INFECTION AND IMMUNITY, Aug. 1980, p. 411-416
0019-9567/80/08-0411/06$02.00/0

Vol. 29, No. 2

ColV Plasmid-Mediated, Colicin V-Independent Iron Uptake System of Invasive Strains of *Escherichia coli*

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Evidence is presented that ColV plasmid-mediated iron uptake, an important component of the virulence of invasive strains of *Escherichia coli*, is independent of colicin V synthesis and activity. A mutant of *E. coli* K-12 deficient in the biosynthesis of enterochelin (strain AN1937) was unable to grow on minimal agar containing the chelating agent α,α'-dipyridyl unless it was harboring the plasmid ColV-K30 (strain LG1315). Acquisition of the active plasmid-specified iron sequestering system was accompanied by marked enhancement of pathogenicity in experimental infections of mice. Mutants of strain LG1315 were isolated that were defective in iron uptake due to plasmid mutations. They were unchanged with respect to colicin production, but were significantly less virulent than the parent strain. Conversely, mutants isolated as defective in colicin V synthesis were normal for the plasmid-coded iron uptake mechanism and showed the same lethality for infected mice as did strain LG1315. Furthermore, mutations in strain AN1937 which render it resistant or tolerant to the bactericidal action of colicin V did not influence the uptake of iron into plasmid-carrying strains. Cross-feeding tests involving plasmid mutants defective in iron uptake identified two plasmid-specified components of the system, an extracellular iron-chelating compound and a nondiffusible product allowing transport of iron across the bacterial cell membrane.

It is clear that there is a correlation between plasmids that specify the production of colicin V (ColV plasmids) and the virulence of invasive strains of *Escherichia coli* (5, 20, 21). The presence of ColV plasmids is associated with the enhanced ability of bacterial strains to survive and proliferate in the tissues and fluids of the body of an infected animal (20, 21). It has recently been shown that an important aspect of the pathogenicity of colicinogenic *E. coli* strains is their ability to sequester iron by an efficient ColV plasmid-specified process that is independent of the chromosome-specified enterochelin system (22, 23). A screening program to evaluate the ubiquity of this plasmid-controlled virulence determinant is being initiated, and to aid the identification of clinical isolates of interest we wished to investigate the functional relationship between the production and action of colicin V and the chelation and uptake of iron by the ColV plasmid-specified process. It is tempting to invoke such a functional relationship because of the observation that one of the outer membrane proteins whose synthesis is induced in conditions of iron stress is the receptor (*cir* protein, 74,000 daltons) for colicins I and V (6, 11). No iron transport system involving this protein has yet been identified (4, 14). The results presented here, however, do not support this hypothesis, but suggest instead that screening directly for plasmids that promote efficient iron uptake may identify some which do not also specify the production of colicin V.

(A preliminary report of this work was presented at the 192nd meeting of the Genetical Society, Leeds, England, April, 1980.)

MATERIALS AND METHODS

Bacterial strains and growth media. Strains of *E. coli* used in this study are described in Table 1. Defined growth medium was M9 minimal salts medium (19) supplemented with Casamino Acids (0.5%, wt/vol), L-tryptophan (20 μg/ml), thiamine (50 μg/ml), and glucose (0.2%, wt/vol). Strain AN1937, deficient in enterochelin biosynthesis, grew poorly on minimal agar (approximately 2 μM Fe^{3+}) unless sodium citrate (10 mM) was added (10); growth was completely inhibited by the addition of the chelating agent α,α'-dipyridyl (160 μM) (17). ColV plasmid-specified iron uptake (22, 23) allowed growth of colicinogenic derivatives on dipyridyl-supplemented medium.

Mutagenesis. Nutrient broth-growth cultures of bacterial strains were washed and suspended in sodium citrate (0.1 M, pH 5.5), and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a dose giving approximately 50% lethality. After treatment, cells were washed with phosphate buffer (0.1 M, pH 7.0) to remove the mutagen.

Ampicillin enrichment. Mutagenized cells were suspended in minimal medium containing sodium cit-

TABLE 1. *Bacterial strains*

Designation	Characteristics[a]
LG1312	Derivative of C600 (1), *cir* (spontaneous)
AN1937[b]	*ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl* (22, 23)
LG1315	Derivative of AN1937 carrying plasmid ColV-K30 (22, 23)
LG1417	Derivative of AN1937, *nalA* (spontaneous)
LG1439	Derivative of LG1417, *cir* (spontaneous)
LG1418	Derivative of LG1417 carrying plasmid ColV-K30, Iu⁻
LG1419	Derivative of LG1417 carrying plasmid ColV-K30, Iu⁻
LG1421	Derivative of LG1417 carrying plasmid ColV-K30, Iu⁻
LG1434	Derivative of LG1417 carrying plasmid ColV-K30, Iu⁻
LG1437	Derivative of LG1417 carrying plasmid ColV-K30, Cva⁻
LG1314	Derivative of AN1937 carrying plasmid ColV,I-K94 (22, 23)
LG1323	Derivative of AN1937 carrying plasmid ColV-H247 (22, 23)
LG1324	Derivative of AN1937 carrying plasmid ColV-P72 (22, 23)

[a] Strain genotypes are as presented by Bachmann et al. (2). Cva⁻ is the phenotype symbol for failure to produce colicin V (15); Iu⁻ indicates a defect in ColV plasmid-specified iron uptake (22).

[b] AN1937 is the same as strain AN193 isolated by I. G. Young.

rate (10 mM). After overnight incubation at 37°C, cells were harvested by centrifugation, washed, and then suspended at a density of 10^7 cells per ml in minimal medium containing dipyridyl (160 μM). Cultures were incubated at 37°C with aeration for 3 to 4 mass-doublings to deplete intracellular iron pools (22, 23), and ampicillin (20 μg/ml) was added to kill growing cells. Incubation was continued for 90 min, and the ampicillin was then removed by washing. This procedure was estimated from reconstruction experiments to result in a 10- to 50-fold enrichment of iron uptake-deficient individuals in a population of predominantly uptake-proficient organisms.

Agarose gel electrophoresis. Plasmid content of bacterial strains was determined physically by agarose gel electrophoresis of cleared lysates. Strains were grown to late exponential phase in nutrient broth and lysed by the lysozyme-ethylenediaminetetraacetic acid-detergent method (7), using the detergent Sarkosyl NL97 (0.5% [wt/vol], final concentration). Lysates were blended briefly on a Vortex mixer and centrifuged at 40,000 × *g* for 30 min to pellet the bulk of chromosomal deoxyribonucleic acid; cleared lysates with a final volume of 400 μl were obtained from 10-ml bacterial cultures. Samples (40 μl) of cleared lysate were loaded onto horizontal agarose slab gels (0.5%, wt/vol) and subjected to electrophoresis at 200 mA for approximately 2 h. The running buffer was 25 mM tris(hydroxymethyl)aminomethane (pH 7.7) containing 20 mM glacial acetic acid and 5 mM ethylenediaminetetraacetic acid. Gels were strained with ethidium bromide, and the deoxyribonucleic acid was visualized by transmitted shortwave ultraviolet light.

Experimental infections. Bacteria were grown overnight at 37°C with aeration in nutrient broth. Cells were washed and suspended to a density of 5×10^7 cells per ml in NaCl (0.9%, wt/vol). Young adult white mice were inoculated intraperitoneally with 5×10^6 bacteria, and the approximate time of death was recorded.

Sources of reagents. Ampicillin, α,α′-dypyridyl, ethidium bromide, lysozyme, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine were obtained from Sigma London Chemical Co. Ltd. Sarkosyl NL97 was purchased from Geigy Industrial Chemicals, and agarose was from Miles Laboratories, Ltd. Nutrient broth and agar were obtained from Oxoid Ltd.

RESULTS

Isolation of ColV plasmid mutants deficient in iron uptake. Our first approach to the question of the relationship between iron uptake and colicin V was to screen for mutants deficient in the former process and to test subsequently for simultaneous loss of colicin activity. Strain LG1315, which is enterochelin deficient but can sequester iron by the ColV-K30 plasmid-specified system, was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and subjected to two cycles of ampicillin treatment, as described above, to enrich for induced mutants unable to grow in liquid minimal medium containing dipyridyl. Cultures were plated on minimal agar supplemented with sodium citrate, and single colonies were then examined for their ability to grow in the absence of citrate on minimal agar containing dipyridyl. Individual clones that were unable to grow on the latter medium were presumed to be defective in the ColV plasmid-coded iron uptake system. Since this requires both plasmid and chromosomal gene products (22, 23), mutants carrying plasmid mutations were identified by transferring the ColV plasmid by conjugation to strain LG1417, a nalidixic acid-resistant derivative of strain AN1937, and checking transconjugants for their ability to take up iron. Cases in which acquisition of the plasmid from an induced mutant did not lead to the ability of the recipient to grow on minimal agar containing dipyridyl were indicative of mutations in the plasmid-specified functions of the iron uptake system (Iu⁻). Four such mutants of independent derivation were isolated, representing about 0.2% of the mutagenized, ampicillin-enriched populations. LG1418, LG1419, LG1421, and LG1434 are the designations of Iu⁻ transconjugants from crosses between the four independent mutants and the recipient strain LG1417. Agarose gel electrophoresis of cleared lysates of these strains showed that they all

carried plasmids of the same molecular size as ColV-K30. Furthermore, all four mutant plasmids were normal with respect to colicin V production and immunity.

Mutants carrying chromosomal mutations rendering the plasmid iron uptake system inoperative were isolated from ampicillin-enriched populations at a frequency of greater than 1%. The characterization of these is of importance in the elucidation of the plasmid iron uptake system, and in determining its relationship to other routes for iron transport, and will be reported elsewhere.

Cross-feeding tests. Wild-type *E. coli* cells excrete enterochelin into the surrounding medium, where it solubilizes and chelates iron (16); the ferric-enterochelin complex is subsequently actively transported across the bacterial cell membrane (12). Furthermore, an enterochelin-producing strain is able to support the growth of *entA* mutant bacteria by cross-feeding in mixed populations. On the other hand, the colicinogenic *entA* strain LG1315 did not cross-feed *entA* mutant bacteria (Table 2), in this case a colicin V-insensitive derivative of strain AN1937. This suggests that either no extracellular chelating agent is produced by strain LG1315, or, if there is an extracellular chelator, its transport into the cell relies on plasmid-encoded functions. These alternatives were investigated using the Iu$^-$ mutants of plasmid ColV-K30 described above. As shown in Table 2, strain LG1315 was able to cross-feed mutants LG1418, LG1421, and LG1434, indicating that it compensated for a defect in chelator function (*iuc*) in these mutants. Moreover, Iu$^-$ strain LG1419 was also able to cross-feed the three presumptive *iuc* mutants; however, strain LG1315 did not cross-feed mutant LG1419. These data suggest that strain LG1419 synthesizes a functional extracellular product, but is in some way impaired in the transport of complexed iron across the cell membrane (*iut*).

It is also shown in Table 2 that derivatives of strain AN1937 carrying plasmids ColV-H247 and ColV-P72 (from human and porcine bacteremic *E. coli* strains, respectively), which were previously demonstrated to promote efficient iron uptake (22, 23), were able to cross-feed the *iuc* class of mutants (LG1418, LG1421, and LG1434) but not the *iut* mutant LG1419. This suggests that the iron chelators specified by plasmids ColV-K30, ColV-H247, and ColV-P72 are similar to the extent of utilizing the same transport mechanism. On the other hand, a derivative of strain AN1937 carrying plasmid ColV,I-K94 (which does not promote iron uptake [22, 23]) was not cross-fed by chelator-producing strains, nor did it cross-feed the *iut* mutant LG1419. These data imply that neither element of the iron uptake system is encoded by plasmid ColV,I-K94.

Isolation of ColV plasmid mutants defective in colicin V synthesis. Cultures of strain LG1315 were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described above and plated, without further enrichment, for single colonies on nutrient agar. Individual clones were screened for loss of colicin production by inoculation onto a lawn of colicin-sensitive bacteria on nutrient agar (21). The plasmid content of possible mutants was checked by agarose gel electrophoresis. Four Cva$^-$ derivatives of strain LG1315 were independently isolated in this way; all showed normal growth, as compared with that of the parental strain, on minimal agar

TABLE 2. *Cross-feeding tests*[a]

Patch inoculum		Bacterial lawn			
Bacterial strain	Relevant characteristics	LG1439 *entA*	LG1418[b] *entA* ColV-K30 *iuc*	LG1419 *entA* ColV-K30 *iut*	LG1314 *entA* ColV,I-K94 Iu$^-$
LG1312	Ent$^+$	+	+	+	+
LG1315	*entA* ColV-K30 Iu$^+$	−	+	−	−
LG1418[c]	*entA* ColV-K30 *iuc*	−	−	−	−
LG1419[d]	*entA* ColV-K30 *iut*	−	+	−	−
LG1323	*entA* ColV-H247 Iu$^+$	−	+	−	−
LG1324	*entA* ColV-P72 Iu$^+$	−	+	−	−
LG1314[e]	*entA* ColV,I-K94 Iu$^-$	−	−	−	−

[a] Lawns of bacteria (10^7 cells per plate) on minimal agar containing dipyridyl were patch inoculated with cultures of the strains indicated. Cross-feeding was observed as a zone of growth of the lawn around a particular patch (+). (−) Indicates no cross-feeding.

[b] Similar results were obtained for strains LG1421 and LG1434.

[c] No growth of patch inocula except on the LG1419 lawn. Similar results were obtained for strains LG1421 and LG1434.

[d] Poor growth of patch inocula.

[e] No growth of patch inocula.

containing dipyridyl, indicating that the plasmid-specified iron uptake system was unaffected. In the case of one of the mutants, the plasmid was transferred by conjugation to strain LG1417. Transconjugant strain LG1437 was also Cva$^-$ but Iu$^+$, confirming that the original mutant did indeed carry a plasmid mutation.

Effect of plasmid mutations on bacterial virulence. The relative virulence of mutants isolated as defective in either iron uptake or colicin V production was determined by inoculating groups of mice with the bacterial strains shown in Table 3 at a dose which gave no killing by the plasmid-free strain AN1937 but which caused death of infected animals within about 36 h with strain LG1315 (Cva$^+$Iu$^+$). A Cva$^-$ mutant displayed the same level of virulence as did strain LG1315, whereas the Iu$^-$ mutants did not kill infected animals when administered at the same dose.

Effect of mutations to colicin V insensitivity on iron uptake. If colicin V were directly involved in iron chelation, mutations leading to insensitivity to the lethal action of colicin V might be expected also to influence the transport of iron across the bacterial cell membrane. Spontaneous colicin-insensitive mutants of strain AN1937 were isolated and characterized by their resistance or tolerance to colicin V and colicin Ia as described by Davies and Reeves (9). Mutants resistant to both colicins were deduced to have lost the *cir* outer membrane receptor protein common for these colicins (11). Mutants tolerant to both colicins were assumed to be *tonB* mutants (13). A third class, mutants tolerant only to colicin V (Cvt), presumably lacks a specific function essential to the action of this colicin (8). Plasmid ColV-K30 was transferred by conjugation to independent isolates of each class of insensitive mutant, and transconjugants were subsequently tested for their ability to grow on minimal agar in the absence of added citrate. As reported previously, *tonB* mutants were unable to sequester iron by the plasmid-encoded process (22, 23). However, colicinogenic derivatives of the other two classes were able to grow on the selective medium, indicating that the mutations they carry do not affect the ColV plasmid-mediated iron uptake system (Table 4).

TABLE 3. *Experimental infections of mice by plasmid mutants*

Bacterial strain	Relevant characteristics	Mortality at 36 h
AN1937	*entA*	0/5
LG1315	*entA* ColV-K30 Cva$^+$ Iu$^+$	5/5
LG1418	*entA* ColV-K30 Cva$^+$ *iuc*	0/5
LG1419	*entA* ColV-K30 Cva$^+$ *iut*	0/5
LG1437	*entA* ColV-K30 Cva$^-$ Iu$^+$	5/5

TABLE 4. *Effect of mutation to colicin V insensitivity on plasmid-mediated iron uptake*

No. of independent colicin V-insensitive isolates tested	Relevant characteristics[a]	Phenotype of colicinogenic derivative[b]
3	*tonB* (V^t, I^t)	Cva$^+$ Iu$^-$
13	*cir* (V^r, I^r)	Cva$^+$ Iu$^+$
7	Cvt (V^t, I^s)	Cva$^+$ Iu$^+$

[a] Superscripts ^{t}, ^{r}, and ^{s} denote tolerance, resistance, and sensitivity, respectively, to colicins V and Ia.

[b] Derivative harboring wild-type ColV-K30 plasmid.

DISCUSSION

Investigations of the correlation between colicinogenicity and virulence in invasive strains of *E. coli* have led to the identification of two independent pathogenic mechanisms associated with ColV plasmids. The molecular cloning has been reported of fragments of plasmid ColV,I-K94 which do not specify colicin synthesis but which confer on the bacterial host the same level of lethality in the experimental infections as the entire plasmid (3). In this case, enhancement of virulence is said to be due to increased tolerance to the bactericidal effects of serum. Moreover, evidence has recently been presented for an efficient iron uptake system specified by a number of ColV plasmids harboured by invasive *E. coli* strains (although not by plasmid ColV,I-K94 [22, 23]). The presence of a plasmid conferred a strong selective advantage to the host bacterial strain in experimental infections unless excess iron was administered in the inoculum. We demonstrate here that the plasmid-specified mechanism for efficient sequestering of iron is also independent of the synthesis and activity of colicin V itself. Mutants isolated on the basis of loss of colicin V production remained proficient in plasmid-mediated iron uptake and were unchanged in virulence. Conversely, mutants screened for defects in the plasmid-coded iron uptake system still produced colicin, but were markedly less virulent than the parental strain.

These data are consistent with the recent observation that transposon Tn1 inactivation of the colicin V gene of a ColV plasmid harboured by a bovine pathogen did not influence the level of pathogenicity for mice of the host bacterial strain (18). It is perhaps arguable that lethality testing may not be a sufficiently sensitive technique to show unequivocally the loss of a possible colicin-related pathogenic function in the presence of a second, independent mechanism. However, the fact that serum tolerance deter-

minants can be cloned separately from the colicin V gene (3) and the observation reported in this paper that iron uptake is independent of colicin V activity suggest that the observed correlation between ColV plasmids and pathogenicity is fortuitous. We are currently beginning to screen directly among clinical isolates for plasmids that specify efficient iron uptake but which are Cva$^-$.

In contrast with enterochelin, the plasmid-specified product to which iron binds cannot be transported across the membrane of strains that do not carry a ColV plasmid. Furthermore, plasmid mutants defective in iron uptake fell into two classes on the basis of cross-feeding tests, thus defining two plasmid-specified functions for the uptake of iron. One class (*iuc*) was cross-fed by a strain carrying a wild-type plasmid and is therefore postulated to lack an extracellular chelating agent for which the cross-feeding strain compensates. The other class (*iut*) was not cross-fed by strains producing extracellular chelator but was itself able to cross-feed the *iuc* mutants; thus, it produces normal chelator but is defective in some aspect, possibly a membrane protein, of the transport of chelator into the cell. It may be significant that the *iut* mutant LG1419 is somewhat leaky, growing poorly on minimal medium containing dipyridyl. The transport function that is mutated in this strain may still have residual activity, or else the chelator produced by the mutant can, in extreme circumstances, utilize an alternative route of entry into the cell.

The process of transport of the plasmid-coded iron chelator requires *tonB* protein (22, 23) and probably other chromosome-specified products, as well as the plasmid-specified *iut* function. It does not, however, require functions specifically involved in the killing action of colicin V. Mutants in the primary binding site for colicin V in the cell membrane (*cir*) and mutants which bind, but are not killed by, colicin V (*Cvt*) were not defective in ColV plasmid-specified iron uptake. These data imply that colicinogenicity and iron uptake are physically as well as functionally independent, and the significance of the inducibility of *cir* protein in conditions of iron stress remains unresolved.

ACKNOWLEDGMENTS

This work was supported by project grant G979/461/C from the Medical Research Council and by funds from the University of Leicester.

We are very grateful to I. G. Young for the enterochelin-deficient mutant used throughout this work, and we apologize for not acknowledging him in previous publications. Thanks are due also to Victoria Shingler for initiating this study as part of an undergraduate project and to I. B. Holland and G. S. Plastow for many helpful discussions.

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