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Detection of "Lost" Plasmids from *Escherichia coli* Using Excess Ampicillin

André-Denis G. Wright and Teresa J. Crease
Department of Zoology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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We have discovered, in two different laboratories, that plasmids from different sources containing antibiotic resistance are sometimes undetectable in broth or plate cultures containing antibiotic after inoculation from frozen glycerol stocks. This "missing plasmid" phenomenon has also been observed and reported from other laboratories around the world via the Internet news group Bionet.molbio.methds-reagnts. Here we report a quick and easy method to obtain detectable yields of "lost plasmids" from frozen glycerol stocks without repeating time-consuming cloning and transformation experiments.

Frozen glycerol stocks of *Escherichia coli* strain XL1-Blue (Stratagene) (1), containing recombinant pBluescript IIKS (Stratagene) (2), were prepared from freshly grown cultures inoculated with streak purified colonies and stored at −80°C for at least 6 to 8 months. These frozen glycerol stocks were then used to inoculate either 10 ml of Luria-Bertani (LB)[1] broth or terrific broth (TB) containing ampicillin (Sigma) (100 mg/liter). Although high cell densities were routinely obtained in the cultures shaken overnight (14 h) at 37°C, little or no plasmid DNA was observed on 0.8% agarose gels after standard plasmid purifications (3–5) from at least 50 of our frozen glycerol stocks. Of these problematic glycerol stocks, 48 contained fragments of crustacean mitochondrial DNA cloned into pBluescript in both orientations. These inserts ranged in size from 3.2 kb to 500 bp. The other two glycerol stocks contained pBluescript with 1.8-kb ribosomal RNA gene inserts from two different species of ciliates.

Four of the "problem" glycerol stocks were also streaked onto LB agar plates containing ampicillin (100 mg/liter). Twenty-four single colonies were randomly picked from the selective plates, lysed in a cracking buffer (0.1 N sodium hydroxide, 10 mM disodium EDTA, 1% sodium dodecyl sulfate, 10% glycerol), and loaded directly onto an 0.8% agarose gel. No plasmid DNA was observed in 9 (37.5%) of the colonies screened.

To test the efficacy of the antibiotic, two aliquots of the same preparation of LB/ampicillin broth and agar plates were inoculated with nontransformed *E. coli* strain XL1-Blue (1). Under the same incubation conditions in which transformed cells grew, cell growth did not occur with nontransformed cells, confirming the activity of the antibiotic. A rigorous test was then carried out with increased concentrations of ampicillin in the growing medium. Test tubes of LB broth containing 100 mg/liter (1×), 200 mg/liter (2×), 500 mg/liter (5×), and 1000 mg/liter (10×) of ampicillin were inoculated with the same 50 frozen glycerol stocks, under the same incubation conditions. Although similar cell densities occurred in all four concentrations of ampicillin, harvests from cultures containing 10× ampicillin yielded the highest amount of plasmid DNA (Fig. 1). A smaller amount of DNA was also recovered from the 5× ampicillin cultures (Fig. 1). Increased plasmid DNA yields were also recovered in 10× ampicillin from transformed cell lines that normally give good yields of plasmid in 1× ampicillin (Fig. 1). Thus, by increasing the concentration of ampicillin in our growing medium, we have been able to successfully improve the yield of the 50 recombinant plasmids that were previously undetectable after routine plasmid DNA harvests.

Ampicillin prevents cell growth by inhibiting metabolic

[1] Abbreviation used: LB, Luria-Bertani.

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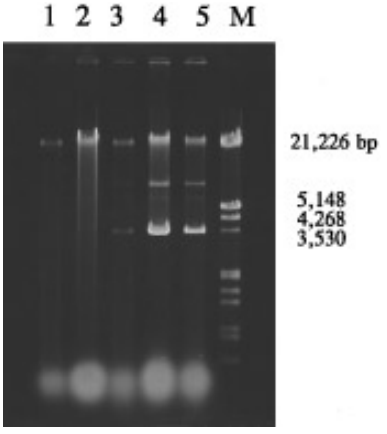

FIG. 1. Agarose gel showing "missing" and detected recombinant pBluescript DNA. A 1% agarose gel was loaded with plasmid DNA harvests from transformed *Escherichia coli* XL1-Blue cells. Lane 1 shows the "missing plasmid" phenomenon from transformed cells grown in LB broth containing a regular dosage of ampicillin (100 mg/liter). Lane 2 shows a plasmid harvest with no plasmid DNA from cells grown in LB broth containing 2× ampicillin (200 mg/liter). Lane 3 shows a small amount of supercoiled plasmid DNA (approximately 3.5 kb) recovered in LB broth containing 5× ampicillin (500 mg/liter). Lane 4 shows plasmid DNA (supercoiled and linear) recovered from cells grown in LB broth containing 10× ampicillin (1000 mg/liter). Lane 5 shows a routine plasmid DNA harvest from cells grown in LB broth containing a regular dosage of ampicillin. Lane M contains a λDNA/*Hin*dIII–*Eco*RI marker, 400 ng. The 1% agarose gel was run in 1× TAE buffer at a constant voltage at 100 V for 1 h.

pathways necessary for cell wall synthesis (6), such that nontransformed bacteria are unable to replicate and remain in stasis. Ampicillin resistance is mediated by β-lactamase produced by the plasmid-encoded *bla* gene (6–8). This enzyme is secreted into the periplasmic space of the bacterium where it hydrolyzes the β-lactam ring of ampicillin and detoxifies the drug (8, 9). During cell replication, enzymes called autolysins hydrolyze the old cell wall to allow for the synthesis of a new one (9). During this normal growth process, β-lactamase may be released into the medium from the periplasmic space. Thus, at each cell wall synthesis, the levels of ampicillin in the medium may be depleted by this free enzyme.

Plasmids carrying drug resistance genes are usually maintained in the microbial population only as long as the drug is present (9) because bacteria without plasmids are usually able to divide much faster than those that do and will eventually take over the culture. Similarly, if the antibiotic concentration is too low, cells with low plasmid copy number may be at an advantage over those with high plasmid copy number as they are able to produce enough β-lactamase to confer drug resistance and are also able to divide faster. Consequently, at low ampicillin concentrations, the more rapid replication of cells with low plasmid copy number, and perhaps even nontransformed cells once the ampicillin is depleted, results in cultures that give low plasmid yields despite having high bacterial cell densities. Increasing the concentration of ampicillin increases the demand for β-lactamase and thus selects for and maintains cells with high plasmid copy number leading to improved plasmid DNA yields.

When fresh glycerol stocks containing "rescued" plasmids are used to inoculate LB broth containing a regular dose of ampicillin (100 mg/liter), the plasmid DNA yield is maintained. It appears that once the plasmid is rescued, it is not necessary to maintain the clones in broth containing excess ampicillin.

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S1 Nuclease Protection Assay Using Streptavidin Dynabeads-Purified Single-Stranded DNA

Florence M. Cammas and Adrian J. L. Clark[1]
Department of Chemical Endocrinology, St Bartholomew's Hospital, Medical College, West Smithfield, London EC1A 7BE, United Kingdom

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The S1 nuclease protection assay is a useful method for determining the position of the transcription start site and the intron/exon structure of a gene. The princi-

[1] To whom correspondence should be addressed. Fax: +44 171 601 8468.

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