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Plasmid Curing From *Escherichia Coli* By Acridine Dyes

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ABSTRACT

In the present study, curing of *Escherichia coli* plasmids in the presence of acriflavine and acridine orange, acridine dyes, were characterized using clinical strains. The bacteria offered tetracycline resistance as a plasmid marker. Growth in the present of acridine dyes initially caused changes in the plasmid profile followed by total elimination of plasmids. Concomitant to this loss of all plasmids, the cured derivatives became sensitive to tetracycline. The viability of *E. coli* was measured using replica plate method. The results obtained demonstrate that the acridine dyes can induces a clear curing with good viability. The ability to cure plasmids provides a useful tool for some genetic analysis and engineering.
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KEYWORDS

INTRODUCTION

The acridines: acriflavine and acridine orange dyes are closely related to the members of the xanthene class. Like the xanthenes, these have a long history of biological application as stains. As far back as 1929, the yellow derivative acriflavine was being used, as a tissue stain to visualize cell nuclei and acridine orange has remained a very popular stain for its use in fluorescence microscopy. Like the xanthenes the acridines are of inadequate stability for more demanding applications such as textile coloration[1,2]. Acriflavine is also used as an active medium for dye lasers[3]. In this paper, acridine dyes properties were exploited for curing of the *E.coli* plasmids. Plasmids are extrachromosomal pieces of double-stranded circular DNA which have the capability to replicate independently of the host chromosome[4]. Bacterial plasmids are known to harbour genes for: (i) resistances to antibiotics and heavy metals, (ii) catabolic pathways, (iii) biosynthesis of certain antibiotics, etc. In many cases, however, characteristics of the host organism conferred by the plasmids[5]. When working with some plasmid containing bacteria, it is often desirable to obtain a plasmid cured derivative[6]. Curing of plasmids from a bacterial strain is a method to substantiate the relationship between a genetic trait and carriage of that specific trait in the plasmid. This allows a direct comparison to be made between the plasmid containing and plasmid cured cells[7]. Some plasmids undergo spontaneous segregation and deletion. However, the majority is extremely stable, and requires the use of curing agents,

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to increase the frequency of spontaneous segregation. Various methods involving chemical and physical agents have been developed to eliminate plasmids[8]. The usefulness of curing agents is unpredictable in many bacterial strains, as there are no standard protocols applicable to all plasmids. Plasmid curing studies have been focused on *Escherichia coli*, *Salmonella typhi murium*, *Staphylococcus aureus* and, to a lesser extent, strains of degradative *Pseudomonads*[9]. The *E.coli* is a model microorganism in bacteriology research. The bacterium is used in many genetic study. First studies about DNA replication and transformation have been obtained from this organism. This non-pathogenic bacterial species replicates under ideal condition rapidly. In the present study, we report a method for curing native plasmids responsible for tetracycline reseistance bye acrine dyes in *E.coli*.

MATERIALS AND METHODS

Culture media

Luria bertani (LB) broth and LB agar plates were used throughout this study. Peptone, yeast extract and agar were from Merck; tetracycline, acriflavine and acridine orange were from Sigma.

Bacterial strains and culture condition

Three clinical uropathogenic *E.coli* were isolated from different hospitals and clinical laboratories of Tehran and identified on the basis of morphology, growth, and biochemical characteristics[10,11]. *E.coli* $DH_5\alpha$ was used for transformation tests. They were grown in LB medium and were stored at -70^0C in the form of 1ml aliquots containing 1.5×10^5 bacteria per ml in the growth phase. In each experiment, an aliquot was thawed, inoculated in to 200ml of LB broth and incubated at 37^0C for 15 to 16h. The organisms were collected by centrifugation and resuspended in LB broth. The concentration of the cell suspension was adjusted with 0.5 Mcfarland turbidity standard then diluted.

Determination of susceptibility

Tetracycline susceptibilities were determined by Kirby Bauer disk diffusion following the definition of National Committee for Clinical Laboratory Standards (NCCLS) for agar diffusion tests[12]. Microdilution tests were carried out to establish the minimum inhibitory con-

centration (MIC) of the acridin dyes, in accordance with NCCLS guidelines[13]. Overnight cultures of bacterial strains were diluted to 10^{-4} in LB broth, aliquots of 50µl transferred to a 96 well microplate, then 50µl of different concentrations of acridine orange and acriflavine solutions were added. The microplates were incubated at 37^0C for 24h, after which MIC values were determined.

Replica plating

Overnight cultures of bacterial strains were diluted to 10^{-4} and 1ml aliquots inoculated into 100ml of LB broth. Various concentrations of acriflavine and acridine orange were added to 5ml of the diluted cultures and incubated at 37^0C for 24h. Dilutions of 10^{-4} were prepared from tubes showing growth and 100µl aliquots were plated on LB agar. The plates were incubated at 37^0C for 24h. The velvet replica from master plates was prepared onto plates containing tetracycline and after 24h incubation, the master and replica plates were compared and the ratio of the plasmid elimination was determined[14].

Plasmid isolation and curing

Plasmid from *E.coli* were isolated as described by alkalilysis method[15] and resolved by electrophoresis through a 1% agarose gel using TAE buffer at 50_V. The DNA was visualized by ethidium bromide (1g/ml) staining for 15min and photographed under short wave UV light. Prior to curing, we determined the sublethal concentrations of the the curing agents for the *E.coli* strains. The sublethal concentration was defined as the highest concentration allowing for the detectable growth of a *E.coli* strain. For curing with acridine dyes, the bacteria were grown to mid log phase and passaged in LB broth containing 70-150µg/ml of acridine dyes maintained at 37^0C under shaking conditions. Cured bacteria were identified by replica plating and further tested by culture in LB medium supplied with tetracycline. The growth cycles were repeated three times to ensure the possibility of plasmid curing by these chemicals and following final passage the bacteria were washed and plated onto LB agar plates. The single colonies thus obtained were screened on an tetracycline plate for the loss of resistance. The tetracycline sensitive colonies were grown in LB and checked for the presence or absence of plasmids by agarose gel electrophoresis.

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Transformation experiments

The *E.coli* DH$_5\alpha$ was transformed with plasmid DNA isolated from the clinical strain used in this study by the calcium chloride procedure[16]. Transformed bacteria were identified by replica plating and further tested by culture in LB medium supplied with tetracycline. The transformed bacteria could be identified using the LB media containing tetracycline. Plasmid DNA was extracted from the transformed bacteria and checked by agarose gel electrophoresis.

Determination of viability and cell numbers

Viable counts of the bacterial were determined using serial dilutions of samples and plate counting method. Bacterial colonies grown on LB agar plates were taken and diluted in sterile LB 0.1ml from each dilution was spread on agar plates containing tetracycline. Similarly, LB agar plates and pure agar plates without tetracycline were also inoculated and incubated. Triplicate plates were prepared for each dilution level.

RESULTS AND DISCUSSION

Elimination of bacterial plasmids is often an essential step in functional analysis of these replicons[17]. Several chemical curing agents have been used in the curing of bacterial plasmids[7]. However, these chemicals proved to be fairly ineffective with regard to the curing of plasmids from the bacteria. The effectiveness of curing methods depends on the nature of the bacterial host and plasmids, in which some methods work better in a system than others[18]. In this study, the clinical isolates were investigated for elimination of tetracycline resistance. This study focused primarily on the tetracycline, because they serve as selective markers in transformation studies of bacteria. The antibiotics have also been listed as antibiotic which are authorized for medicine. The strains exhibited varying degrees of resistance to tetracycline. *E.coli* DH$_5\alpha$ cells were used for transformation. The presence of isolated plasmids, which carries a functional tetracycline, was easily evidenced in their host by the growth of the colonies on the plates countaing tetracycline. The bacteria were grown in the presence of 70-150µg/ml of acriflavine and acridine orange. After 24h incubation the MIC values were obtained(TABLE 1). The MICs, which ranged from

TABLE 1 : MIC values of the compounds against clinical isolates

Strains	Acroflavine(µg/ml)	Acridine orange(µg/ml)
E.coli 1	80	90
E.coli 2	70	80
E.coli 3	90	80

70 to>125µg/ml for acridine orange and acriflavine, were determined to be dependent on the strain involved. In our initial approach we sought to obtain rapidly qualitative information concerning the possible existence of acridine dyes effects at sub-lethal concentrations of the dyes and the possibility that exposure to this dyes might induce plasmid loss so as to avoid the difficult task of determining the optimum concentration of the dyes required to elicit the desired effect. LB gradient plates were prepared, containing a acridine dyes gradient ranging from concentrations approx. 10-fold higher to 10-fold lower than the MIC. Following 24h of incubation the colonies from master plates were transferred by velvet replica plating method onto tetracycline containing LB agar. The smaller colonies displayed irregular margins, suggesting that the antibiotic causes alterations of cell permeability and unusual growth. The plasmid profiles of the various strains were analysed by agarose gel electrophoresis and was shown to differ for various strains. When these plasmid containing strains were exposed to plasmid curing compounds, the plasmid DNA profile became different for the majority of the extracts as shown by agarose gel electrophoretic properties. To establish a quantitative relationship between possible acridine dyes induced plasmid loss and cell viability, *E.coli* cells carrying plasmid were plated on LB plates containing different concentrations of the acridine dyes. After 16h incubation at the chosen temperature (37^{0}C), some colonies appeared on plates containing sub-lethal concentrations of the antibiotic and these were transferred, directly or by replica plating, on LB plates with and without tetracycline. Since equal colony counts were obtained in all cases in the presence and absence of the selecting agent and since all colonies were of normal size, we concluded from this experiment that, following exposure to acriflavine, no cell had completely lost its plasmid without concomitant loss of its viability. Under our experimental conditions, growth in various concentrations of acridine orange(Figure 1) and acriflavine(Figure 2) led to the cure of plasmid carrying *E.co*li.The results clearly dem-
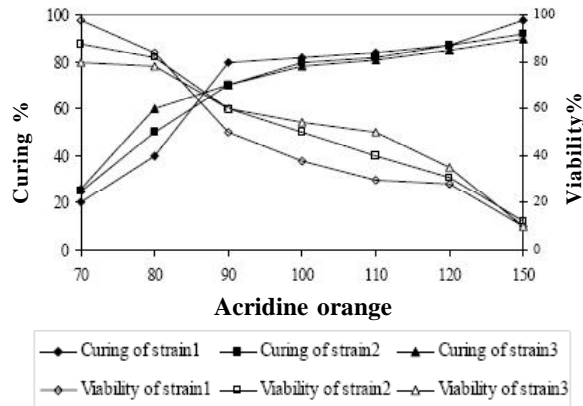
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Figure 1 : Percentage of curing and viability of clinical *E.coli* strains after treating with 70-150μg/ml of acridine orange
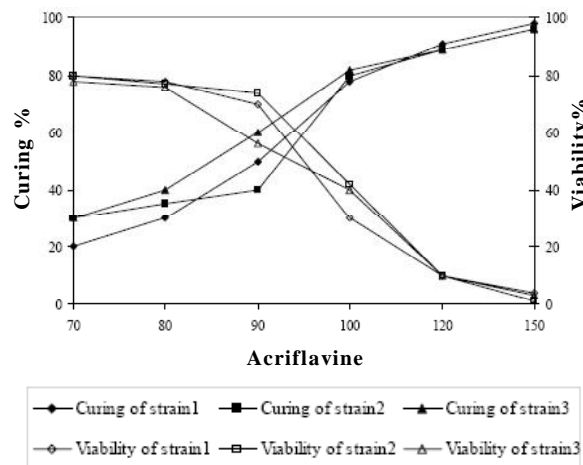

Figure 2 : Percentage of curing and viability of clinical *E.coli* strains after treating with 70-150μg/ml of acriflavine

onstrate that the dyes indeed causes a substantial, yet incomplete, loss of plasmid at 37⁰C. Higher concentrations probably have more drastic effects on plasmid loss but are accompanied by a corresponding drastic reduction of cell viability. *E.coli* DH$_5\alpha$ was transformed with the plasmid DNA preparation obtained from clinical strains. Transformed colonies that were resistant to tetracycline were obtained. Having established that the clinical strains under study had acquired plasmid-borne antibiotic resistance, the efficiency of resistance in curing the strain was investigated and compared to that of acridine orange and acriflavine. The procedure is relatively simple and it can also be applied when the target plasmid does not carry any phenotypically detectable marker genes. The method seems to be applicable in different bacteria and strains.

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