Naturally Occurring Plasmid Carrying Genes for Enterotoxin Production and Drug Resistance

Abstract. Escherichia coli *strain 86, isolated from a piglet with diarrhea, carries plasmid-linked genes for resistance to tetracycline, streptomycin, and sulfonamides and for production of heat-labile and heat-stable enterotoxin. Results of (i) genetic experiments involving conjugal transfer and phage P1-mediated transduction and (ii) physical experiments involving electron microscopic examination of plasmid DNA and heteroduplex analysis show that a single conjugative plasmid carries the genes for drug resistance and production of enterotoxin.*

Genes controlling the production of heat-labile (LT) and heat-stable (ST) enterotoxin of *Escherichia coli* are located on plasmids called Ent plasmids (*1*). Since strains with Ent plasmids often carry additional plasmids, including R factors (*2*), and since recombination among different plasmids has been demonstrated (*3*), it seems possible that such recombinations in nature would lead to the formation of plasmids carrying genes for enterotoxin production and for drug resistance. In this report, we describe such a plasmid, which carries genes for LT and ST production, conjugal transfer (Tra), and resistance to tetracycline (Tc), streptomycin (Sm), and sulfonamides (Su). Phenotypically the strain is LT^+, ST^+, Tc^R, Sm^R, Su^R, and Tra^+.

Initially 100 enterotoxigenic strains of *E. coli* isolated from piglets with diarrhea were tested for resistance to a variety of antimicrobial agents and for conjugal transfer of such resistance genes. Ninety were resistant to one or more of these agents and in about half of them resistance genes could be transferred conjugally to strain 711, a multiauxotrophic, nalidixic acid–resistant derivative of *E. coli* K12. From one of these strains, number 86, resistance to Tc, Sm, and Su was transferred with high frequency to strain 711, and there was 100 percent cotransfer of all plasmid-controlled traits (Tc^R, Sm^R, Su^R, LT^+, ST^+, and Tra^+) following selection for transfer of either Tc^R or Sm^R. We describe here further experiments that establish that a single plasmid named pCG86 carries the genes for the above six plasmid-controlled traits.

In gene transfer experiments involving conjugation or transduction, selection was carried out for either Tc^R or Sm^R recombinants, with the use of either rich (Neopeptone) or minimal agar media containing Tc at 20 μg/ml and Sm at 30 μg/ml. The general procedures for growing cells, performing matings and transductions with phage P1, and scoring the phenotypes of progeny colonies have been described (*4*). The unselected plasmid-determined characteristics were resistance to sulfadiazine (added at 100 μg/ml), Tra, LT, and ST production. LT production was tested routinely by the Y1 adrenal cell culture assay (*5*), with the use of a modified procedure with microtiter plates (*6*). Both LT and ST formation were checked occasionally by the intestinal loop assay (*7*). In these spot checks there was complete agreement between the results of cell culture and intestinal loop assays and all LT^+ cultures were ST^+, all LT^- were ST^-.

After the transfer of pCG86 to strain 711, the plasmid was transmitted conjugally to a number of other K12 derivatives. In all cases, selection for either Tc^R or Sm^R progeny resulted in 100 percent cotransfer of all the other scored plasmid traits. The recipients used in these matings included eight F^- strains, two of which were recombination deficient (*recA*), an Hfr strain, and a number of strains carrying plasmids belonging to different incompatibility groups. The frequency of transfer was 1 to 2 percent of the added donor cells in all cases. Thus, in these experiments involving the analysis of hundreds of recombinant colonies, the traits Tc^R, Sm^R, Su^R, LT^+, and Tra^+ appeared as though they were present on a single genetic unit.

Transduction experiments have been carried out with phage P1 lysates prepared from six different strains carrying plasmid pCG86. Recipients were multiauxotrophic F^- strains that did not carry any known plasmid. With selection for either Tc^R or Sm^R progeny, the transduction frequency was low, about 1 percent that of selected chromosomal genes. In all transductions, some progeny colonies inherited all the other plasmid-determined traits (Table 1). With selection for Tc^R colonies, the frequency of transductants receiving all other plasmid traits is somewhat higher than with selection for Sm^R colonies. With either selection all the known plasmid genes are cotransducible. Since in generalized transduction with phage P1 presumably a single DNA segment is transmitted by a phage particle and since double transductions by separate transducing particles are rare, the results of these experiments provide strong evidence that all

Table 1. Transduction of genes located on plasmid pCG86. Donor: 289-1; *thi⁻ pro⁻ trp⁻ ilv⁻ his⁻*/pCG86. Recipient: 335: *thi⁻ pro⁻ trp⁻ met⁻ his⁻*.

Selected marker from donor	Phenotype of transductants	Colonies (No.)
Tc^R, 22 colonies	Tc^R Sm^R Su^R* LT^+ Tra^+	9
	Tc^R Sm^R LT^- Tra^+	6
	Tc^R Sm^S Su^S LT^- Tra^-†	7
Sm^R, 25 colonies	Tc^R Sm^R Su^R LT^+ Tra^+	4
	Tc^S Sm^R Su^R LT^+ Tra^+	20
	Tc^R Sm^R Su^R LT^- Tra^+	1

*The phenotypes of Sm and Su were always the same and no segregation was observed. †This class most likely represents Tc^R mutants of strain 335, since no plasmid DNA could be demonstrated in these colonies.

Table 2. Measurement of plasmid DNA molecules by electron microscopy.

Source of plasmid DNA	Size (kilobases)	Molecules measured (No.)
Original strain 86	114 ± 5 (85)*; 83 ± 3 (10); 62 ± 3 (4); 32 ± 2 (1)	120
Transconjugants†		
pCG 86/711	114 ± 5 (85); 84 ± 3 (14); 32 ± 2 (1)	125
pCG 86/KL 320	114 ± 5 (80); 84 ± 3 (19); 32 ± 2 (1)	100
pCG 86/711 *recA*	114 ± 6 (75); 84 ± 4 (23); 32 ± 3 (2)	100
pCG 86/AB 2463	114 ± 5 (70); 84 ± 3 (28); 32 ± 3 (2)	100
Transductants		
All plasmid traits present	114 ± 5 (83); 84 ± 3 (17)	65
Tc^S, otherwise all plasmid traits present	94 ± 4 (100)	107

*Number in parentheses represents percentage of total number of molecules measured in the preparation. †The indicated host strains are all K12 derivatives. Strain AB2463 is *recA*, like 711 *recA*. The host strain for the transductants was strain 711.

the genes determining the scored traits are located on a single plasmid.

Isolation of plasmid DNA and characterization of plasmid DNA molecules, including heteroduplex molecules by electron microscopic examination, have been described (*8*). The sizes of plasmid DNA molecules in preparations of the original strain 86 and various K12 strains carrying pCG86 are listed in Table 2. Included are strains that have received pCG86 by conjugation and by transduction. In strain 86, four sizes of plasmids are seen, whereas three sizes are present in the strains which have received all the scored plasmid-determined traits. The transductant strain which is Tc^S has plasmid molecules of only one size.

In interpreting these findings we shall first consider the difference between strain 86 and the K12 strains. Plasmid molecules whose length is 62 kilobases are present in strain 86 and absent in the other strains. It is likely that the 62-kilobase plasmid carries the genes for the production of K88 antigen and raffinose fermentation. These traits are present in strain 86, but not in the other strains, and the genes determining these two traits are known to be located on a single plasmid (*9*).

To explain the presence of three plasmid sizes in strains carrying all the known pCG86 genes we may consider two possibilities: either there are three plasmids that are always transmitted together or it is only the large plasmid that is transmitted and that dissociates into two smaller ones after transfer. The latter possibility appears to be more likely, because the two smaller plasmids are present at lower frequencies in the plasmid preparations than the large one and, as pointed out above, it is unlikely that during transduction more than one plasmid molecule is transmitted. Furthermore, in physical terms, phage P1 DNA is 90 kilobases long and in transducing particles the DNA substituted for phage DNA is usually of a similar size. It is therefore most unlikely that a transducing particle would contain three DNA molecules with size 114, 84, and 32 kilobases. In fact, we think that the reason for the low transduction frequency of pCG86 is the rarity with which the 114-kilobase DNA molecule is packed into the P1 phage coat.

The transductant selected for Sm^R, which is Tc^S, contains only one size of plasmid DNA—94 kilobases. Thus, it can be concluded unambiguously that at least the genes determining Sm^R, Su^R, LT^+, ST^+, and Tra^+ are carried on a single plasmid. In heteroduplex experiments we have found complete homology between the 94-kilobase molecule and a corresponding 94-kilobase segment of the 114-kilobase molecule present in the other strains. We have also found that the 114-kilobase plasmid, but not the 94-kilobase plasmid, contains a 7.0-kilobase segment bounded by inverted repeat segments each of 1.4 kilobase. It has been shown with other plasmids that the gene for Tc^R is part of a transposon (*10*). This *tet* transposon has the same physical dimensions as the 7.0-kilobase segment bounded by the inverted segments. From these observations we conclude that the 94-kilobase plasmid was generated from the 114-kilobase plasmid, presumably during the period of phage growth after infection by phage P1.

Our results appear to be the first report of a plasmid carrying genes for drug resistance and enterotoxin production. This plasmid is readily transmissible by conjugation among strains of *E. coli*. Presumably the plasmid was formed as a result of recombination between an R factor and an Ent plasmid. The likelihood of such an event depends on the frequency with which these parent plasmids occur in nature, and it is well known that the widespread use of antibiotics has resulted in a greatly increased frequency of R factors among natural isolates. Our results show that genes for drug resistance are spread in nature not only by being part of an R factor, but also by becoming incorporated into other plasmids, in this case a plasmid carrying genes which contribute to pathogenicity.

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Bovine Protoporphyria: The First Nonhuman Model of This Hereditary Photosensitizing Disease

Abstract. *Protoporphyria, a photosensitizing disease documented only in humans, was transmitted as a recessive trait to seven female calves. Cutaneous lesions were extensive, and erythrocyte and fecal protoporphyrin concentrations exceeded by far those of human protoporphyria. Average ferrochelatase activity was decreased to one-half of normal in the liver of carriers, and to about one-tenth of normal in liver, kidney, heart, spleen, lung, and marrow of protoporphyrics.*

We discovered four calves and one adult cow with clinical and chemical findings characteristic of protoporphyria (EPP). They constitute the first known animal model for any of the hereditary human porphyrias other than so-called "erythropoietic porphyria" (EP, congenital, or Gunther's porphyria). Of special interest was the finding of a marked uniform decrease in activity of ferrochelatase (heme synthetase) in liver, heart, kidney, marrow, and other tissues studied.

Within a few days after birth, all the protoporphyric calves actively avoided sunlight, crawled under haystacks, or sought shade even behind fence posts. Within a few weeks, skin over the snout, ears, and back became edematous, erythematous, fissured, partially alopecic, and scabbed. When presented at the University of Minnesota, the four heifers ranged in age from 2 to 3 months. A tentative diagnosis of protoporphyria was made because of the apparently painful photosensitivity, without the discoloration of teeth or urine (*1*) which characterizes EP in humans (*2*), cows (*3*), pigs (*4*), cats (*5*), and squirrels (*6*).

The affected calves were sired by the