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Initial Stage of DNA-Electrotransfer into *E. coli* Cells[1]

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The mechanism of electrotransfer of DNA into *Escherichia coli* cells was investigated under conditions optimal for genetic transformation or transfection. Simple mixing in 10% polyethylene glycol 6000 did not cause binding of DNA to the recipient bacteria. When subjected to a high electric field, however, 90-98% of the input plasmid or phage DNAs were complexed with the cells. By application of the electric field, a significant amount of biotin-labeled DNA was bound onto the recipient surface, as detected by fluorescein isothiocyanate-coupled avidin. When subjected to a high voltage pulse, DNA molecules were rapidly attracted toward the anode. Concurrently, the electric field induced the orientation of bacterial cells, along the field lines and their movement toward the anode. Since the bacterial movement was relatively slow, a substantial fraction of DNA molecules must strike the cathode-facing end or side of the recipient cells. Irrespective of the high efficiency of DNA transformation, the voltage pulse did not induce release of alkaline phosphate and β-galactosidase. The electrotransferred DNA first remained sensitive to Tris-EDTA treatment, and became refractory to spheroplasting only after incubation at 37°C. These results indicate that the infecting DNA is electrophoretically plugged to the outer membrane loosened by the voltage pulse.

Key words: DNA transformation, *E. coli* cells, electrotransfer, voltage pulse.

In spite of extensive application of electro-transformation and -transfection to analyses of structure and function of nucleic acids, as well as to genetic cloning (*1-4*), the mechanism underlying electrotransfer of DNA into living cells remains to be fully elucidated. Thus, opinions differ on whether binding of DNA to the cell surface is prerequisite for electrotransfer of DNA (*5-7*). And, it is not settled whether transfecting DNA molecules penetrate freely into cells through putative pore (*8, 9*) or are driven by electrophoresis (*7, 10*), electroosmosis (*11*), lateral diffusion (*5*), or actively transported by cellular metabolism (*12*). In addition, differences in electric parameters, recipient cell species (mammalian cells *vs.* Gram-negative bacteria), and nucleic acid molecules (minute plasmids *vs.* huge composite DNA) complicate comparison of the results obtained from each system.

During transfection of *Escherichia coli* and *Bacillus subtilis* with phage- or plasmid-DNA, we frequently observed conspicuous crowding of the bacterial cells on the anode surface of the electroporation cuvette, when high field strength (17.5 kV/cm) was applied in deionized and distilled water (ddH_2O). This fact indicates that even the bacterial rods are negatively charged in ddH_2O and electrostatically driven to the anode. Based on this observation and previous experimental results (*7, 13*), we have inferred that nucleic acid molecules are rapidly attracted toward the anode, and that en route they collide with bacterial cells and are inserted into an envelope of the cathode-facing ends or sides of the cells loosened by local Joule heating. The present study was undertaken to delineate each step of electrotransfer of DNA to *E. coli* cells.

MATERIALS AND METHODS

Bacteria and DNA—Strain C of *E. coli* was used as the recipient. The bacterial cells were grown at 37°C with shaking in LB medium supplemented with 5 mM $MgCl_2$. The growth was monitored and the cells were harvested as previously described (*13*). For transformation experiments, superhelical pBR322 DNA was used, whereas double-stranded replication form I DNA (RF) of ϕX174 or α3 was employed for transfection. DNA-propidium iodide complex was prepared by incubating 10 μg/ml RF with 500 μg/ml propidium iodide (Calbiochem) in 8 mM Tris-HCl (pH 7.5) at 4°C for 24 h, followed by filtration through a Millipore microfilter (UFCP3TK50). Biotin-labeled pBR322 was prepared using a nick translation kit (United States Biochem.) and biotin-16-dUTP (Boehringer), and purified by centrifugal filtration with SUPREC-02 (Takara Shuzo, Kyoto).

Electroporation—The cells were washed three times with, and suspended in, chilled ddH_2O at a density of about 10^{11} cells/ml. Fifty microliters of the suspension was mixed first with 10 μl of DNA (usually 1 μg/ml, in 10 mM Tris-1 mM EDTA, pH 7.5), then with 50 μl of chilled 20% polyethylene glycol (PEG)6000. An aliquot (usually 50 μl) of this mixture was pipetted into a chilled Bio-Rad electroporation cuvette (interelectrode distance = 0.1 cm) and subjected to a single voltage shock, using a Gene Pulser™ apparatus (set voltage, 1.75 kV; initial field strength, 17.5 kV/cm; resistor, 600 Ω; set capacitance, 25 μF). Immediately after the pulse, 150 μl of LB medium was added and

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the sample was incubated at 37°C for 10 min (for transfection) or 30 min (for transformation), diluted, then plated (*14*). Mean survival of the bacteria after the electroporation procedure was about 50%.

DNA-Binding and Enzyme Release—For determination of the amount of DNA bound to *E. coli*, the DNA recipient-cell mixture was centrifuged at 10,000 rpm for 3 min at 4°C, and the infectivity remaining in the supernatant was determined by the standard electroporation method described above, while the pellet was resuspended in 10% PEG 6000, diluted, incubated for 10 or 30 min at 37°C, then plated. Binding of biotin-labeled pBR322 was studied as follows. Immediately after the pulse, 150 μl of 25 μg/ml avidin-fluoresceine isothiocyanate (Boehringer) in 10 mM Tris-HCl (pH 7.2) was added into the bacteria-DNA mixture in PEG 6000, followed by incubation at 37°C for 30 min. The mixture was diluted 50-fold into DABCO solution composed of 5% 1,4-diazabicyclo[2.2.2]octane, 50% glycerol, and 10 mM Tris-HCl (pH 8.0) and put onto a film of 1.5% agarose on a slide glass. This was covered with a coverglass, sealed, and the mounted sample was analyzed using an Olympus fluorescence microscope AH2-QRF equipped with a Hewlett Packard 9000 series computer and soft ware. When release of the marker enzymes and UV absorbing materials was to be analyzed, centrifugation was performed at 14,000 rpm for 10 min at 4°C. UV absorbing materials, alkaline phosphatase and β-galactosidase were assayed as described previously (*15, 16*). Electric resistance of the DNA-cell mixture was measured using a built-in ohmmeter in a Shimazu GTE-10 apparatus.

Movement of DNA and Bacteria—The propidium iodide (PI)-labeled RFI (9.1 μg/ml) was mixed with PEG 6000 and subjected to a voltage pulse at 17.5 kV/cm (resistance: 600 Ω; capacitance: 3 or 25 μF) in a Bio-Rad electroporation cuvette (interelectrode distance: 0.1 cm), then rapidly photographed under UV illumination from the upper side of the cuvette, using a Polaroid MP-4 apparatus. Densitometric analysis of the photographs was carried out with a computerized image processing system composed of an image scanner (GT-6000, Epson, Tokyo), an Apple Macintosh Quadra 800, and NIH Image software developed by W. Rasband at the U.S. National Institutes of Health. Cellular movement in the presence or absence of DNA was similarly analyzed directly after electroporation, but without UV illumination.

Orientation of the bacterial cells was investigated as follows: Washed cells of *E. coli* C were electroporated in 0.32% low-melting agarose (Nippongene, Toyama) under the conditions of 17.5 kV/cm, 3 or 25 μF, 600 Ω, 40°C, and immediately ice-cooled. (Even after this treatment, about 50% of the input bacteria retained colony-forming ability.) The solidified gel was placed on a coverglass and microphotographed with Olympus PM-10AD system attached to an Olympus microscope IMT-2. Fifty-four films were examined to compute orientation of the bacterial cells. The principle is outlined in Fig. 5.

Others—Treatment with 2,4-dinitrophenol (DNP), sodium azide, or dicyclohexylcarbodiimide (DCCD) was performed as follows: the electroporated sample was mixed at 0°C with each inhibitor (final concentration, 5 mM). When DCCD dissolved in dimethylsulfoxide was used, the solvent was added to the control. After 10 min, each mixture was incubated at 37°C for 10 min, chilled, and subjected to Tris-EDTA treatment. For the Tris-EDTA treatment or plasmolysis, the electroporated mixture was incubated at 0 or 37°C for 10 min (followed by chilling at 0°C), centrifuged in the cold, suspended in chilled 50 mM Tris-5 mM EDTA (pH 7.5) or 20% sucrose-0.1 M Tris-HCl buffer (pH 8.0), and kept at 0°C for 10 min. When the cells were to be converted into spheroplasts, lysozyme (200 μg/ml) and EDTA (2.5 mM) were added to the sucrose-Tris buffer.

RESULTS

Some Properties of the Electrotransfer System—It is essential for analysis of electrotransfer mechanisms to use a system with high efficiency and/or frequency of DNA transformation (transfection). Under our standard conditions, the transforming efficiency of pBR322 was $\sim 10^{10}/\mu g$ (Fig. 1), and the frequency (number of transformants/total colony-forming units) was $3\text{-}4 \times 10^{-2}$. When, however, the input DNA was increased to 9 μg/ml, the frequency reached $8\text{-}9 \times 10^{-1}$. At DNA concentration of 18 μg/ml, nearly all colony-formers were transformants. In subsequent experiments, the standard electric and cellular conditions were employed, unless otherwise specified.

Electrotransfer of DNA without Preadsorption onto the Recipient Cells—In a previous paper (*7*), it was suggested that a free DNA molecule is required for the electrotransfer, because addition of Mg^{2+}, which facilitates binding of DNA onto the bacterial surface (*17*), is rather detrimental to the voltage-mediated transfection. Under the present conditions, the amount of DNA adsorbed onto the recipient cells was negligibly small when a voltage pulse was not

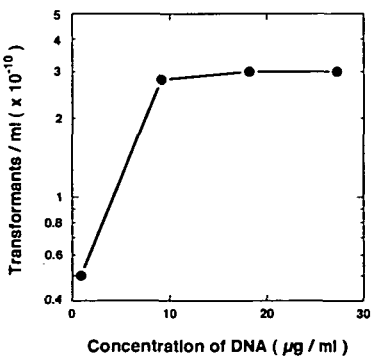

Fig. 1. **Yield of transformants at the higher DNA concentrations.** Indicated amounts of pBR322 were mixed with *E. coli* C cells (8.3×10^{10}/ml) in 10% polyethylene glycol 6000 at 0°C, then subjected to a voltage pulse under the standard conditions. Cellular viability after the pulse was ~42%, and transformation frequency at a DNA concentration of 9.1 μg/ml was 86%.

TABLE I. **Voltage-dependent association of transforming DNA with cells.** Cells of *E. coli* C and pBR322 DNA were mixed in chilled 10% polyethylene glycol, divided into two portions and subjected to a voltage pulse or kept at 0°C. Each mixture was centrifuged and transforming units in the pellet were determined after resuspension in LB. The DNA remaining in the supernatant was assayed by standard electrotransformation method.

Condition	Transforming units per ml		
	Supernatant	Pellet	Supernatant/pellet
Without pulse	2.9×10^5	1.0×10^5	2.9×10^3
With pulse	7.3×10^6	2.6×10^8	2.8×10^{-2}

applied. When pBR322 (or RFI DNA of ϕX174 or α3) and *E. coli* C cells were mixed at 0°C in 10% PEG 6000 and centrifuged, the transforming (or transfecting) activity was recovered exclusively from the supernatant (Table I). After application of a high voltage pulse, however, almost all of the activity was transferred to the bacterial pellet fraction.

Similar results were obtained by fluorescence labeling of DNA before and after electroporation (Fig. 2). The fraction of the cells with the higher fluorescence intensity (caused by binding of avidin-fluoresceine isothiocyanate) significantly increased, upon electroporation of the recipient bacteria in the presence of biotin-labeled pBR322 DNA. Without electroporation or in the absence of DNA, the cellular fluorescence intensity remained low.

Rapid Movement of DNA Toward Anode—By application of an electric field strength of 100 V/cm, ϕX174 RFI DNA intercalated with propidium iodide (PI) migrated, through 0.3% agarose, to reach the anode of the electroporation cuvette (interelectrode distance = 0.1 cm) within 1 min. This electric field and other conditions employed in conventional electrophoresis of DNA are, however, remote from those of electrotransfection (electrotransformation). When the field strength was increased to 17.5 kV/cm and 10% PEG was used, as in the standard electrotransfer experiments, a significant amount of the PI-intercalated DNA moved toward the anode before the time constant (τ) reached 1.6 ms (Fig. 3). Prolongation of τ to 11.3 ms resulted in accumulation of a large portion of the DNA around the anode.

Movement of Bacterial Cells—As mentioned in introduction, we have frequently observed macroscopic crowding of the cells of *E. coli* and *B. subtilis* around the anode after application of a voltage pulse (field strength, 17.5 kV/cm; τ, 11.4 ms; in ddH_2O). A short pulse (τ = 1.6 ms) of high field strength hardly affected the distribution of the bacteria (Fig. 3). When τ reached 11.3 ms, the cellular distribution distinctly shifted to the anode side, indicating that the bacterial cells are negatively charged in 10% PEG as in ddH_2O, and electrostatically attracted to the anode, though the rate of bacterial movement is considerably lower than that of the DNA molecules. The cellular mobility was not particularly influenced by addition of the ϕX174 RF or pBR322 DNA.

Application of high field strength causes electric dipole moment and rotation of *E. coli* cells (*18, 19*). In addition, electric pulling of the bacteria through liquid is expected to promote cellular orientation, to minimize hydrodynamic resistance. In order to quantify the population of oriented bacteria, cells of *E. coli* C were subjected to a voltage pulse in 0.32% low-melting agarose at 40°C and rapidly solidified as detailed in "MATERIALS AND METHODS." Fraction of the cells oriented parallel to the electric field direction was about 20% when τ was 1.2 ms, and 65% at 9.5 ms (Fig. 4). These figures are probably underestimated, because random micromovement of cells might occur in the several seconds from the end of the discharge until solidification of the gel. Moreover, bacterial orientation may occur more

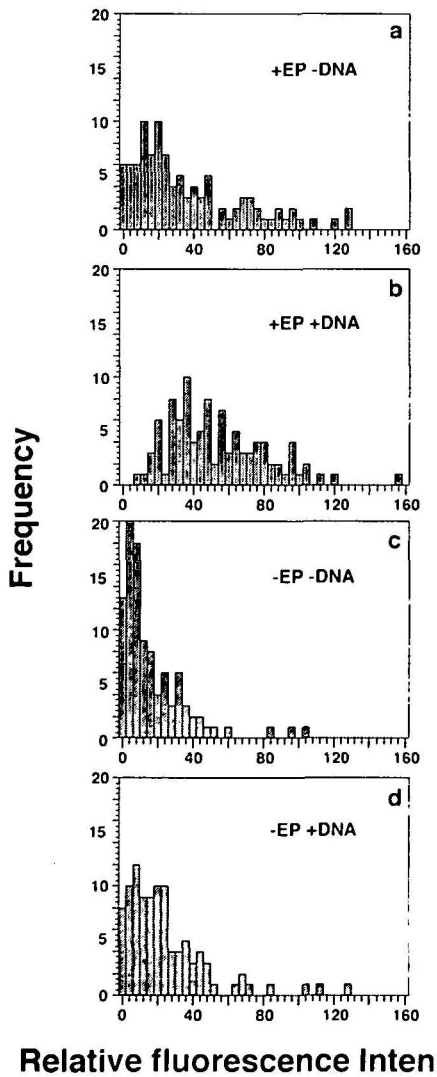

Fig. 2. **DNA-cell association as detected by fluorescence labeling.** Cells of *E. coli* C were subjected to a voltage pulse in the absence (a) or presence (b) of biotin-labeled pBR322 and incubated with avidin-FITC. FITC intensity of the cells was then analyzed under a fluorescence microscope. Cells mock-pulsed in the absence (c) or presence (d) of DNA were similarly treated as controls.

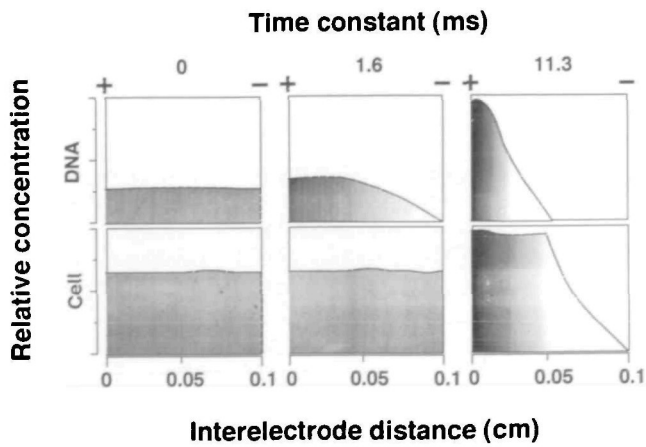

Fig. 3. **Effect of pulse duration on the movement of DNA and cells.** The RFI molecules (intercalated with PI) or cells of *E. coli* C were exposed to a voltage pulse at 17.5 kV/cm in a 0.1-cm cuvette. Other conditions were as specified in "MATERIALS AND METHODS."

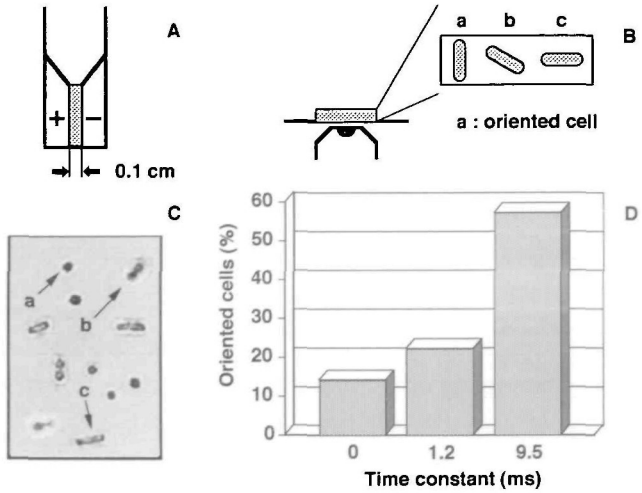

Fig. 4. **Voltage-induced orientation of the bacterial cells.** Cells of *E. coli* C were exposed to an electric field strength of 17.5 kV/cm at 40°C in melted agarose and promptly solidified by cooling. After microphotography, cellular orientation was analyzed.

easily in 10% PEG 6000 or ddH_2O than in the melted agarose.

Release of Cellular Constituents—Electroporation, like Ca^{2+} treatment, causes leakage of a small amount of UV-absorbing materials from the cells of *E. coli*: the amount (determined by optical density at 260 nm) increased about fivefold, upon application of the standard voltage pulse. These substances were soluble in cold 0.5 N perchloric acid, indicating that their molecular weights were low. In addition, some electrolytes were released by electroporation, as evidenced by small decrease in electrical resistance of the cell-DNA mixture (from 1.3 to 1.0 kΩ). Moreover, permeation of orthonitrophenyl-β-galactoside (estimated by cytoplasmic β-galactosidase activity) was increased twofold by the pulse. The cellular permeability to this chromogenic compound was 1.5 times higher in the treated cells at 20 s after the pulse. The presence of DNA during the pulse neither promoted nor inhibited the permeation of orthonitrophenyl-β-galactoside. On the other hand, alkaline phosphatase (periplasmic marker enzyme released by conversion of cells into spheroplasts or by osmotic shock) and β-galactosidase (cytoplasmic marker enzyme liberated by cell lysis) were not released upon the voltage pulse. It is thus evident that, irrespective of the efficient transfer of infective DNA and RNA, the voltage pulse inflicts injury that makes the membrane permeable to low-molecular-weight substances, but does not form pores that allow release of proteins and nucleic acids.

Localization of the Electrotransfected DNA in the Cells—The yield of the transfectants were not affected by addition of DNase, to the recipient-α3 RFI complex within 30 s after the voltage pulse (unpublished observation). It is, however, unknown whether the DNA penetrates into the outer membrane, periplasmic space or cytoplasm upon the pulse. In subsequent experiments, the yield of transfectants, rather than recovery of radioactivity or fluorescence, was followed directly in order to exclude nonspecific or abortive uptake of DNA. The "electroporated" bacteria were sensitive to such treatments as centrifugation and/washing (unpublished observation), and this vulnerability

TABLE II. **Effect of various treatments on the plaque yield of *E. coli* electrotransfected with RFI DNA or voltage-pulsed after α3 infection.** In Exp. I, cells of strain C were electrotransfected with α3 RFI as described in "MATERIALS AND METHODS." In Exp. II, the bacteria were infected with α3 phage at a multiplicity of 0.05 at 37°C for 5 min in LB supplemented with 5 mM $CaCl_2$, chilled, washed and exposed to a voltage pulse under the standard conditions. The bacterial suspensions were incubated for 10 min at 0 or 37°C with or without metabolic inhibitor (5 mM) then subjected to each specified treatment at 0°C.

Preincubation at/with		Treatment	Relative plaque yield	
			Exp. I Electrotransfected	Exp. II Phage-infected
0°C	None	None	1	1
	None	Tris-EDTA	7.9×10^{-3}	3.5×10^{-2}
	None	Plasmolysis	1.2×10^{-4}	—
	None	Spheroplasting	5.6×10^{-4}	—
37°C	None	None	1	1
	DNP	None	1.3	—
	NaN_3	None	9.1×10^{-1}	—
	None	Tris-EDTA	2.7×10^{-1}	1.9×10^{-1}
	DNP	Tris-EDTA	3.3×10^{-1}	—
	NaN_3	Tris-EDTA	2.8×10^{-1}	—
	DCCD	None	2.3×10^{-3}	5.2×10^{-2}
	DCCD	Tris-EDTA	1.5×10^{-4}	3.6×10^{-3}
	None	Plasmolysis	1.1×10^{-1}	1.8×10^{-1}
	None	Spheroplasting	1.0×10^{-1}	1.8×10^{-1}

is a major obstacle to interpretation of the experimental results. Therefore, the cells preinfected with α3 phage were subjected to the electric pulse and used as the control. Upon the voltage pulse, plaque yield of the phage-infected cells was reduced to ~44%, but this was sufficiently high for the present purpose. When the pulsed bacteria were treated with chilled 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA, the plaque yield was drastically reduced (to less than 0.8% of the untreated control), whereas that of the phage-infected cells was decreased to 1.8-3.5% (Table II). Incubation at 37°C for 10 min before the Tris-EDTA treatment conspicuously increased the yield of the transfectants (to 27% of the untreated control). The presence of 5 mM DNP or sodium azide during the incubation did not affect the recovery of the transfectants. Although incubation with DCCD resulted in severe reduction in the transfecting efficiency, subsequent treatment with Tris-EDTA did not particularly reduce the infectivity yield, as compared with the phage-infected cells.

A minor fraction of the cells electrotransfected with α3 RFI produced plaques, even after plasmolysis and spheroplasting. Upon incubation at 37°C for 10 min, this fraction reached the level comparable to that in the phage-infected bacteria (Table II). Insensitivity to the spheroplasting step strongly suggests penetration of the viral DNA into the cytoplasm or at least into the inner membrane.

DISCUSSION

Under the present conditions, association between DNA and *E. coli* does not take place unless a voltage pulse is applied. Preadsorption of DNA onto the bacterial cell is thus not essential for its effective transfer to the recipient. Although Mg^{2+} caused extensive adsorption of DNA to *E. coli* cells (*17*), the efficiency of electrotransfection was rather decreased by addition of the cation (*7*). It seems likely that Mg^{2+} mediates binding of DNA to nonspecific

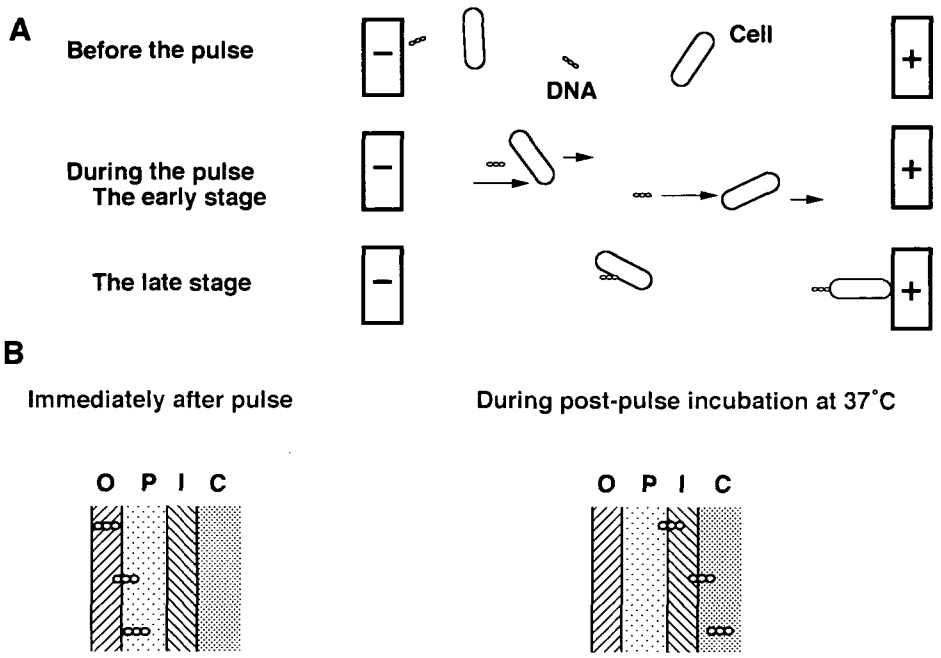

Fig. 5. **Processes of the electrotransfer of DNA.** Before the electric pulse, DNA and cells were randomly distributed between the electrodes. During the early stage of the pulse, DNA and Cells were oriented and drawn toward the anode. Owing to their smaller size, DNA molecules moved much faster than the bacteria. At the late stage of the pulse, DNA struck the cathode-facing side or end of the cell and was inserted into the outer membrane (O) or driven further into the periplasm (P). During the subsequent incubation at 37°C, DNA penetrated through the inner membrane (I) into cytoplasm (C).

sites on the cell surface, and a very limited fraction of the bound DNA is exposed to converged electric field lines.

The present experiments demonstrate that both DNA molecules and the cells of *E. coli* are drawn toward the anode upon application of a high electric field. As *E. coli* cells are bacillar, the flow, in addition to the electric dipole moment, ought to cause orientation of the bacteria to reduce hydrodynamic resistance. Microscopic analysis indeed showed that at least 65% of the bacteria were positioned parallel to the electric field a few seconds after the voltage pulse. Bacterial orientation due to electric dipole moment (*18, 19*) has been observed by Eynard *et al.* (*12*) under mild pulsing conditions (at 2–2.7 kV/cm, lasting 7 ms). In the present experiments of DNA transfer (the field strength is 17.5 kV/cm and τ is about 10 ms), orientation of the recipient bacteria may be determined by combination of the hydrodynamic resistance and the dipole moment. Despite the rapid cellular orientation, it is not known whether DNA collides exclusively with the cathode-facing end or cap (*7, 12*). The bacterial density is so high and DNA is so rapidly drawn electrically that collision may occasionally occur before the cellular orientation is complete (Fig. 5A). It is natural that DNA molecules also undergo rapid orientation parallel to the electric field (*20*).

Very little is known about the transport mechanism after collision of DNA with the recipient cells. It is unlikely that DNA freely penetrates into cytoplasm through a putative pore, whether driven by electrophoresis or electroosmosis. Such a pore would inevitably cause release of enzymes and lysis of *E. coli*, because of the high turgor pressure of the bacterial cytoplasm. Although the electric pulse induced leakage of some electrolytes and UV-absorbing substances, neither alkaline phosphatase nor β-galactosidase was released into the surrounding medium. The voltage pulse probably has a dual effect: rapid driving of DNA toward the anode and permeabilization of the surface of the recipient. Localized melting of the membrane lipid by Joule heating may also contribute to the membrane loosening. High efficiency of the electrotransfer is due to specificity. Thus, the very site where DNA strikes is permeabilized almost simultaneously with the collision. After partial insertion into the outer membrane, DNA may be transferred into the cytoplasm during subsequent incubation of the recipient cell at 37°C (*12, 13, 24*), as in the Ca^{2+}-dependent transfection (*17, 23*). The marked sensitivity of the pulsed cell-DNA complex to Tris-EDTA treatment, which is known to remove about 50% of lipopolysaccharide from *E. coli* outer membrane (*21*), indicates surface localization of the electrotransferred DNA at this stage. By subsequent incubation at 37°C, this complex became refractory to spheroplasting as well as to Tris-EDTA treatment, indicating penetration of the DNA into the inner membrane or cytoplasm (Fig. 5B). This translocation is no longer voltage-dependent, and it is insensitive to such metabolic inhibitors as DNP, azide or DCCD. The transition from membrane lipid crystal to liquid might be involved in the temperature-dependent penetration process. The primary barrier to free nucleic acid is the bacterial outer membrane, whereas cytoplasmic membrane is relatively permeable, as evidenced by the sensitivity of spheroplasts to transfecting DNA or RNA. The latter stage of DNA transfer, penetration through the cytoplasmic membrane, is dependent on temperature and may be common to the three systems, *i.e.*, electrotransfer, Ca^{2+} treatment (*22, 23*) and spheroplast transfection.

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