous BF23-r mutant of RJ179; colicin E-r	This laboratory
PC639	Spontaneous $\phi 80vir$ -r mutant of RJ179; colicin M-r	This laboratory
PC640	Spontaneous $\phi 80vir$ -r mutant of PC264; colicin M-r	This laboratory
CSH57	Multiply auxotrophic; SM ^r ; colicin E-s; colicin M-r	J. Miller, University of Geneva, Switzerland
PC263	arg ⁺ derivative of CSH57	This laboratory
W3110	Prototrophic; colicin E-s	Plasmid Reference Center, Stanford University, Calif., USA
A592	<i>tolA</i>	B. Bachmann, Yale University, New Haven, Conn., USA
A593	<i>tolB</i>	B. Bachmann
Gillel	<i>tolD</i>	B. Bachmann
PC259	RJ179 (R64-11 <i>drd</i>)*	This laboratory
PC620	RJ (R64-11 <i>drd</i>) (ColE1-K30)*	This laboratory
PC621	RJ179 (R64-11 <i>drd</i>) (ColE2-P9)*	This laboratory
PC622	RJ179 (R64-11 <i>drd</i>) (ColE3-CA38)*	This laboratory
PC252	RJ179 (pPC101) (pPC104)	See text
PC617	PC264 (pPC102) (pPC107)	See text
PC651	W3110 (pPC101)	See text
PC652	W3110 (pPC102)	See text
PRC593	W3110 (Col E1-K53)	Plasmid Reference Center
PRC594	W3110 (ColE2-P9)	Plasmid Reference Center
PRC595	W3110 (ColE3-CA38)	Plasmid Reference Center
PRC625	W3110 (ColE4-CT9)	Plasmid Reference Center
PRC596	W3110 (ColE5-099)	Plasmid Reference Center
PRC608	W3110 (ColE6-CT14)	Plasmid Reference Center
PRC597	W3110 (ColE7-K317)	Plasmid Reference Center
PRC499	Produces colicin B-K260; colicin B-r; colicin M-r	Ruth Barker, Ninewells Hospital, Dundee, UK
PRC628	Produces colicin A-CA31	R. Kadner, University of Virginia, Charlottesville, Va., USA
PC637	PC264 (pPC107)	See text
PC650	PC263 (pPC107)	See text
PC647	Spontaneous $\phi 80vir$ -r mutant of PC637	See text
BF23	Phage using vitamin B12 receptor, <i>btuB</i> gene product	R. Kadner
$\phi 80vir$	Phage using ferrichrome receptor, <i>tonA</i> gene product	N. Murray, University of Edinburgh, UK

Abbreviations: s, sensitive; r, resistant; Raf⁺, raffinose utilization; Tc, tetracycline; SM, streptomycin; NaI, nalidixic acid.

* Plasmid donors kindly supplied by Professor D. Sherratt, University of Glasgow, UK.

Transformation procedure. Strain W3110 was made competent for transformation by the method of Mandel & Higa (1970) except that cells were washed with cold 0.1 M-MgCl₂ prior to the CaCl₂ wash. After mixing with plasmid DNA from PC252 or PC617, cells were plated on nutrient medium containing a colicin extract of the DNA donor strain. Survivors were screened for the production of the appropriate E colicin.

Preparation of colicin E3 antiserum. A crude colicin E3 extract was prepared from *E. coli* RJ179 ColE3-CA38 grown with shaking at 37 °C in LB medium. At an *A*₅₅₀ of 0.2, mitomycin C (0.5 µg ml⁻¹) was added to 1 l of culture. After incubation for a further 60 min the cells were harvested by centrifugation at 7000 *g* for 15 min. The cell pellet was resuspended in 16 ml 10 mM-phosphate buffer pH 7.0 and was sonicated for four periods of 30 s, with 60 s cooling in between, in an ice bath. The sonicated cells were centrifuged at 100 000 *g* in a Beckman 65

rotor for 40 min to remove unbroken cells, cell membranes etc. and the supernatant was used as a crude colicin E3 extract to raise antiserum.

Two New Zealand White rabbits were injected at multiple sites on the back with 0.5 ml colicin E3 extract (10 mg total protein) mixed with 0.5 ml of Freund's complete adjuvant. At 14 d intervals the rabbits received booster intra-muscular injections of extract, mixed with incomplete Freund's adjuvant. After 42 d blood was removed from the ear vein of both rabbits and allowed to clot. After overnight incubation at 4 °C the serum was pooled and stored in portions at -20 °C.

RESULTS

Properties of strain J

Strain J was isolated from chicken caeca. Colicin E and M activities were shown by the stab/overlay method. Stab inoculation of strain J gave a large zone (diameter, 12–15 mm) with a colicin E-sensitive indicator, a smaller zone with a colicin E-resistant, colicin M-sensitive indicator, and no zone with a colicin E-resistant, colicin M-resistant indicator. Strain J was found to be resistant to tetracycline and to ferment raffinose; raffinose also served as a sole source of carbon. Agarose gel electrophoresis of DNA isolated from strain J showed seven plasmid bands, called pPC101–pPC107 from smallest to largest (Fig. 1).

Transfer of colicin E activity to E. coli K12

Strain J was mated with RJ179, and plated on nalidixic acid to counterselect the donor. One of the few surviving recipient colonies was colicin E⁺, and agarose gel electrophoresis of DNA isolated from this strain (PC252) showed two plasmid bands, corresponding to pPC101 and pPC104 (data not shown). We assumed that the smaller plasmid was the colicin E plasmid by analogy with other colicin E plasmids, and that the larger was the mobilizing conjugative plasmid. Subsequent transformation of strain W3110 with DNA from this strain yielded an E colicin producing strain which carried pPC101 but not pPC104. We have not yet found a phenotypic character which is conferred by pPC104.

Fig. 1. Line drawing of an agarose gel preparation of the plasmids isolated from strain J. The seven plasmid bands represent plasmids pPC101 (bottom) to pPC107 (top). The pointers indicate the positions of the molecular weight markers: ColE3-CA38, 4.6 MDal; R64-11, 72.3 MDal. Chr denotes chromosomal DNA.

Table 2. *Colicinogenic immunity between ColE⁺ derivatives of W3110*

Strains used as indicators*	ColE plasmid carried by W3110								
	E1-K53	E2-P9	E3-CA38	E4-CT9	E5-099	E6-CT14	E7-K317	pPC101	pPC102
E1-K53	—	+	+	+	+	+	+	+	+
E2-P9	+	—	+	+	+	+	+	+	+
E3-CA38	+	+	—	+	+	+	+	(+)	+
E4-CT9	+	+	+	—	+	+	+	+	+
E5-099	+	+	+	+	—	+	+	+	+
E6-CT14	+	+	+	+	+	—	+	(+)	+
E7-K317	+	+	+	+	+	+	—	+	+
pPC101	+	+	+	+	+	+	+	—	+
pPC102	+	+	+	+	(+)	+	+	+	—
W3110	+	+	+	+	+	+	+	+	+

+, Clear zone of inhibition; (+), cloudy zone of inhibition (reduced in size relative to W3110 plasmid-free strain); —, no zone (very narrow zone sometimes observed with E3⁺ stab on E3⁺ indicator and E5⁺ stab with E5⁺ indicator).

* See Table 1 for details of strain designation.

Transfer of a second colicin E activity from strain J

Conjugation experiments showed that Raf⁺, the ability to use raffinose as a sole source of carbon, could be transferred from strain J to PC264. This *btuB* recipient was used to avoid killing by E colicin in the mating mixture. We expected that the Raf⁺ plasmid might mobilize pPC101 and screened Raf⁺ progeny for colicin E activity. Of fourteen Raf⁺ colonies tested, five were colicin E⁺. The E colicin of two of them was inactive against PC252 which carries pPC101, suggesting that these transconjugants also carried pPC101. The E colicin of the other three, however, was active against PC252, which placed it in a different immunity group. Agarose gel electrophoresis of Raf⁺ colicin E⁺ strains of the latter type, such as PC617, showed two plasmid bands which on the basis of mobility correspond to pPC102 and pPC107. pPC107 was the only plasmid observed on gels with Raf⁺ colicin E[−] transconjugants indicating that it is the Raf⁺ plasmid and pPC102 is the second ColE plasmid. Transformation of W3110 with plasmid DNA from PC617 yielded colicin E⁺ progeny carrying pPC102, but not pPC107.

Immunity testing with standard ColE strains

Strains PC651 and PC652 which are W3110 transformants carrying pPC101 and pPC102, respectively, were cross-tested against the seven standard colicin E producing strains (Table 2). All indicator strains were sensitive to the colicin produced by PC651, except the colicin E3⁺ and colicin E6⁺ strains which were only partially sensitive. PC651 was sensitive to all E colicins tested. In the case of PC652, its E colicin was active on all other colicin E⁺ indicators. PC652 was sensitive to all the E colicins except E5, to which it was only partially sensitive.

Tests against colicin tolerant indicators

Chromosomal mutants able to grow in the presence of E colicins but still *btuB*⁺ are termed tolerant. *TolA* and *tolD* mutants are tolerant to colicins E2 and E3, but not to E1; *tolB* mutants are tolerant to all three (Bachmann & Low, 1980). We therefore tested the tolerance of strains A592 (*tolA*), A593 (*tolB*), and G11el (*tolD*) to all colicin E⁺ strains including pPC651 and pPC652. A592 and G11el were sensitive to colicin E1, but tolerant to all other E colicins. A593 was tolerant to all nine E colicins.

Antigenic relatedness of E colicins

Antiserum prepared to colicin E3 extract was able to protect a sensitive strain against the killing activity of the colicin E3⁺ strain (Fig. 2). We found that this antiserum could also protect

Fig. 2. The effect of antiserum to crude colicin E3 extract on the activity of various *E colicins*. The stab/overlay method was used, but before the *btuB*⁺ indicator was overlayed, a 10 µl drop of antiserum was applied to the right of each stab and allowed to soak in. Top left, PC651 (pPC101); top right, PRC593 (ColE1); bottom left, PRC595 (ColE3); bottom right, PRC608 (ColE6).

Table 3. *Colicin production and immunity determined by plasmid pPC107*

Indicator strain	Producer strain				Phage $\phi 80$ vir*
	PC637	PC650	PC647	PRC499	
PC264	—	+	+	+	s
PC263	—	—	—	+	r
PC637	—	—	—	—	s
PC650	—	—	—	—	r
PC647	—	—	—	—	r
PRC499	—	—	—	—	NT

NT, Not tested. +, Colicin zone; —, no colicin zone.

* Drop of phage suspension (approx. 10^7 p.f.u. ml⁻¹) applied to lawn. $\phi 80$ vir-s = *tonA*⁺ and colicin M-sensitive; $\phi 80$ vir-r = *tonA* and colicin M-resistant.

against the *E colicins* of PC651 and PC652. It also protected against all the *E colicins* of the standard strains except colicin E1. It did not protect against colicin A.

Other properties of pPC107

Transconjugants selected for Raf⁺ and carrying pPC107 alone gave a colicin zone if they were *tonA*⁻, lacking the colicin M receptor, but gave no zone if they were *tonA*⁺ (Table 3). This characteristic of colicin M was reported by Fredricq & Smarda (1970) who also found that colicin B activity and colicin M activity were usually determined by the same plasmid. We found that although transconjugants carrying pPC107 did not produce colicin B, they were completely immune to this colicin. A transmissible Raf⁺ plasmid was described which also determines hydrogen sulphide production (Ørskov & Ørskov, 1973). Neither strain J or *E. coli* K12 transconjugants carrying pPC107 produce H₂S in API 20E tests.

Plasmid pPC105 carries tetracycline resistance and also mobilizes strain J ColE plasmids

Selection for tetracycline-resistance (Tc^r) transconjugants from a mating between strain J and RJ179 (in the presence of trypsin) yielded colicin E⁻ and colicin E⁺ colonies. The colicin E⁺ colonies were of three classes when tested as producers against pPC101- and pPC102-carrying indicator strains (Table 4). Classes 1 and 2 were active on one of the indicators, class 3 was active on both.

Plasmid DNA isolated from representative Tc^r transconjugants was electrophoresed on agarose gels. On the basis of relative mobilities, Tc^r colicin E⁻ transconjugants carried pPC105

Table 4. *Classes of colicin E producers among tetracycline-resistant transconjugants*

The number of colonies is shown in parentheses.

	Stab culture		
	Class 1 (9)	Class 2 (1)	Class 3 (7)
pPC101	—	+	+
pPC102	+	—	+

+, Clear zone of inhibition; —, no zone of inhibition.

alone, class 1 colicin E⁺ carried pPC105 and pPC101, class 2 colicin E⁺ carried pPC105 and pPC102; and class 3 carried pPC105, pPC101 and pPC102 (data not shown). This class was immune to both of the new E colicins. In separate experiments we also found that pPC104 and pPC105 could mobilize pPC101 and pPC102 together, as well as separately.

DISCUSSION

Strain J, a natural isolate of *E. coli* from chickens has seven plasmids, five of which have been transferred to *E. coli* K12. pPC101 and pPC102 are ColE plasmids with molecular weights of 6.7 kb and 7.8 kb, respectively (R. James, unpublished) and are mobilized by the transmissible plasmids pPC104, pPC105 and pPC107. pPC107 confers tetracycline resistance and pPC107 confers raffinose utilization as well as colicin M production and immunity to colicins B and M. Our major interest in strain J is the two E colicins it produces. We isolated transformants of *E. coli* strain W3110 which produce each of the new E colicins separately and tested them against the seven E-colicin type strains provided by the Plasmid Reference Center.

The E colicin of PC651 falls into a different immunity group from colicins E1, E2, E4, and E7 based on lack of cross-immunity. Both the colicin E3⁺ and E6⁺ strains are only partially sensitive to the colicin of PC651, giving smaller, turbid zones compared to the clear zones characteristic of lack of immunity. However, PC651 is fully sensitive to colicins E3 and E6, and the producers of these colicins are sensitive to each other's colicins. There are several possible explanations of these results. One is that colicin E3 immunity (and colicin E6 immunity) can protect to some extent against the colicin of PC651, but not vice versa. Another possibility is that the ColE3⁺ and the ColE6⁺ strains carry a separate gene for immunity to the colicin of PC651 in addition to immunity to the colicin they produce.

Males & Stocker (1980) described a strain of *E. coli* which produces only colicin E7 but is immune to colicin E7 and colicin E2. In this case immunity to colicin E2 was shown to be distinct from E7 immunity and determined by a separate plasmid, ColE2_{imm}-K317 (Watson *et al.*, 1981).

Chak & James (1984) have shown that in the case of the colicin E3⁺ strain PRC595, immunity to the colicin of PC651 is carried by ColE3-CA38 and is distinct from colicin E3 immunity. ColE3-CA38 has been cloned into pBR322 and this recombinant plasmid confers colicin E3 production and immunity to colicin E3 and the colicin of PC651. The latter immunity was abolished when transposons were inserted in one region of this plasmid without affecting colicin E3 production or immunity. The genetic basis of the immunity of the colicin E6⁺ strain, PRC608, to the colicin of PC651 is being investigated.

We believe that the colicin of PC651 defines a new immunity group, E8. The colicin will be designated colicin E8-J. The E colicin of PC652, which is active on representatives of all seven standard colicin E⁺ strains tested and PC651, defines a further immunity group, E9. The colicin of PC652 will be designated colicin E9-J. The partial immunity of this strain to colicin E5 may be due to a separate E5 immunity gene, and this possibility is being explored. Our limited studies show that E8-J and E9-J are similar to colicins E2 to E7 and different from colicin E1 on the basis of antigenicity and their activity on certain colicin-tolerant mutants. Mock & Pugsley (1982) have reported antigenic similarities between colicins E2 to E7 by immunoprecipitation

using antibody to purified colicin E3-CA38. It is not apparent why the E5 immunity of PC652 or the E8 immunity of ColE3-CA38 is only partial whereas the colicin B immunity of pPC107 is complete. Watson *et al.* (1981) noted that E2 immunity conferred by Col E2_{imm}-K317 is not completely effective in protecting indicator lawns from colicin E2 by the stab/overlay method. Pugsley & Reeves (1977) found that some colicin B⁺ strains were also immune to colicins they did not produce, and occasionally this immunity was partial. Perhaps for certain plasmids immunity without production of the corresponding colicin is at a lower level than when colicin is produced. Strains carrying ColE8-J and ColE9-J will be deposited with the Plasmid Reference Center, Stanford University, Stanford, California 94305, USA.

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