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Colicin resistance associated with resistance factors in *Escherichia coli*

By A. G. SICCARDI

*Medical Research Council, Microbial Genetics Research Unit, Hammersmith Hospital, Ducane Road, London W.*12

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1. INTRODUCTION

Transmissible drug resistance amongst the *Enterobacteriaceae* results from the presence of extrachromosomal elements known as resistance factors, or R factors (see Watanabe, 1963). These carry genes determining resistance to one or more antibiotics, and also others which enable their host to conjugate and transmit their resistance to new bacteria. R factors found in different parts of the world differ to some extent in the frequencies with which they confer resistance to different antibiotics (see Watanabe, 1963; Mitsuhashi, 1965); and, as more antibacterial drugs have progressively come into use, R factors have in turn appeared giving resistance to a larger number of these drugs (Lebek, 1963; Anderson & Datta, 1965; Anderson & Lewis, 1965; Williams Smith & Halls, 1966). This leads to the conclusion that, whatever the ultimate origin of the drug resistance determinants on an R factor, the emergence of transmissible resistance to a particular drug and the progressively increasing frequency with which it occurs are the result of selective pressure due to extensive use of the drug. Enterobacteria in their natural environment must be exposed to colicins. This might be expected to have led by a similar process to the appearance of transmissible colicin resistance, and colicin resistance determinants analogous to those conferring resistance to antibiotic drugs might then be carried as part of an R factor. In the present paper, it is reported that, in fact, when derivatives of *Escherichia coli* strain K12 were examined after receiving a number of different R factors from naturally occurring *Salmonella* strains, many were found also to have acquired resistance to one or more colicins. The colicin resistance was evidently associated with the R factor, and experiments to characterize it indicated that it often differed in some respects from previously recognized types of colicin resistance.

Insensitivity, or 'indifference' (Monk & Clowes, 1964), to colicin is recognized as arising in either one of two ways in a sensitive bacterium. First, the bacteria may not take up the colicin because the surface receptor is absent: resistance of this kind is complete, and arises in a sensitive strain as the result of a mutation in a chromosomal gene. Second, colicinogeny gives a degree of immunity (Fredericq, 1956) to the colicin produced: here insensitivity is not complete and is also much more specific than the resistance associated with loss of the surface receptor. Several colicins may share the same receptor but have different immunity patterns which

allow them to be distinguished from each other. Since colicinogeny is determined by extrachromosomal *col* factors which are transferred from one bacterium to another by conjugation (Fredericq, 1954; Ozeki, Howarth & Clowes, 1961), when colicin resistance was found to be acquired with an R factor, this could have merely been due to simultaneous acquisition of a *col* factor. The production of colicin was not always observed, however, in the colicin-resistant R^+ bacteria, and, moreover, the colicin insensitivity associated with many of the R factors differed in character from the immunity conferred by a *col* factor. It could also be distinguished from the resistance due to loss of the colicin receptor, for, although it was of high degree, it was not absolutely complete.

2. MATERIALS AND METHODS

Culture media. These were either Oxoid Nutrient Broth No. 2, or the same broth solidified with 1·25% (w/v) Davis N.Z. agar.

Bacteria. These are listed in Table 1, and fall into two groups: derivatives of *E. coli* K12 tested for acquisition of colicin resistance with an R factor; and standard colicinogenic strains used for production of the colicins.

R factors. Derivatives of *E. coli* K12 carrying 150 different R factors transferred by conjugation from salmonellae were kindly provided by Dr Naomi Datta. In a random sample of sixty of these R factors, about half were fi^+ and half fi^- (Watanabe *et al.*, 1964).

Bacteriophages. Phage W31 (Watanabe & Okada, 1964), phage BF23 (Fredericq, 1946; Clowes, 1965), and phages T1, T3, and *λvir* were kindly supplied by Professor B. A. D. Stocker, Dr R. C. Clowes and Dr S. W. Glover, respectively.

Tests for colicin sensitivity and colicinogeny. These were made by conventional techniques (Fredericq, 1958). Strains CL.123, CL.121, CL.122, SL.903, RC.519, CL.131, CL.129 and CL.120, producing colicins A, B, D, E1, Ib, K, H and V respectively, were each inoculated as a stab into a nutrient agar plate, allowed to grow overnight at 37°C. and killed with chloroform vapour. About 10^8 bacteria of the strain to be tested for sensitivity were then inoculated over the surface of the whole plate in 3 ml. of soft (0·6%, w/v) agar overlay, and the plate was reincubated for a further 24 hours. A fully sensitive R^- control strain of *E. coli* K12 was included with each batch of R^+ strains tested. Each of the colicinogenic stabs inhibited growth of the sensitive strain, the diameter of the zone being characteristic of the particular stab. Resistance was indicated by continuous growth over the stab, and partial resistance by a reduction in the size of the zone, which often also showed slight turbidity resulting from a limited amount of growth.

Tests for colicinogeny of the R^+ strains were made by inoculating the strain as a stab and using sensitive *E. coli* K12 as indicator in the overlay. If the strain turned out to be colicinogenic, it was further tested with the specifically colicin I-resistant strain CL.147, to see if the colicin produced belonged to the I group.

Tests for phage restriction by R^+ or col^+ *bacteria.* Drops of serial dilutions of the phage preparations were spotted on nutrient agar plates previously spread with the bacteria to be tested. The efficiency of plating of the phage was determined by

Table 1. *Bacterial strains*

Strain	Species	Synonym and characters	Reference
RC.709*	*E. coli*	K12 J5-3 (*pro.*$^-$*met*$^-$*.lac*$^+$*.S*^{s}) F$^-$ (acridine cured)	Clowes & Rowley (1954)
RC.24*	*E. coli*	K12 129; derived from W677 (*thr*$^-$*leu*$^-$*.B*1$^-$*.lac*$^-$; F$^-$) *fim*$^-$*.S*^{r}	Maccacaro, Colombo & Nardo (1959)
58.161*	*E. coli*	K12 *met*$^-$*.S*^{s}; F$^+$	Lederberg (1947)
58.161/*sp**	*E. coli*	K12 58.161 defective F$^+$	Hayes (1953); Meynell & Datta (1966)
CL.147†	*E. coli*	Mutant of strain ϕ resistant to colicin I	Gratia (1925); Ozeki *et al* (1962)
Colicin-producing			
CL.123†	*E. freundii*	CA.31.‡ Produces colicin A	Fredericq (1965)
CL.121†	*E. coli*	CA.18.‡ Produces colicin B	Fredericq (1965)
CL.122†	*E. coli*	CA.23.‡ Produces colicin D.	Fredericq (1965)
RC.903*	*S. typhimurium*	LT2 *cys*36 (*col* E1). Produces colicin E1-30	Ozeki *et al* (1962)
RC.519*	*E. coli*	K12 58.161/*sp* (*col* Ib). Produces colicin Ib-P9	Monk & Clowes (1964); Stocker (1965)
RC.902*	*S. typhimurium*	LT2 *cys*36 (*col* Ib). Produces colicin Ib-P9	Stocker (1965)
CL.235†	*E. coli*	K12 58.161/*sp* (*col* Ia). Produces colicin Ia-CT2	Stocker (1965)
SL.1082†	*S. typhimurium*	LT2 (*col* Ia). Produces colicin Ia-CT2	Stocker (1965)
CL.131†	*E. coli*	K235.‡ Produces colicin K	Fredericq (1965)
CL.129†	*E. coli*	CA.58.‡ Produces colicin H	Fredericq (1965)
CL.120†	*E. coli*	CA.7.‡ Strain V. Produces colicin V	Gratia (1925); Fredericq (1965)

* Strains kindly provided by Dr R. C. Clowes.
† Strains from the collection of the Guinness-Lister Unit, and kindly provided by Professor B. A. D. Stocker.
‡ Strain numbers of Dr P. Fredericq: personal communication to Professor B. A. D. Stocker.

comparing the number of plaques on the R$^+$ or *col*$^+$ strain with the number on its R$^-$*col*$^-$ counterpart.

Isolation of R$^-$ segregants. R factors may be spontaneously lost during growth of an R$^+$ culture, but, while the frequency with which R$^-$ segregants appear is sometimes as high as 0·1% (see Watanabe, 1963), it is often very much lower. A relative increase in the R$^-$ fraction of a culture can be obtained by the penicillin-screening technique (Watanabe & Fukasawa, 1961) if the R factor confers resistance to a bacteriostatic antibiotic and the R$^+$ bacteria are still sensitive to penicillin. In the present experiments, using R$^+$ cultures with resistance to tetracycline, R$^-$ segregants were obtained by adding ampicillin to a concentration of 250 μg./ml. to the R$^+$ culture growing exponentially in 10 μg./ml. tetracycline.

3. RESULTS

(i) *Colicin resistance of R+ derivatives of* Escherichia coli *K12*

All the 150 R factors were tested for their ability to confer resistance in strain 58.161/*sp* to colicins A, B, D, E1, I, K, H, and V. Altogether, about half the R factors produced some alteration in the colicin sensitivity of the host, reducing the diameters of the zones of inhibition around one or more of the colicinogenic stabs. With forty-one (27%) of the R factors, the zone was completely abolished around the colicin I-producing strain RC-519. Thirty of the R factors were also tested in strain RC.709 and twelve of these in strains RC.24 and 58.161 F+, with exactly the same results as in strain 58.161/*sp*. Resistance to colicin I was in many cases accompanied by resistance or partial resistance to other colicins, most often H, K and A. For example, one particular R factor conferred apparently complete resistance to colicins, I, A and H, allowing growth to occur over the colicinogenic stabs, and reduced the width of the zone around the stab producing colicin K.

Bacteria carrying the forty-one R factors associated with resistance to colicin I were further examined, first for colicinogeny and, second, for the nature of their colicin resistance. With twenty of the forty-one R factors, the strain produced a colicin which was evidently colicin I since it attacked the universally sensitive strain K12, but not the specifically colicin I-resistant indicator, CL.147. There are two types of colicin I (Stocker, 1965) which both attach to the same surface receptor on the bacterium, but which are each determined by two different *col* factors, Ia and Ib, which give no cross immunity (Fredericq, 1965). Using bacteria carrying *col* Ia or *col* Ib as indicators, the colicin produced by all the colicinogenic R+ strains was identified as being of type Ib. With the remaining twenty-one R factors associated with resistance to colicin I, no colicinogeny could be demonstrated, either in R+ derivatives of *E. coli* K12 or the *Salmonella* strains from which they had originally been transferred.

The amount of colicin which diffuses from a colicinogenic stab depends partly on whether it is readsorbed by the bacteria (Monk & Clowes, 1964). Thus, if the strain used as colicin producer is a *Salmonella*, which lacks the surface receptor for colicin I, it gives a wider zone of inhibition than *E. coli* K12 which readsorbs part of the colicin produced. By the use of the two kinds of colicinogenic bacteria, it is also possible to distinguish between an indicator strain which is merely immune to the limited extent conferred by colicinogeny, and one which has the absolute resistance associated with absence of the receptor (Table 2). The former shows a zone of inhibition around the strongly-producing *Salmonella*, but no zone around the weakly-producing *E. coli*, while the latter shows no zone around either. To investigate the colicin insensitivity acquired with R factors, the 41 R+ derivatives of *E. coli* strain K12 which appeared to be resistant to strain RC.519 in the initial tests were further tested for their reactions to stab inocula of *E. coli* and *Salmonella* strains each carrying a *col* Ia or *col* Ib factor. Table 2 shows that the R+ strains fall into four classes: eleven were colicinogenic for *col* Ib (Class 1); nineteen had the same level of insensitivity as the colicinogenic strains, but were not demonstrably

Table 2. *Sensitivity to colicin Ia and Ib of* E. coli *K12 carrying different R factors*

Indicator			Colicin-producing stab					
			Colicin Ib		Colicin Ia			
Strain	R factor carried	Colicin produced	RC.519 (weak)	RC.902 (strong)	CL.235 (weak)	SL.1082 (strong)	Resistance	Found with
Control strains								
RC.709	none	none	+	+ +	+	+ +	none	—
58.161/*sp*	none	none	+	+ +	+	+ +	none	—
CL.147	none	none	—	—	—	—	complete	lack of receptor
RC.519	none	Ib	—	+	+	+ +	low Ib	*col* Ib
CL.235	none	Ia	+	+ +	—	+	low Ia	*col* Ia
R⁺ strains								
RC.709R⁺ or 58.161/*sp*R⁺	Class 1	Ib	—	+	+	+ +	low Ib	11 R factors
RC.709R⁺ or 58.161/*sp*R⁺	Class 2	Ib	—	±	—	±	high Ia, Ib	9 R factors
RC.709R⁺ or 58.161/*sp*R⁺	Class 3	none	—	+	+	+ +	low Ib	19 R factors
RC.709R⁺ or 58.161/*sp*R⁺	Class 4	none	—	±	—	±	high Ia, Ib	2 R factors

Inhibition: + + width of zone typical of strong producer (see text) acting on fully-sensitive indicator; + typical of weak producer (see text) acting on fully-sensitive indicator; ± very narrow zone of partial inhibition inconstantly seen.

colicinogenic (Class 3); nine were colicinogenic for *col* Ib, but showed a much higher level of insensitivity than is ordinarily conferred by colicinogeny, and were at the same time as insensitive to colicin Ia (Class 2); and the remaining two reacted similarly, but were non-colicinogenic (Class 4). In their insensitivity to colicin Ia as well as Ib, the R^+ strains in Class 2 and Class 4 thus resembled bacteria which had lost the colicin receptor. However, in distinction to resistant bacteria of this kind, the R^+ bacteria were not absolutely insensitive: with repeated testing, definite narrow zones of partial inhibition could sometimes be observed around the colicinogenic salmonellae.

Behaviour of R^- segregants. One problem with the colicinogenic R^+ bacteria was whether the determinants of colicinogeny and drug resistance were part of the same structure; that is, if they were always jointly inherited, or if segragation could occur. Colicinogeny is a relatively stable character, and no *col*$^-$ segregants were obtained in the present experiments. However, bacteria which had lost the R factor were isolated from R^+ strains belonging to each of the four colicin-insensitivity classes, and then examined for their reactions to the strongly and weakly colicin Ia and Ib producing stabs. Table 3 shows that, in every case, when the R factor was lost, the strain also lost any insensitivity it had, over and above the immunity to colicin Ib conferred by its colicinogeny. When the original R^+ strain was not colicinogenic, its R^- segregant became fully colicin-sensitive.

Table 3. *Colicin resistance of R^- segregants*

R^+ parents			R^- segregants			
R^+ class	*col*	Colicin resistance	No. tested	*col*	Colicin resistance	Resistance lost with R
1	Ib	low Ib	3	Ib	low Ib	none
2	Ib	high Ia, Ib	3	Ib	low Ib	high Ib, Ia
3	—	low Ib	3	—	none	low Ib
4	—	high Ia, Ib	2	—	none	high Ib, Ia

(ii) *Phage restriction by R^+ strains*

A number of R factors and *col* factors are known to restrict the multiplication of certain phages in their host bacteria (Watanabe *et al.*, 1964; Strobel & Nomura, 1966). Thirty derivatives of *E. coli* K12 strain RC.709 each carrying a different R factor, including fourteen of the forty-one conferring resistance to colicin I, were tested as hosts for phages W31, BF23, T1, T3, and λ*vir*. All the fourteen R factors, whether *fi*$^+$ or *fi*$^-$ (Watanabe *et al.*, 1964), associated with any sort of insensitivity to colicin I reduced the efficiency of plating of phages W31 and BF23 to $c.10^{-3}$ of the control value observed on RC.709 R^-. This held whether or not the R^+ bacteria were also colicinogenic (Table 4). At the same time, none of the other R factors restricted either phage. The R^- segregants tested for a change in reaction to colicin I (Table 3) were also examined for the effect of loss of the R factor on phage restriction. Table 4 shows that those which were still *col* Ib$^+$ retained the ability to

restrict phages W31 and BF23, but those which were not colicinogenic and had lost all their original colicin I insensitivity with the R factor no longer restricted the phages.

Table 4. *Phage restriction by R^+ bacteria and R^- segregants*

R^+ class	*col*	Colicin resistance	Restriction of phages W31 and BF23*	R^- segregants: Resistance	R^- segregants: *col*	Restriction of phages W31 and BF23*
1	Ib	low Ib	+	low Ib	Ib	+
2	Ib	high Ia, Ib	+	low Ib	Ib	+
3	—	low Ib	+	—	—	—
4	—	high Ia, Ib	+	—	—	—

* + indicates that efficiency of plating is reduced to *c.* 10^{-3} of the value on the R^-col^- strain; — indicates absence of restriction.

Phages T1, T3 and *λvir* were all restricted by one of the R factors which restricted phages W31 and BF23. They were also, individually or in various combinations, restricted by some other R factors.

Phage restriction by a bacterial strain may either result from the presence of a plasmid, or be a character of the bacterium itself. It has been reported that a bacterial gene responsible for restriction in *E. coli* K12 is situated near the genes determining synthesis of threonine and leucine, and r^-m^- mutants have been isolated which have lost the normal ability of the wild-type to restrict and modify phages grown on other strains (Colson, Glover, Symonds & Stacey, 1965). Since the restriction brought about by a plasmid might not operate directly but by conferring additional specificities on the inherent restricting mechanism of the host, an R factor was tested for restricting ability after transfer to a non-restricting bacterial mutant. The R factor which restricted all the phages, W31, BF23, T1, T3 and *λvir*, was transferred to an r^-m^- mutant of *E. coli* K12, kindly supplied by Dr S. W. Glover; restriction occurred in this strain no less then in the original host, indicating that the R factor had indeed imposed an independent mechanism for restriction on the bacterium.

4. DISCUSSION

Epidemiological surveys have shown that after a particular antibiotic has been in use for a certain length of time, R factors carrying resistance to it make their appearance. The expectation has now been fulfilled that a naturally occurring antibacterial agent of widespread distribution, such as a colicin, should equally be associated with the existence of transmissible resistance determinants. The use of antibiotics in human and veterinary medicine is a relatively recent development; thus the emergence of transmissible resistance to colicins should logically have preceded the detection of R factors. Colicin resistance, such as was detected in the present experiments, might therefore be found on R factor-like plasmids without drug resistance genes, but only R factors proper, known to carry antibiotic resistance were examined here.

A large proportion of 150 R factors transferred to *E. coli* strain K12 from *Salmonella* strains were found to bring with them a decrease in sensitivity to one or more colicins. Insensitivity to colicin I was particularly frequent, perhaps because the R factors had been obtained from *Salmonella* strains which commonly carry *col* factor I (Ozeki, *et al* 1962); and was chosen for further investigation because in the initial tests it appeared to be complete, in distinction to resistance to the other colicins. The findings for colicin I might very likely apply to other colicins also, however, for the apparently complete resistance later turned out to be due to the use of a weakly-producing test strain.

Two distinct types of insensitivity to colicin Ib were conferred on *E. coli* K12 by an R factor. The first was similar to the immunity associated with colicinogeny, being of low degree and restricted to colicin Ib itself. The second resembled resistance due to loss of the receptor, being of higher degree and also including resistance to colicin Ia. It was, however, not complete. R factors associated with insensitivity of either kind were found, either accompanied by *col* factor Ib or alone.

A number of plasmids restrict the growth of certain phages: examples are the restriction of phages W31 and BF23 by *col* factors (Watanabe & Okada, 1964; Strobel & Nomura, 1966), and restriction of *λvir*, T1 and T7, separately or in various combinations and to different extents by a number of different R factors (Watanabe *et al.*, 1964). It is interesting that all four sorts of colicin I-resistant R^+ strains, and these strains only, restricted the growth of phages W31 and BF23. The restriction of these two phages is unlikely to be due to one single mechanism, for other plasmids are known to restrict only one or other of the two: for example, the F factor restricts phage W31, not but BF23, and *col* factor E2 restricts phage BF23 but not W31 (Watanabe & Okada, 1964; Stocker, 1965; Strobel & Nomura, 1966). If phage restriction, insensitivity to colicin, and drug resistance are all present on a single R factor, this suggests that it may have recombined with a *col* factor in the past. This idea is supported by one *Salmonella* strain which was discovered to be *col* Ib^+ as well as R^+ although it transmitted the R factor with colicin resistance and phage restriction but without the colicinogeny. It might be argued that the characters of colicin resistance and phage restrictions were not actually carried on the R factor but on a separate *col* factor which was now defective for production of colicin. However, *col* factor I is known to be eliminated only at low frequency (Clowes, Moody & Pritchard, 1965), so, if these characters are in fact on a defective *col* factor, this must have become integrated with the R factor to explain their loss simultaneously with the determinants of drug resistance and at the higher frequency more typical of an R factor.

Recombination between a *col* factor and an R factor cannot, however, account for the kind of colicin resistance found with some R factors, for this was greater than the immunity given by a *col* factor, and extended to Ia as well as Ib. Penicillin resistance brought about by R factors has been shown to be due to production of penicillinase (Datta & Kontomichalou, 1965), and there is some evidence, both biochemical (Okamoto & Suzuki, 1965) and biological (Tsukamoto, Miyake & Sato, 1965), that R factor resistance to other antibiotics is also brought about by the

production of enzymes which inactivate the drug. The resistance to colicin I, observed here, might thus also be due to an enzyme inactivating the agent: the fact that it could be overcome by a sufficient concentration of colicin, and covered both Ia and Ib supports this idea, which is under further investigation.

SUMMARY

Derivatives of *E. coli* K12 which had received a number of different drug resistance factors by conjugation from *Salmonella* strains were found also to have acquired insensitivity to colicins. The insensitivity associated with some R factors was similar in character to immunity conferred by colicinogeny, while that given by others more closely resembled the higher level of resistance due to absence of the colicin receptor. It was, however, not complete. Many of the R factors also restricted the growth of various phages.

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