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In Situ Genetic Transformation Of Bacteria In Fresh Water Ponds And In Beaker Microcosms

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ABSTRACT

First and second order correlation of receptor responses in twin pairs of distinct groups of receptors by pairs of successive time-quanta of about 0.04 seconds, constitute vision's luminosity channel. A response is due to one or more light quantum absorptions in a time-quantum. A group consists of 1 R or G cone and 0-100 rods. The retinal twin units contain the three gain controls of the system. Action potentials from the myocardium produce the time-quanta. The extra cellular fluid, blood, and glia cells conduct them to the sensory organs and the motor systems. The small involuntary eye tremor scans the retinal image of the environment in synchrony with the time-quanta. The limits of perceptual hyperfunctions for time and space correspond to the free space between adjacent groups and the rise time of the action potentials. Two or more light quantum absorptions in a time-quantum in a receptor elicit a color signal. The B cone produces a color signal already for any quantum absorption. These signals fill the perceptive products of the luminosity channel with color. In anomalous color vision, some twin units have different cones instead of equal ones.

KEYWORDS

Plasmid;
Aquatic environment;
Mg^{2+};
EDTA;
Transformation;
Bacteria.

FULL PAPER

INTRODUCTION

Gene transfer in bacteria in natural ecosystems has gained much attention with the growing concern about the fate of genetically engineered microorganisms released into the environment. Natural competence in bacteria is a physiological state which permits the uptake of exogenous DNA. Natural competence can be differentiated from artificial competence, the latter resulting from physicochemical treatments which force the uptake of the transforming DNA. Strains of many genera of gram-positive and gram negative bacteria are known to develop natural competence. There have been a few reviews dealing with genetic transformation, competence development and DNA uptake processes[4,5]. The development of genetic competence is usually monitored by the detection of transformants. Competence is physiologically regulated and inducible in naturally transformable bacteria[19]. The problem with studies of natural competence development is finding the relevant environmental parameters which trigger this induction. Lorenz and Wackernagel[8] have reviewed the known parameters. The bacteria have to be metabolically active, and a shift to unbalanced growth, e.g., by nutrient limitation, can trigger competence development in many gram-negative bacteria. For example, azotobacter vinelandii, a typical soil bacterium, is best transformable after growth in minimal medium[15] while acinetobacter calcoaceticus, as ubiquitous opportunistic human pathogen found on the skin develops competence in complex as well as minimal media[16]. Metal ions, such as Ca^{2+} and Mg^{2+}, are key elements for competence induction. Ca^{2+} (1 mM) were shown to be necessary for cell growth and competence induction in Streptococcus pneumoniae[20]. Ca^{2+} (0.5 to 1 mM) gave optimal competence development and Mg^{2+} was required for the transformation of A. vinelandii[21].

In situ experiments are necessary to prove the naturally occurring transform ation in aquatic environment. Natural transformation studies in aquatic ecosystems are rare. Hence the present work was undertaken to study the occurrence of natural plasmid transfer using a shuttle plasmid pWH1520 on gram negative and gram positive bacteria such as *Escherichia coli, Pseudomonas putida, Bacillus megaterium* and *Bacillus subtilis.*

MATERIALS AND METHODS

Bacterial strains and plasmid DNA

Bacterial strains used in this study were *E.coli* DH5α, *P.putida* KT 2440, *B.megaterium* BM 80 and *B.subtilis* VT 1660. They were obtained from School of Biotechnology, Madurai Kamaraj University, Madurai, India and *E.coli* XLB 1 bearing a shuttle plasmid pWH1520 (Amp r, tet r in gram negative organism and tet r in gram positive organism) obtained from School of Biological Sciences, Madurai Kamaraj University, Madurai, India.

Media and antibiotics

Bacterial strains were grown in Luria Bertani medium. The bacterial strain harboring plasmid pWH1520 was selected by supplementing antibiotics viz, Ampicillin, tetracycline. The broth cultures were grown at 370C in a rotary shaker platform at the aeration speed of 150-200 rpm. Agar cultures were grown by incubating the plates at 37°C for 16hr.

Experimental design

The transformation experiment was carried out in situ, in pond water in Kadavur and in Sellur and was also done in beaker microcosm in the laboratory [23]. The plasmid pWH1520 was extracted from *E.coli* XLB 1 following alkaline denaturation method[18]. The recipient cells were grown to log phase from a single colony. Cells in a particular growth phase were harvested and washed once with sterile distilled water. The freshly prepared plasmid DNA (1 μg) was mixed with the cells (5 x 105 cfu/ml) thus prepared and coated onto nitrocellulose filters. One filter was incubated in situ in the pond directly for 24 hr. Water samples were collected from the same ponds and used as a beaker microcosm for seeding another filter under laboratory conditions. After incubation, the filters were recovered and the bacterial cells were resuspended in sterile water under aseptic conditions and were plated on to antibiotic supplemented agar. The plates were incubated at 37°C, and the transformants were scored after 16 hr. Parameters such as, age of culture, pH of water, addition of EDTA and magnesium ions on transformation were

also studied.

Natural transformation

The nitrocellulose filters coated with a mixture of plasmid DNA and bacterial cells were incubated separately in immersed condition in pond water both in situ and in beaker microcosm in polythene bags. The environmental conditions of the pond water at Majagram in Kadavur were temperature 28°C and pH 7.4 and the pond water at Sellur recorded a temperature of 29°C and the pH was at 6.6. The whole set ups were incubated for 24 hr. After incubation, the nitrocellulose filters were taken out of the bags aseptically. They were cut into pieces and were put in four separate tubes with 1ml of sterile water. The cells were resuspended from the filters by vortexing and aliquots of transformed cultures were plated onto the ampicillin and tetracycline supplemented media for *E.coli* and *P.putida* and tetracycline supplemented medium for *B.subtilis* and *B.megaterium*. The plates were incubated at 37°C, and the transformants were scored after 16 hr.

Age of bacterial culture

Bacterial strains were grown to different phases, after optical densities were measured at 600nm. Appropriate volumes were filtered, mixed with 1µg of plasmid DNA and were coated on to different nitrocellulose membranes. They were incubated in situ at the pond in Majagram and in the beaker microcosm for 24 hr. Then the cells were recovered from the nitrocellulose membranes by briefly vortexing. Aliquots of the recovered cells were plated on to selective agar.

Effect of addition of magnesium ions

The cells were cultured to mid log phase, harvested and coated on to nitrocellulose membranes as described above. To the beaker microcosm set up concentrations of 5 and 10 mM of Mg^{+2} were added. They were prepared by dissolving appropriate quantities of magnesium chloride to get a stock of 100mM. After 24 hr of incubation, the cells were recovered and resuspended in sterile distilled water and aliquots were plated on to selective agar.

Effect of addition EDTA

The cells were cultured to mid log phase, harvested and coated on to nitrocellulose membranes as in the previous experiment. To the beaker microcosm set up, concentration of 5 and 10 mM of EDTA was added. After 24 hr of incubation, the cells were harvested and suspended in sterile distilled water and aliquots were plated onto selective agar.

RESULTS

Effect of pH on plasmid transfer

The number of transformants obtained in experiments carried out at different natural locations such as., Kadavur pond and Sellur pond with different pH ranges showed that Kadavur pond with pH 7.4 gave higher frequency of natural transformation than Sellur pond with acidic pH 6.6 (TABLE 1, Figures 1 and 2).

Effect of growth phase of culture on plasmid transfer

TABLE 1: Number of transformants obtained in both Sellur pond (pH 6.6) and Kadavur pond (pH 7.4) and the beaker microcosm (n=6)

Recipient organisms	*In situ*		Beaker microcosm	
	Sellur	Kadavur	Sellur	Kadavur
E coli	164	174	173	182
P putida	187	211	195	216
B.subtilis	197	213	205	221
B megaterium	184	206	201	227

(In control plates there was no colonies were observed)

When different phases of bacterial cultures such as early, mid, late log phases and stationary phase, were used. The number of transformants scored for mid log phase of *E.coli* and *P.putida* were 213 and 174 respectively, was higher than any other phase in these organisms. Whereas, for gram positive organisms such as, *B.subtilis* and *B.megaterium* the maximum number of transformants scored was in the late log phase, viz., 268 and 256 for *B.subtilis* and *B.megaterium* respectively (Figures 3 and 4).

Effect of Mg^{2+} ion concentration on plasmid transfer

FULL PAPER

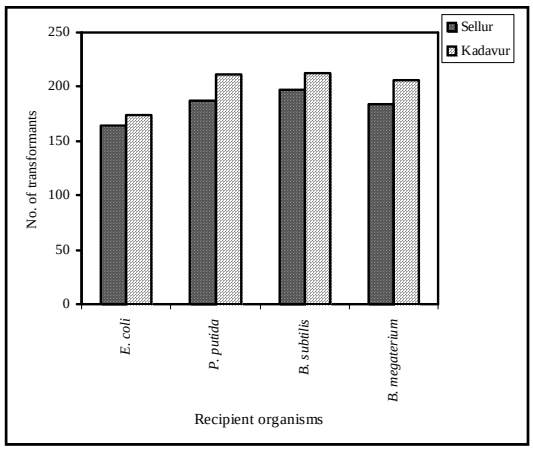

Figure 1: Number of transformants obtained both in Sellur pond (pH 6.6) and in Kadavur pond (pH 7.4)

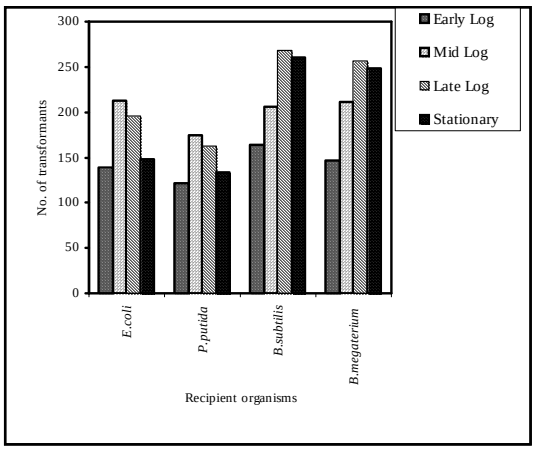

Figure 3: Effect of growth phase of culture on *in situ* transformation in pond ecosystem

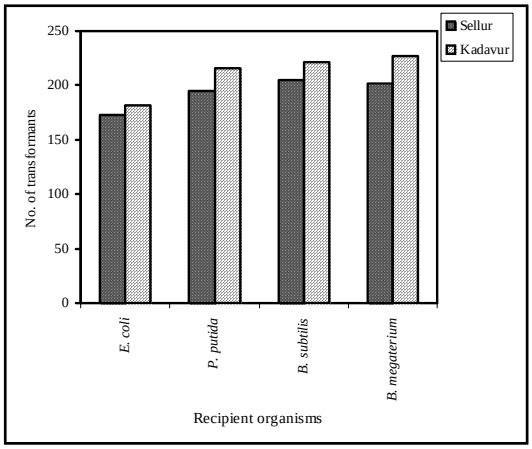

Figure 2: Number of transformants obtained in beaker microcosm using pond water collected both from Sellur pond (pH 6.6) and Kadavur pond (pH 7.4)

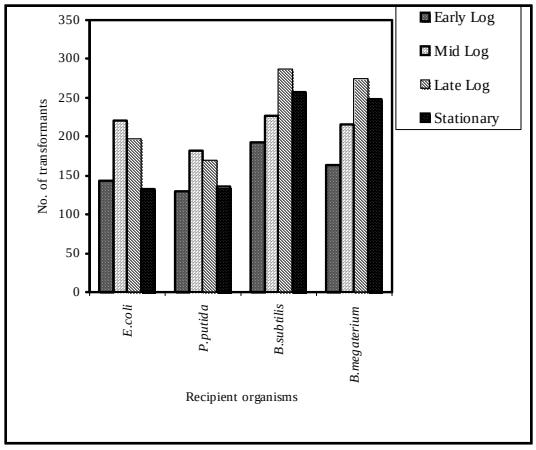

Figure 4: Effect of growth phase of culture on transformation in beaker microcosm

With the addition of magnesium ions, the plasmid transfer frequency increased up to 3 times. *E.coli* gave the maximum number of transformants viz., 819 and 910 at 5 and 10 mM of Mg^{2+} ions respectively (Figure 5).

Effect of EDTA on plasmid transfer

Addition of EDTA drastically reduced the number of transformants. The maximum number of transformants was scored for *B.subtilis* viz., 33 and 21 at 5 and 10 mM respectively (Figure 6).

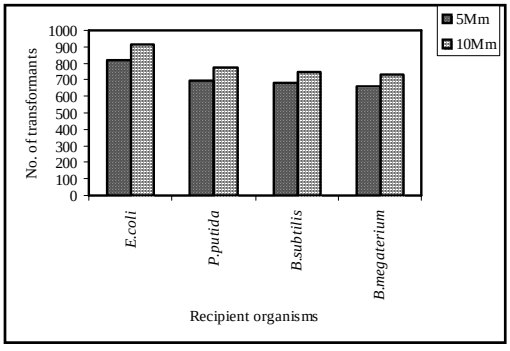

Figure 5: Effect of Mg^{2+} ion concentration on the transformation of pWH1520 in beaker microcosm

DISCUSSION

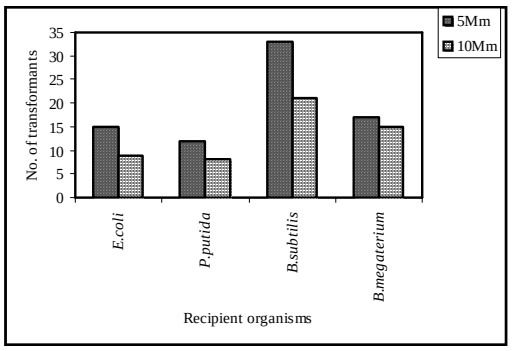

Figure 6: Effect of EDTA ion concentration on the transformation of pWH1520 in beaker microcosm

Until recently, *E.coli* was thought to be unable to develop natural competence, i.e., genetic transformation could be achieved only artificially with the aid of nonphysiological concentrations of calcium ions or by other treatments. The idea that the induction of competence in *E.coli* is physiologically but not physicochemically regulated was put forward by Reusch et al.[17]. Our study also confirmed the existence of the natural competence of *E.coli* in aquatic environment.

The present study reveals that bacterial transformation occurs in pond water. The frequency of plasmid transformation in natural ecosystems and in the beaker microcosms was comparable. The number of transformants obtained for the experiment conducted at Kadavur pond was higher than that of the Sellur pond. The reason for this could be due to the water pH. The Kadavur pond had a pH about 7.4 and the Sellur pond had a pH of about 6.6. This was in accordance with previous natural transformation studies with *E.coli*. Where there was an increase in competence with increase in pH[1]. The reason for this could be due to the fact that competent factor was more active at alkaline pH, even though it was constitutively expressed[8], irrespective of pH conditions. Moreover, our results also agree with the fact that *E.coli* can develop genetic competence under environmental conditions when in contact with surface water originating from calcareous regions. Calcium plays an important role as a regulatory mediator in prokaryotes[12,13]. Calcium concentrations above 1 mM, which are often found in spring water and river water, are sufficient to make *E.coli* cells competent. Our results on the age of culture and transformation frequency also corroborate the fact.

Growth phase of culture also plays a central role in the competence development[6]. Surface factors like c, com e, com f etc are expressed at different times in different bacteria. These factors are important for transformation. Bacillus strains exhibits maximum competence in the late log phase whereas, gram negative bacteria exhibits maximum competence in the late log phase. This could be due to differential temporal expression of these receptors in different bacteria. The development of competence in *E.coli* is coincident with de novo synthesis and incorporation of poly-beta-hydroxybutyrate (PHB) into the cytoplasmic membranes. In 1995, it is also known that the genetic competence of *E.coli* requires PHB-calcium polyphosphate complexes[7].

Addition of Mg^{2+} ions to the beaker microcosm increases the transformation efficiency. The number of transformants scored was up to 3 times more than the normal number. The DNA uptake requires Mg^{2+} ions and other monovalent ions. The Mg^{2+} ions are required for the translocation of the DNA in to the cytoplasmic membrane. Some bacteria produce transformasomes which are vesicles on the cell surface. The adherence to the transformasomes may require Mg^{2+} ions. The monovalent ions like Na^{+} in the medium can help the plasmid DNA to achieve a DNase resistant state. Metal ions, such as Ca^{2+} and Mg^{2+}, are key elements for competence induction. Ca^{2+} (1 mM) was shown to be necessary for cell growth and competence induction in *Streptococcus pneumoniae*[20]. Ca^{2+} (0.5 to 1 mM) gave optimal competence development and Mg^{2+} was required for the transformation of *A.vinelandii*[15,11]. Morris et al.[12] reported that the intracellular calcium concentration in *E.coli* is tightly regulated at 0.1 mM, a level similar to that in eukaryotic cells. A lot of evidence suggests the role of calcium in the regulation of its cell cycle. The artificial transformation of *E.coli* by incubation in highly concentrated solutions of $CaCl_2$ is well known[2,10,11]. These facts suggested the possibility of natural competence development of *E.coli* during incubation in natural water samples originating from calcareous regions (rivers, springs, and mineral waters) which contained different Ca^{2+} concen-

FULL PAPER

trations. It is evident from the present study that *E.coli* (laboratory strains) becomes competent for transformation with pWH1520 plasmid DNA under conditions which prevail in certain natural aquatic ecosystems and that the competence is maintained for several weeks in resting cells.

Addition of EDTA decreases the transformation drastically. EDTA binds to divalent metal ions, like Ca^{2+}, Mg^{2+} etc. these divalent metal ions play an important role in transformation. As discussed earlier, Mg^{2+} ions are required for the translocation of DNA. There are specific Ca^{2+} transporters which are important for competence. Mutants lacking Ca^{2+} transporters cannot be transformed[20]. Therefore in natural environment, Ca^{2+} induces competence in bacteria. It is also understood that the transformation related surface structure requires calcium ions for assembly. Hence addition of EDTA severely impairs transformation. With similar experimental conditions, *B.subtilis* has the highest number of transformants in spite of the addition of EDTA. This indicates that among the 4 bacterial strains used for this study, *B.subtilis* was the most competent bacteria that possessed natural competency. Our results proved that transformation does occur in natural aquatic environments such as pond ecosystems. Moreover, several factors like water pH, the age of the bacterium and the metallic ion concentration in water affect the frequency of plasmid transformation. Almost all bacteria are naturally transformable at one stage or the other[3]. Therefore the bacteria can uptake DNA and propagate its genetic information either when DNA is present in dissolved state or when it is associated with particulate matter or with other living cells[22]. The continual release of DNA by bacterial populations and their relatively long persistence in the environment provides extra cellular gene pool in the habitat in spite of the ubiquitous presence of DNases[8].

The transfer of genetic material in natural environments such as in pond water is useful for the bacteria in developing of genetic diversity and it provides the raw material for natural selection and consequent evolution of bacteria.

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