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Original Research Article

Transformation of plasmid DNA in environmental *Escherichia coli* isolates without $CaCl_2$ induced competence

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A B S T R A C T

It is suggested that natural transformability of all forms of bacteria including archaebacteria has evolved early in phylogeny. In this study, the natural transformability of environmental isolates of *E. coli* without artificially inducing cell competent with $CaCl_2$ treatment was investigated. About 60 *E. coli* isolates were obtained from the water bodies of Jahangirnagar University, Bangladesh. Among them 12 isolates were found sensitive to ampicillin and 03 of them were selected for this study. Transformation experiments were carried out with both $CaCl_2$ treated competent cells and non-competent cells of environmental and laboratory strains of *E. coli* using ampicillin resistant pUC18 plasmid DNA at log phase and stationary phase of growth. Two of the three isolates (Eco-22, Eco-31) were found transformable after $CaCl_2$ induced competence in the log phase. On the other hand, in natural transformation assays without $CaCl_2$ competence, transformants were obtained only in log phase of Eco-31 at low frequency ($5.5x10^{-7}$), but not in strain Eco-22. In stationary phase of growth none of these strains showed transformability with and without $CaCl_2$ induced competence.

K e y w o r d s

Natural transformation, Plasmid DNA, *Escherichia coli*, Competent state

Introduction

Like other physiological processes natural transformation is normally shown in a wide range of bacteria (Stewart, 1989). Natural genetic transformation of bacteria is characterized by the uptake of free extracellular DNA by a recipient and incorporation it into its genetic information (Lorenz and Wackernagel; 1994). A new phenotype is produced by this process (Baur *et al.*, 1996). In natural transformation, competent stage is encoded by cell's genes.

On the other hand, in artificial transformation cells are made competent by physical, chemical or enzymatic manipulation (Stewart, 1989). In most of natural environment bacterial cells are continually encountered by different environmental stress, including nutrient limitation, varying pH and temperature and, in soil by fluctuations of available water (Lorenz and Wackernagel, 1994). These factors make them vulnerable to transformation.

Although horizontal gene transfer can occur by various means, transformation is assumed to play a major role in the spread of antibiotic resistance resulting from the extensive use and abuse of antibiotic for different purposes. Transfer of plasmid-encoded resistance to antimicrobial agents and spread of virulence genes from one bacterial strain to another, which leads to a new antibiotic resistant phenotype, is a significant public health concern. The likelihood of spread of resistance and virulence genes in natural habitats has fascinated much attention for scientific studies (Stewart, 1992). In developing countries like Bangladesh, a significant proportion of diarrheal cases is caused by *Escherichia coli*, which may obtain and spread virulent genes in the environment through the natural transformation. The sediment in alluvial flood plain together with high population density and poor hygiene and sanitation conditions can provide a suitable condition for natural transformation. Pond and river sediments also hold great possibility to contain high concentrations of extracellular DNA because of very high microbial load. Although there are many reports on the presence of highly pathogenic and drug resistant *E. coli* in our environment, the possibility of natural transformation to occur in these habitats has not yet been studied well. The aim of this study was to assess the transformability of natural strains of *E. coli* by plasmid DNA under different transformation conditions and compare their transformation frequency with a laboratory strain.

The information that will be gained through this study may help to recognize the environmental risk factors causing widespread transfer of harmful bacterial genes related to human disease or genes that determine antibiotic resistance in bacteria. This knowledge may also help to lessen spread of harmful (including toxic chemicals and heavy metal resistance) genes in the environment by such mechanism.

Materials and Methods

Bacterial strains

Ampicillin-sensitive environmental isolates of *Escherichia coli* from different aquatic sources of Jahangirnagar University, Bangladesh and a laboratory strain of *E. coli* K12 DH5α were used in this study. After dilution of the sample, repeated streaking on MacConkey and nutrient agar plates was done for isolation of the samples. In order to confirm the identity of the test organisms, which were suspected to be *E. coli,* Gram-staining was performed and different cultural and biochemical characteristics were observed according to standard procedure (Gerhardt, 1981).Isolated colonies from a plate were patched onto nutrient agar containing ampicillin (50µg/ml) by sterile tooth picks. In a single plate 50 colonies were patched by placing a numbered grid with 50 sectors under the plate. After patching, plates were incubated overnight at 37°C. After incubation, the ampicillin sensitive organisms were determined by observing clear section on the nutrient agar plates containing ampicillin (50µg/ml).

Source of Plasmid DNA

Plasmid pUC18 (obtained from icrdd,b, Dhaka) encoding resistance to ampicillin was used as transforming vector in this study. Plasmid DNA extraction was carried out using the Birnboim and Doly method (Birnboim and Doly; 1979). Extracted plasmid was analyzed by electrophoresis on 0.7% agarose gel. After staining the gel with ethidium bromide, it was examined in a UV transilluminator.

Transformation assay

Standard protocol of $CaCl_2$ induced transformation was used for transformation of plasmid DNA in *E. coli* strains according to Sambrook *et al.* (1989) before this, the transforming plasmid DNA was extracted from a laboratory strain and stored it at -4°C. The bacterial strains, subjected for transformation (both natural and artificial), was inoculated into 20ml LB in 250ml flask, then shacked at 37°C for 3 hours for log phase and overnight for stationary phase. Cells were harvested when visual examination (compared with McFarland standard solution) revealed that approximately a density of 10^8cfu / ml has been attained for late log phase and 10^{10}cfu/ml for stationary phase. The content was divided into two equal parts and centrifuged at 3,000 rpm for 10 minutes. Supernatant was discarded and pellet was suspended in 2ml of 50mM $CaCl_2$ and kept in the freeze at 4°C for 30 minutes. 200µl cell suspension was taken in each of two microcentrifuge tubes next day. pUC18 DNA was added in one tube for treatment and in another tube small amount of PBS (no DNA) was added, which is used as a control. Tubes were kept at ice for 30 minutes then heat shocked at 42°C for 2 minutes. After this, tubes were doused in ice bath for 2 minutes. Cells were transferred in 10 ml LB and shook at 37°C for 1h in water bath. Finally, these were centrifuged and spreaded on ampicillian containing nutrient agar plates. The plates were incubated at 37°C and observed for natural transformation (without $CaCl_2$ induced) assay, all the steps were identical except the use of ice-cold $CaCl_2$ solution

Confirmation of transformation

Putative transformant colonies (i.e. ampicillin resistant colonies) were picked randomly from each selection plate and sub-cultured on nutrient plate containing ampicillin (50 µg / ml) and streaked on MacConkey agar media. The presence of transforming plasmid in the putative transformants was examined by isolating plasmid DNA by the method of plasmid extraction. The isolated extracts were electrophoresed in 0.7% agarose gel stained with ethidium bromide. The gel was analyzed in an UV-transilluminator and was photographed using a digital camera. For all transformation procedure same conditions and identical processes were carried out to make the results of different experiments comparable.

Results and Discussion

According to the results of the antibiotic sensitivity test and biochemical tests from 60 environmental *Escherichia coli* pond isolates, three were taken for the study. All the three isolates were sensitive to antibiotics ampicillin.

Plasmid profile analysis revealed that both *E. coli* pond isolates- Eco-22 and Eco-31 do not contain any plasmid. Eco-27 contains multicopy of plasmid (Fig. 1). The plasmids are different in mobility and hence in size. It appears to be close to plasmid pUC18. The yield of plasmid pUC18 was used as transforming DNA for our experiments.

Both competent and non-competent cells of log phase and stationary phase were used in transformation. No ampicillin resistant colony (transformants) was found in stationary phase. Two of the three isolates (Eco-22, Eco-31) were found transformable after $CaCl_2$ induced competence in the log phase, but their transformation frequencies were relatively lower than laboratory strain, DH5α (Table 1). On other hand, in natural transformation assays without $CaCl_2$

competence, transformants were obtained only in log phase of Eco-31 at low frequency ($5.5x10^{-7}$), but not in strain Eco-22 (Table 1).

Transformation was confirmed by plasmid isolation from transformants and electrophoresis of purified DNA. For validation of the experiment a control (pUC18 DNA) and the DNA of recipient simultaneously ran in adjacent lanes. In Figure 3 offerings the plasmid profile of transformants and control, which were recovered from transformants of non-competent.

A present, antibiotic resistance has been a tremendous health problem. To get rid of this problem it is important to understand its evolution. Lack of data about the transformation of resistance gene has hindered our effort to do it. In the study, we examined whether transformation of *Escherichia coli* occurs in a natural environment. Due to lack of natural settings, it is difficult to assay transformation in laboratory condition. For this, it is required to set an alternative condition close to the environmental parameters. Many studies had been carried out under these alternative conditions such as sand or marine sediments (Frischer *et al.,* 1990; Stewart and Sinigalliano, 1990). The lack of reliable longitudinal data sets of the competence development in natural bacterial populations is mainly due to experimental difficulties (Nielsen *et al.,* 1997) To the limit of our knowledge a very few studies have been performed without $CaCl_2$ induced transformation, which is very similar to natural condition. Since several transformable bacteria develop competence during late exponential or stationary phase, it seems that these bacteria can also express competence when entering similar growth phases under natural conditions (Bertolla *et al.,* 1997; Nielsen *et al.,* 1997).

We selected four different *E. coli* strains for our experiment- three are natural wild type *E. coli* isolates from pond water and the other is laboratory strain of *E. coli* K12 DH5α. Both competent and non-competent log phase and stationary phase cells were used in transformation. No ampicillin resistant colony (transformants) was found in stationary phase. Thus we can say transformation did not occur in stationary phase. As population of logarithmic phase cells are more active in metabolic activity and other physiological characteristics, there is more chance of up taking the plasmid DNA at this stage compared to stationary phase when cells might produce different types of inhibitory metabolites.

Natural transformation was found in laboratory strain of *E. coli* K12 DH5α and one environmental isolate (Eco-31) without $CaCl_2$ competence. On the other hand, transformation was found in other natural *E. coli* isolate (Eco-22) only when cells were pretreated with $CaCl_2$. Transformation frequencies of both competent and non-competent cells appeared to be high in laboratory strain *E. coli* K12 DH5α (Table 1). No transformants were found in case of wild type *E. coli* pond isolate Eco-27.There might be various reasons behind this. Outer cell wall perhaps is the main barrier of natural transformation in natural isolates of *E. coli*. As wild type *E. coli* strains usually contain very complex cell wall where lipopolysaccharide coated outer membrane surrounds the underlying layer of peptidoglycan, which might be impermeable to plasmid. On the other hand, the cell wall of laboratory strain *E. coli* K12 DH5α is highly modified that probably contains little of cell wall material which offers little resistance to entry of extracellular plasmid DNA.

Table.1 Transformation frequency of laboratory strain and environmental isolates at log phase

Used Strain		Used plasmid	No. of cells used in transformation (cfu/ml)	No. of transformant	Transformation frequency
DH5α K12	Competent	pUC18	10^8	500	$5.0x10^{-6}$
	Non-competent			150	$1.5x10^{-6}$
Eco-31	Competent	pUC18	10^8	110	$1.1x10^{-6}$
	Non-competent			55	$5.5x10^{-7}$
Eco-22	Competent	pUC18	10^8	25	$2.5x10^{-7}$
	Non-competent			0	0
Eco-27	Competent	pUC18	10^8	0	0
	Non-competent			0	0

*Transformation frequencies are expressed as number of transformant colonies relative to the total number of cells plated.

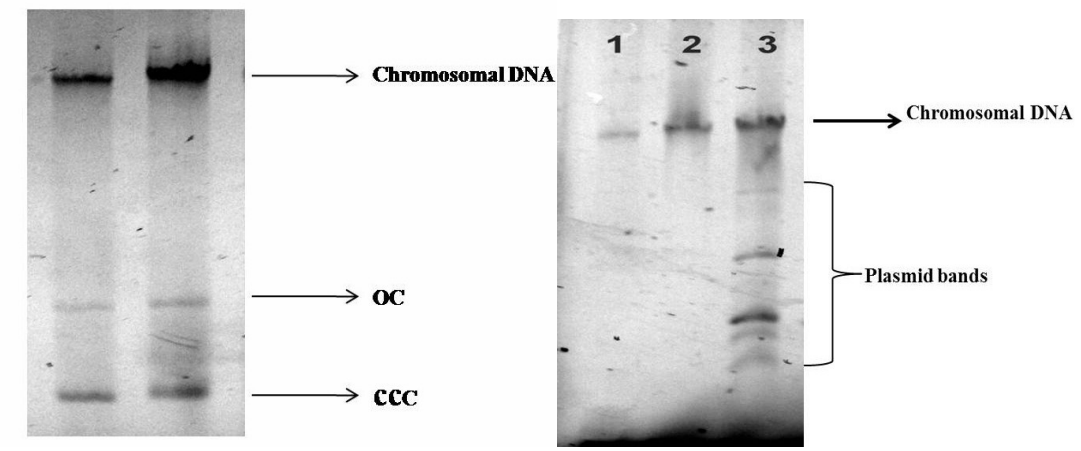

Figure.1 Agarose gel electrophoresis pattern of plasmid extract of *E. coli* pond isolates (left) which were subjected to transformation. Lane 1 Eco-31; Lane 2 Eco-22; Lane 3 Eco-27. Agarose gel electrophoresis profile of plasmid DNA preparation from *E. coli* K-12 DH5α carrying plasmid pUC18 (right). Here, OC= open circular; CCC=covalently closed circle.

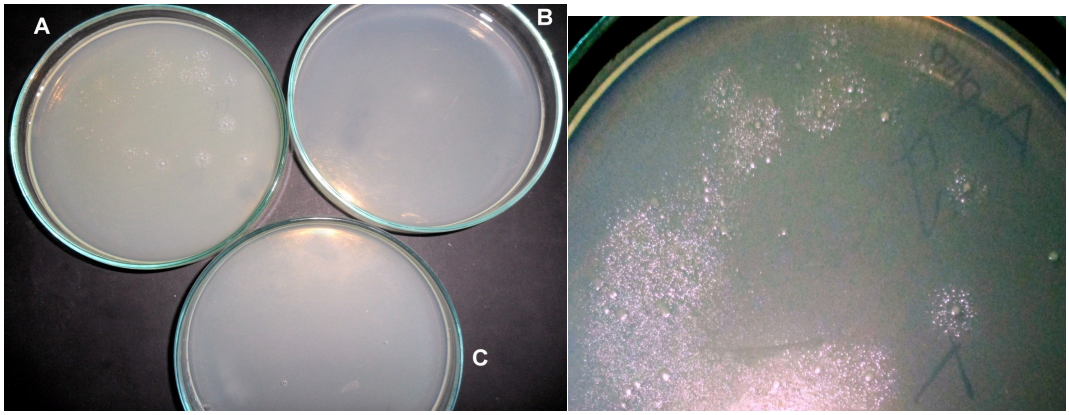

Figure.2 Putative transformants of Eco-22 strain (log phase without $CaCl_2$ induction) on nutrient agar plate containing 50µg/ml ampicillin (left). Here, A= competent plate, B= non competent plate and C= control plate (no plasmid DNA). Photograph showing individual transformants. Some microcolonies appeared around each large transformant colony (right).

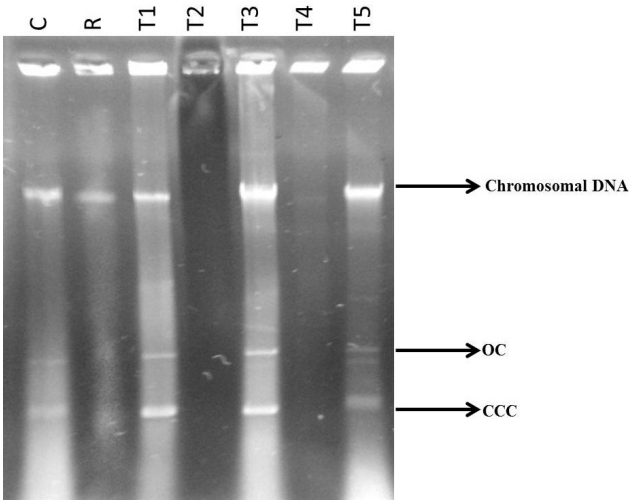

Figure.3 Agarose gel electrophoretic pattern of plasmid DNA extract of *E. coli* pond isolate transformants. Here, C= Control (pUC plasmid), R= Recipient (pond isolate), T_1-T_5=Transformants, OC= Open circular, and CCC=Covalently closed circle.

Moreover, plasmid profile analysis revealed that wild type *E. coli* pond isolates (Eco-27) had already harbored plasmid of nearly equal molecular size with pUC18 in their structure. In some situations two plasmids with nearly equal molecular size are unable to coexist in the same host as they compete with each other for the same host replication apparatus leading to loss of one or the other. This plasmid incompatibility might act as a barrier in transformation of plasmid pUC18 in the *E. coli* pond isolate (Eco-27). Some of the putative transformants were also obtained with no plasmid band in them though they were ampicillin resistant (Figure 3). So their ampicillin resistance might be due to spontaneous

mutation or due to chromosomal integration of gene responsible for ampicillin resistance. When the plates containing *E. coli* transformants were kept for more than one day in the incubator, some microcolonies appeared around each large transformant colony (Figure 2). It happened because transformed bacteria secrete β-lactamase that inactivates ampicillin present in medium. All transformants did not allow microcolony development perhaps because of inefficient secretion of β-lactamase.

Natural transformation of some common enteric bacteria has been studied in sterile sediment by Jahan *et al.* (1999) But natural transformation in environmental isolate of *E. coli* in such habitats has not been reported in Bangladesh. The result of this study appears to be a useful demonstration of natural transformation in an environmental isolate of *E. coli.*

Different use patterns of antimicrobial agents are expected to have some effect on the distribution of antimicrobial resistance phenotypes. The occurrence of antimicrobial resistant bacteria presents a major hazard to public health, because it lessens the effectiveness of antimicrobial treatment, which leads to increased morbidity, mortality and healthcare expenses. The main cause is the abuses of antimicrobial agent in human and animal medicine, as well as in aquaculture and agriculture. Environmental *E. coli* isolates from aquatic sources show lower extent of antibiotic resistance than other enteric bacteria like *Salmonella, Shigella* and *Vibrio* spp. Limited transformability of *E. coli* is the main cause of this, which will be an area that may be of interest to study. To fully identify the possible correlation of plasmid transformability and extent of antibiotic resistance in aquatic environmental *E. coli* isolates further studies are necessary.

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