Isolation and Characterization of ColE2 Plasmid Mutants Unable to Kill Colicin-Sensitive Cells

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Summary. After transfer from a mutagenized host, twenty one ColE2 plasmid mutants were isolated after screening 10,000 clones for abnormal colicin production. Analysis by SDS polyacrylamide slab gel electrophoresis of proteins synthesized after mitomycin C-induction of mutant cultures, indicates that all but two of the mutations are in the structural gene for colicin E2. Of these, nine produce fragments of colicin in both whole cells and minicells and some are suppressed by nonsense suppressors.

Studies with a nonsense mutant producing only a small colicin E2 fragment (ColE2-421) suggest that colicin E2 is not involved in plasmid DNA replication, in the control of its own synthesis, or required for cell death when cells become committed to colicin production. The two plasmid mutants outside the colicin gene segregate plasmid-free cells at 33°, 37° and 43°. One segregates fairly rapidly (about 4% per generation) though the colicin-producing cells make normal amounts of colicin, whilst the other segregates more slowly and the colicin-producing cells make much reduced amounts of colicin.

Introduction

The non-conjugative plasmid ColE2 is maintained in Escherichia coli at about ten copies per bacterial chromosome and is normally isolated as a covalently closed circle of duplex DNA of molecular weight about 4.2 × 10^6 (Bazaral and Helinski, 1968), which is sufficient DNA to code for about 200,000 daltons of protein. In common with most other bacteriocins encoded by small plasmids (Luria, 1973; Kool et al., 1975), colicin E2 synthesis is normally repressed, less than 1% of a ColE2-containing population synthesizing measurable amounts of colicin at a given time, unless the cells are treated with agents that interfere with DNA synthesis such as mitomycin C, when greater than 90% of the cells can be induced to synthesize colicin. During such induced synthesis, two other proteins are co-ordinately synthesized with colicin E2, and chromosomal protein synthesis is selectively inhibited; these three ColE2-specified proteins account for almost half of the plasmid coding capacity (Tyler and Sherratt, 1975).

There are particular problems in both the isolation and characterization of mutants of small plasmids present in multiple copies per cell. Difficulties in isolation arise because recessive mutations will be masked by wild-type plasmids in the cell; after sufficient growth to ensure segregation of pure mutant clones, the apparent frequency of plasmid mutants will have dropped many fold. Moreover, mutations in many genes may be "lethal" for the plasmid, necessitating the isolation of conditional mutants. Characterization of plasmid mutants by recombination and complementation is again complicated by multiple plasmid copies in a cell, our present inability to construct cells containing known numbers or ratios of two different ColE2 mutants, and the likely stochastic segregation of plasmids from such a cell if it could be constructed. Here we report a procedure that produces satisfactory levels of ColE2 plasmid mutants, most of which were found to be in the colicin structural gene.

Materials and Methods

Bacteria

The strains used are listed in Table 1.

Media and Labelling Conditions

Defined media consisted of M9 salts containing 0.4% glucose (Clewell and Helinski, 1972) supplemented with the necessary re-
Concentrations: Difco vitamin-free casamino acids 1%; amino acids 40 μg/ml; thymine 20 μg/ml; thiamine 2 μg/ml; tetracycline.

The requirements for each experiment. Difco agar (1.75%) was added for solid medium. Other supplements were added at the following concentrations: Difco vitamin-free casamino acids 1%; amino acids 40 μg/ml; thymine 20 μg/ml; Colicin E2 extract 10% (v/v). L-broth, L-agar, nutrient agar (Kennedy, 1971), Brain Heart Infusion medium, buffered saline with gelatine (BSG), (Kool et al., 1974) have been described elsewhere.

For labelling proteins 14C-uniformly labelled amino acids (1 μCi/ml; 57 mCi/mAtom; Radiochemical Centre, Amersham) were added to cells growing in M9-minimal medium. For labelling DNA 3H-thymidine (10 μCi/ml; 23 Ci/mmol; Radiochemical Centre, Amersham) was added to M9-casamino acids medium.

### Stab Tests

Stab tests for determining the production of colicin E2 were as described previously (Kennedy, 1971).

### Colicin E2 Extract

A culture of W3110 ColE2 was grown in L-broth with aeration to about 5 x 10⁸ cells/ml. Mitomycin C (0.1 μg/ml) was added and the culture grown overnight. Cells were removed by centrifugation and the supernatant thoroughly mixed with chloroform. The chloroform was allowed to settle out and 10 ml aliquots of supernatant were stored at −20°C. 1/10 volume of this added to agar or liquid media gave at least a 10-fold excess for killing of E-colicin sensitive cells.

### Transfer of Plasmids

ColE2 plasmids were mobilised by R64drd1 into R64drd11 which confers resistance to tetracycline (10 μg/ml) and streptomycin (10 μg/ml). Equal volumes of exponentially growing donor and recipient cultures (about 10⁶ cells/ml in L-broth) were mixed and incubated statically for 60 min and then plated on the appropriate selective medium. Wild type ColE2 + donor cultures produce sufficient colicin to ensure that all surviving R64 + recipients also contain ColE2 (which confers immunity). When transferring mutant ColE2 plasmids unable to synthesize colicin, crude E2 colicin was added 50 min after mating. To isolate ColE2 + R64 recipients, matings were interrupted at 5 min and 10 min and plated onto colicin plates containing a counterselective agent against the donor.

#### Mutagenesis and Isolation of Mutants

After nitrosoguanidine mutagenesis (Miller, 1972), W3110 ColE2 R64 + donors were grown to stationary phase at 33°C in L-broth. 10-fold dilutions of these cultures were made into pre-warmed L-broth and aerobic growth continued for 2 h before mating with an equal volume of M72 recipients at 33°C for 3 h. Mating mixtures were vortexed for 30 s and diluted 20-fold into L-broth containing streptomycin (500 μg/ml), tetracycline and colicin E2 extract. After growth to stationary phase at 33°C, the culture was diluted and plated at 33°C onto nutrient agar containing streptomycin (500 μg/ml) and tetracycline to give about 20 colonies per plate.

10,000 single colonies from four independent mutageneses were picked and tested for immunity and colicin production after growth at 33°C and 43°C. Spontaneous colicin production of stabs (30 per plate) was tested by overlaysing the killed bacteria (chloroform) with a lawn of colicin-sensitive bacteria in soft agar. The size of the “zone of killing” was used as a semi-quantitative estimate of colicin produced. Presumptive mutant clones were streaked for single colonies on selective minimal medium and six single colonies were retested for immunity to E2 colicin and colicin E2 production. The plasmid nature of such mutants was verified by conjugal transfer to a new recipient (CSH 27 spc-r) from which all subsequent experiments were carried out.

#### Sodium Dodecyl Sulphate (SDS) Slab Gel Electrophoresis and Sample Preparation

This was as described previously (Tyler and Sherratt, 1975). All gels were run to a distance of 10 cm from the origin.