Electrooptics Studies of *Escherichia coli* Electropulsation: Orientation, Permeabilization, and Gene Transfer

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ABSTRACT Fast optical transient signals are suitable approaches to the investigation of the behavior of bacteria during an electric pulse. In a previous work, by a dual approach taking advantage of a video method and a fast kinetic study of the light transmitted across a cell suspension, we showed that a field-induced orientation phenomenon was affecting the rod-shaped bacteria during the pulse (Eynard et al., 1992. *Eur. J. Biochem.* 209:431–436). In the present work, time courses of electro-induced responses of bacteria during a single square-wave pulse are analyzed. Observations of both the orientation step and the permeabilization process are relevant. These two steps are affected by the addition of DNA. They both obey to a first-order kinetic. The conclusion of this work is that *Escherichia coli* permeabilization and transformation are multistep processes: orientation (step 1) is followed by an envelope alteration (step 2), all steps being affected by plasmid addition. In the case of *E. coli*, a rod-shaped bacteria, the orientation process (step 1) brings the cell parallel to the field direction. The pulse duration must be longer than the orientation characteristic time ($\approx$ 1 ms) to trigger an effective permeabilization and its associated events. The permeabilization process (step 2) is associated with a field-induced dipole effect.

INTRODUCTION

External electric fields can transiently create nonspecific cell membrane permeability (for a review see Tsong, 1991). This observation is valid for all kinds of organelles or cells, like chromaffin granules (Neumann and Rosenheck, 1972); erythrocytes (Kinosita and Tsong, 1977); mammalian cells (Zimmermann, 1982); plant protoplasts (Mehrle et al., 1985), even when a wall is present (bacteria (Hamilton and Sale, 1967); yeast (Ganeva et al., 1995a); or intact plant cells (Sabri et al., 1996)). Membrane permeabilization induced by external electric fields (i.e., electropermeabilization) occurs by a change in the membrane integrity due to the enlargement of the transmembrane potential by the external field. Several theoretical models have been proposed to explain these phenomena in pure lipid systems (Chizmadzhev et al., 1995; Kakorin et al., 1996; Weaver and Chizmadzhev, 1996). Models have been extended to cell membranes by taking into account experimental observations of electropermeabilization of lipid vesicles (Teissié and Tsong, 1981). But structural characteristics of the field-induced "transient permeation structures" remain unknown.

Nevertheless, pulsing the cells has become a convenient way to mediate direct gene transfer to recipient cells (i.e., electrotransformation). The electrical transfer of DNA was first performed by Neumann in 1982 in the case of mammalian cells (Neumann et al., 1982). This technique was observed to be valid for pulsing of intact walled systems such as bacteria (Dower et al., 1988). Our previous work has shown that the efficiency of electrotransformation of *Escherichia coli* is strongly correlated to the level of electropermeabilization (Sixou et al., 1991), although the mechanisms were different (Eynard et al., 1992). A systematic investigation of *E. coli* electrotransformation was described in a series of three papers by Tsong's group (Xie et al., 1990; Xie and Tsong, 1990, 1992). They suggested that plasmids were first absorbed to the bacterial surface before being able to cross the permeabilized envelope. It is therefore clear that fast kinetic studies of the events affecting bacteria during the pulse were needed. Using a short time resolution of the events, we previously showed that a key step was present during the pulse (Eynard et al., 1992), as observed with other walled systems such as yeast (Ganeva et al., 1995b). Conductometric measurements showed that a fast outflow of cytoplasmic ions took place during the pulse (Eynard et al., 1992). But for technical reasons, pulsing conditions were different from what is used in gene transfer experiments and were not suitable for observing the effect of plasmids.

It is well known that electrooptic methods are very well suited for kinetic studies. It was shown that the application of electric fields to dilute suspension resulted in an orientation of asymmetrical particles with their long axes along the lines of the field. Such electro-induced anisotropy of a cell suspension gave rise to electric birefringence (Kerr effect) (O'Konski and Zimm, 1950; O'Konski and Haltner, 1956), dichroism responses (Allen and Van Holde, 1971), light scattering variation (Baloch and Van De Ven, 1990), and turbidity change (Cerda et al., 1981). The first analysis of the effects of a pure electric field was made by O'Konsky and Zimm, in 1950, using either alternative current or square waves on the tobacco mosaic virus. They quantified orientation relaxation by analysis of birefringence relaxation. A number of anomalies in the Kerr effect led them to propose orienting mechanisms different from those due to

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permanent or induced dipoles, such as ion atmosphere polarization (O'Konski and Haltner, 1957).

More recently, a series of papers reported the electrooptical signals associated with lipid vesicle electropermeabilization (Kakorin et al., 1996, 1998; Neumann and Kakorin, 1996; Tönsing et al., 1997). Our conclusion was therefore that fast optical transient signals were the most suitable approach to the investigation of the behavior of bacteria during the pulse. Our previous approach showed that a field-induced orientation phenomenon was affecting the rod-shaped bacteria during the pulse (Eynard et al., 1992). This was obtained by a dual approach taking advantage of a video method, in which direct observation at the single-cell level was obtained, and by fast kinetic study of the light transmitted across a cell suspension.

In the present work, time courses of electro-induced responses of bacteria during a single square wave pulse were analyzed. Studies were done in the range of nonpermeabilizing to permeabilizing field intensities as determined by ATP leakage measurements. The effect of DNA addition was observed. Results showed that electrooptical studies provide information not only on the orientation kinetics but also on the extent of permeabilization. This result was recently observed in an investigation about electrical breakdown of *E. coli* (Ershler et al., 1992). Conclusions of this work are at variance with our results and earlier studies (Rudd et al., 1975). However, a theoretical interpretation of this work (Khlebtsov, 1994) gave a critical analysis of the conclusions and showed that, in fact, results of Ershler and collaborators and ours lead in the same direction.

Because in *E. coli* electropermeabilization envelope resealing was a slow process and cytoplasmic leakage was present after the pulse (Eynard et al., 1992), it could not be considered a fully reversible process in the second range. As a consequence, birefringence or linear dichroism approaches in which two successive pulses under crossed polarization were needed (Kakorin et al., 1996) were not possible in the case of bacteria. In the present study, changes in turbidity gave the time constants of the events affecting the suspension. Orientation phenomena were unambigously discriminated, by parallel experiments in which the suspension behavior was monitored by video through a microscope (Eynard et al., 1992).

MATERIALS AND METHODS

Cells and plasmids

The strain of *E. coli* was CB 0129, derived from K12 (W1485 F^- $thia^-$, Leu^-, Thy^-, sup E42, deo B or C).

An aliquot of an overnight culture was diluted in fresh culture medium (LB) at an absorbance of 0.05 (650 nm). Cells were grown to an absorbance of 0.4 (650 nm) (middle log growth phase) and harvested by centrifugation. Cells were washed three times in the pulsing buffer (PB): 1 mM Tris, 270 mM sucrose (pH 7.6), resuspended at an absorbance of 10 at 650 nm (5 mg dry weight/ml), kept on ice, and used within 3 h at the suitable dilution in the same buffer.

All procedures were carried out at room temperature. A low ionic content was used to limit the pulse-associated Joule heating. The plasmid used in this study was pBR 322. The plasmid was purified by routine procedures as previously described (Sixou et al., 1991) and resuspended in pure water.

Power generator

Square wave pulses were delivered by a CNRS electropulsator (Jouan, St. Herblain, France). The voltage intensity, the pulse duration, the number, and the time between the pulses were all independently adjustable. Pulse shape was monitored by a 15-MHz oscilloscope (Enertec, St. Etienne, France). Electropulsation procedures for determination of permeabilization, video microscopy studies, or turbidity analysis are described in specific sections. In all of the work, a single pulse of 24 ms duration was used at various field strengths to analyze all events in same conditions.

Turbidity analysis

Turbidimetrics measurements were based on the perturbation of the light signal transmitted by a suspension of nonspherical cells (such as rod-like *E. coli* cells) during a single square wave electric pulse. The changes were followed on-line using a fast spectrophotometer designed by our group (Fig. 1).

The light emitted by an arc lamp (XBO 75W; Osram, Munich, Germany) with a stabilized power supply (Spotlight; Cunow, Cergy pontoise, France) was focused on the entrance slit of a monochromator (Jobin Yvon, Longjumeau, France). The wavelength was set at 650 nm. The output beam was split by a semitransparent plate. A part of the light was focused on a reference photomultiplier (model 9781; EMI, Ruislip, England) whose output signal monitored the fluctuations of the incident beam. The major part of the light was focused on a pulsing chamber. Its two walls parallel to the direction of the incident beam were made of stainless steel and were connected to the electropulser. The width between these two parallel electrodes was 4 mm. The chamber (600 μl) was filled with the bacterial suspension ($\sim 2 \times 10^8$ cells/ml). After crossing the chamber, the light was focused on a photomultiplier (EMI 9781). The signal and the reference were amplified and filtered (bandwidth up to 1 MHz). They were then balanced by the use of a differential amplifier. The resulting signal was fed into a transient recorder (Gould, Valley View, OH). Then only the change in transmitted light was recorded. The output signal was displayed on a chart recorder or stored on a microcomputer connected to a printer (Hewlett Packard, Palo Alto, CA).

Video microscopy studies

The set-up was described previously (Eynard et al., 1992). A very flat pulsing chamber was built by gluing two 0.1-mm-thick stainless steel parallel foils onto a glass slide with a 1-mm interelectrode space. The preparations were placed on the slide between the two electrodes and covered with a glass coverslip. The two electrodes were connected to the electropulsator. This chamber was set on the microscope stage (Leitz, Wetzlar, Germany), and the cell suspension was observed before, during,

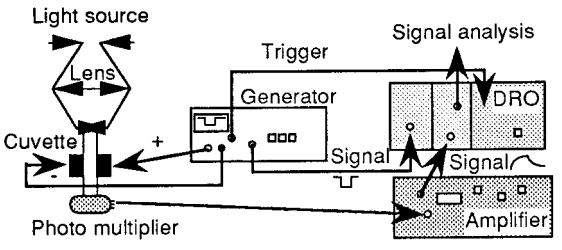

FIGURE 1 Diagram of the experimental set-up used to study the change in light transmitted by a bacterial suspension during an electric pulse. Details are given in the text.

and after the electropulsation by phase contrast under an oil immersion objective (magnitude 100 Leitz objective). A video monitoring set-up consisting of a color video camera (JVC, Yokohama, Japan) associated with a color monitor (JVC) was connected to the microscope. The cell suspension images were recorded on-line with electropulsation with a videotape recorder (Philips, Endoven, The Netherlands).

Analysis of the orientation processes was achieved by digitalization of the pictures from printouts of the screen and determination of angle (ϕ_a) between the long axis of each bacterium and the macroscopic direction of field lines (assumed to be normal to the electrodes). Angles were measured with a digitizing pad and linked to a micro computer with homemade software. For each experiment, partition of the total population in the angle classes (width 10°) was achieved, and the weighted average was calculated for each population. For each experiment, the weighted average was determined both for control (the last picture before application of the field; ϕ_a is 45°) and pulsed cells (Eynard et al., 1992).

ATP leakage

We measured the kinetics of the ATP leakage with the luciferin-luciferase (L-L) complex (Sigma, St. Louis, MO) by mixing 300 μl of pulsed bacteria diluted 25-fold with 100 μl of the L-L complex at 4 mg/ml. The light emission was measured directly in a luminometer (LKB, Turku, Finland). The detection of the luminescence intensity emitted by the luciferin-luciferase complex was a direct assay of the ATP leakage. Results were expressed in relation to the total ATP cell content, determined by disruption of the cell envelope by dimethyl sulfoxide.

THEORY

Besides the trivial Joule heating effect, the primary effect of an external field on a cell is the induction of a transmembrane potential, which is added to the resting potential difference (Bernhardt and Pauly, 1973; Neumann, 1989). The theoretical analysis made use of the Laplace equation. It was shown that the induced potential difference was controlled by the shape and size of cells, pulse duration and strength of the external field, and the conductance of the external buffer. In the case of spherical cells (mammalian cells), a rather simple expression was obtained:

$$\Delta\Psi E = 1.5g(\lambda)rE\cos\Theta$$

in which $g(\lambda)$ is a physiological factor, r is the cell radius, and E is the electric field intensity. The cosine dependence of $\Delta\Psi_E$ was experimentally shown on spherical cells (Gross et al., 1986).

For nonspherical organisms, the form factor f (1.5 above) is complicated by the nonsymmetry of the cell (Bernhardt and Pauly, 1973). Values of the generated potential difference, at the position of maximum value, depend on the shape and size of cells and on their orientation relative to the electrical field. For a long prolate cell, with three semiprincipal axes, and an axial ratio 1:1:3 (assimilated to *E. coli*), the form factor is smaller (1.12) than for spherical cells if the long axes of the cell and field are parallel, and higher (1.8) if they are perpendicular. In other words, when the cell long axis is parallel to the field lines (in a homogeneous field), the form factor is smaller, but the equivalent radius (half the long axis) is longer.

The magnitude of the induced potential difference is then controlled by the orientation of the rod relative to the field direction. But because of the induced dipole the rod is submitted to a torque and turns during the field pulse. Its orientation changes, and as a consequence the intensity of the induced potential difference changes as well. In the case of *E. coli*, where the axial ratio is (1:1:3), the value of the induced potential difference at the locus facing the electrodes, for the same field strength, is two times larger if the long axis is parallel to the field ($rf = 3 \times 1.2$), than when it is in the perpendicular orientation ($rf = 1 \times 1.8$).

RESULTS

Turbidimetry of the field effect on bacteria

In the present study, the fast kinetic spectrophotometer was used to monitor kinetics of changes in transmitted light (I_t) during a 24-ms pulse from 0 to 3.75 kV/cm. I_t depends strongly on the applied field strength (Fig. 2). As long as E is smaller than 1.25 kV/cm, a monotonic increasing phase (IP) is observed up to a plateau value (I_{max}). For higher field intensities, the plateau is followed by a transmitted light decrease (DP) down to the final transmitted light value (I_{fin}). The plateau becomes shorter with an increase in the field strength and even disappears. The amplitudes of the two parts of the signal, characterized, respectively, by I_{max} and I_{fin}, were controlled by the field strength.

Fig. 3 shows that I_{max} increases with the field strength up to 1 kV/cm, then keeps a plateau value up to 2 kV/cm and then decreases. By video monitoring, orientation is observed to be controlled by the field strength (Fig. 3), and similar behavior of the field dependences of I_{max} and ϕ_a is clearly observed. I_{max} increases with the orientation process (whereas ϕ_a decreases with orientation). Then, in the turbidity signal, IP appears to report the orientation process induced by the field on the rods. No correlation between ϕ_a and I_{fin} is present. DP appears to be associated with a mechanism other than orientation.

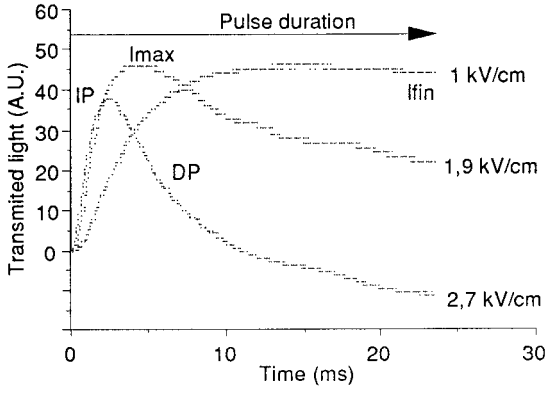

FIGURE 2 Kinetics of change in transmitted light during a 24-ms pulse at different field strengths. IP: A monotonic increase was observed up to a plateau value at 1 kV/cm. For 1.9 kV/cm, the plateau was followed by a transmitted light decrease (DP). The plateau became shorter at 2.7 kV/cm.

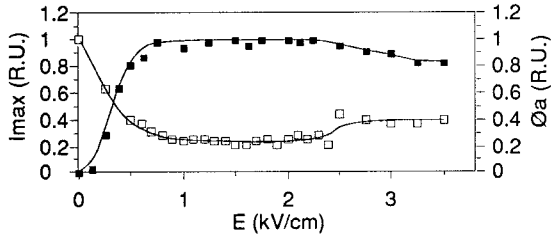

FIGURE 3 Amplitudes of I_{max} and orientation of the bacterial population (ϕ_a) as functions of the field strength. Pulsing parameters were a single pulse of 24 ms duration. I_{max} (*in black*) is the plateau value of the turbidity signal. Orientation was analyzed by measuring the angle between the field direction and the long axis of bacteria by direct video monitoring of cells. ϕ_a (*in white*) was the weight-averaged value of the orientation cell distribution.

DP can be detected only for field intensities larger than a threshold (Fig. 3, 1.25 kV/cm). Moreover, its amplitude becomes larger over 2.5 kV/cm. The existence of a threshold for DP leads us to study correlations between DP and the electropermeabilization of the bacterial suspension. ATP leakage from electropulsed bacteria depends strongly on the field strength (Fig. 4 *a*). No leakage is observed below a critical value (permeabilization threshold $E_p \sim 1$ kV/cm). Then there is a slow increase in the permeabilization between E_p and E_e (defined as the electropermeabilization expansion threshold). A sharp increase is observed above E_e. Representation of the permeabilization as a function of the reciprocal of E (Fig. 4 *b*) shows two linear adjustments, one at low field strength between 1 kV/cm (E_p) and 3 kV/cm (E_e) and another above E_e. DP evolved in the same way and in the same range as electropermeabilization; these correlations lead us to associate DP with events that change the cell envelope to the permeabilized organization.

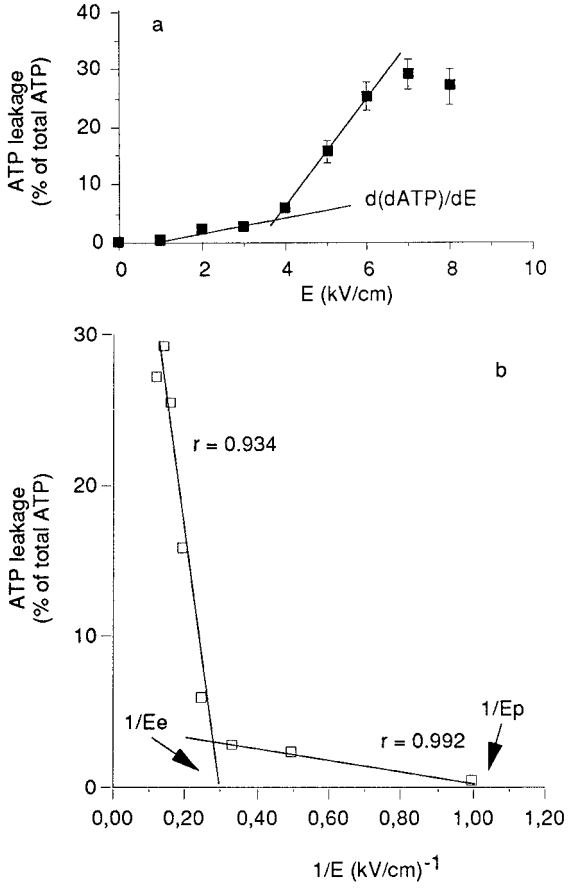

FIGURE 4 ATP leakage of *E. coli*. The pulsing parameters were a single pulse of 24 ms duration at various amplitudes. The amount of the leaked ATP was measured with the luciferin-luciferase complex. Total ATP cell content was determined after dimethyl sulfoxide disruption of the cell envelope before mixing with the L/L complex. (*a*) ATP leakage of *E. coli* as a function of field amplitude (E in kV/cm). (*b*) ATP leakage of *E. coli* as a function of the reciprocal of E.

Kinetic parameters of *E. coli* electropermeabilization

From the turbidity changes, our conclusion is that the first effect of the field is to orient the bacterium while the permeabilization is delayed to a later time after the onset of the field. This is supported by the well-established facts that the field effect on membrane is strongest on the part of the cell facing the electrodes and that it depends linearly on the length of the bacterium (Neumann, 1989). As a consequence, the highest field effect is obtained when rods are parallel to the field lines, i.e., after the orientation process.

Potential modulation and therefore permeabilization occur preferentially on the cell surface in front of electrodes (Tekle et al., 1994; Gabriel and Teissié, 1997). In the case of bacteria, modulation of the membrane potential starts during the orientation phase. The two phenomena are in competition. Our hypothesis is that during the orientation of rods, the cell surface offered to permeabilization changes continuously; therefore, no efficient permeabilization can be observed. Permeabilization can be effective only at the end of orientation, when the rod has a fixed position parallel to the field.

These observations allow us to describe the orientation and permeabilization of *E. coli* as a sequential model:

A	→	B	→	C
Intact bacteria		Oriented bacteria		Permeabilized bacteria

Turbidity changes were mathematically analyzed using the kinetic scheme derived from our model and described in the Appendix. Two rate constants, k_1 and k_2, are obtained; k_1 is relative to the orientation process, and k_2 is relative to the permeabilization process. The dependence of the two rate constants on E was analyzed (Fig. 5).

At low field strength (below E_p), k_1 is small (below 250 s^{-1}), orientation is slow, and bacteria are parallel to the field lines only for long pulse duration ($1/k_1 = 4$ ms). At these field ranges, k_2 is close to 0, as predicted by the definition of E_p as the threshold for permeabilization event.

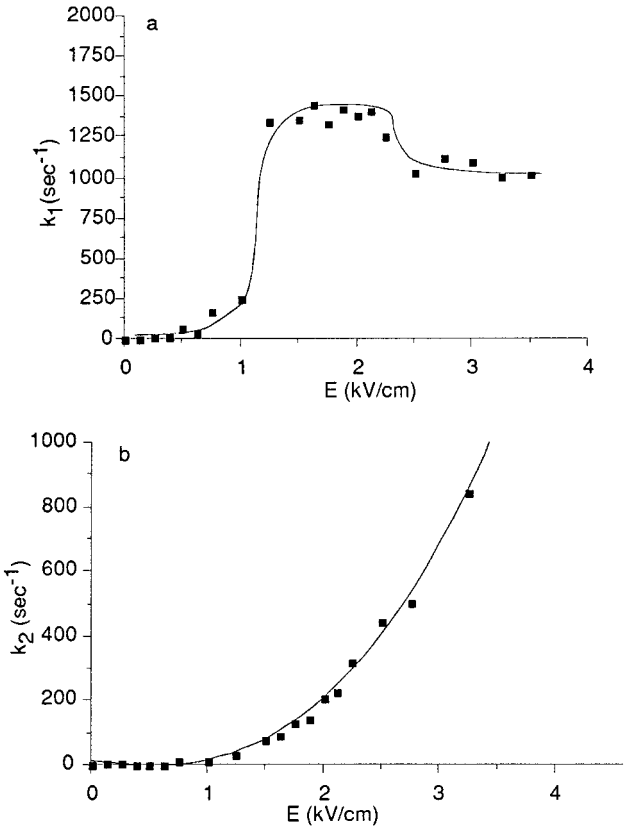

FIGURE 5 Rate constants k_1 and k_2 as functions of electric field strengths. Pulsing parameters were a single pulse of 24 ms duration. (*a*) k_1 is relative to the orientation process. (*b*) k_2 is relative to the permeabilization process. Both are obtained by the kinetic scheme described in the Appendix.

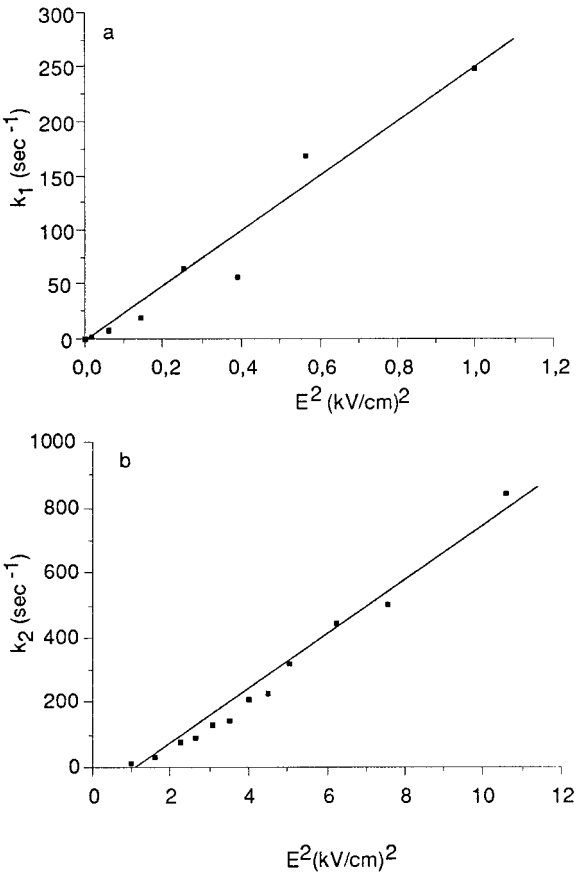

FIGURE 6 Rate constants as functions of the square of the field strengths. (*a*) k_1 is plotted for values below *Ee*. (*b*) k_2 is plotted for values larger than *Ep*.

For field strengths between the two thresholds (E_p and E_e), k_1 is constant at $\sim$1400 s^{-1}, whereas k_2 increases up to 400 s^{-1}, while remaining smaller than k_1. In this field strength range, ATP leakage is small (less than 10% of the maximum leak; Fig. 4 *a*) and occurs in a bacterial population with its long axes oriented parallel to field lines. No plasmid entry is detected (Sixou et al., 1991).

Beyond E_e, k_1 decreases and reaches a plateau value of $\sim$1000 s^{-1}, whereas k_2 increases sharply. In this field amplitude range permeabilization becomes efficient for small molecules (more than 15% of total ATP leakage; Fig. 4 *a*) and for plasmid transfer (Sixou et al., 1991).

k_1 and k_2 are not linearly dependent on the electric field strength. Below E_p, k_1 is a linear function of the square of the field intensity (Fig. 6 *a*), in agreement with theoretical prediction for a phenomenon driven by the electric field (Neumann, 1989). Above E_p, k_2 is a linear function of the square of the field intensity (Fig. 6 *b*). The permeabilization expansion appears as a field effect on an induced dipole on the rod by the external field.

Effect of plasmid addition on orientation and permeabilization

In gene transfer processes mediated by electropulsation, plasmids interact with the cell envelope before, during, and after the pulse. Before the pulse, plasmids are absorbed to the cell surface (Xie et al., 1990). A critical step takes place during the pulse (Eynard et al., 1992), as shown by the lack of transformants when the plasmid is added after the pulse. The translocation of the plasmid across the envelope after the pulse is rather slow (Eynard et al., 1992).

When bacteria are pulsed in a plasmid-containing buffer, no increase in ATP leakage is observed (data not shown). The permeabilization to small molecules by electropulsation is not enhanced by the plasmid, although it is observed that the inflow of macromolecules is enhanced in the case of Cos cells (Sukharev et al., 1992). The electrooptics processes are affected by plasmids. Results for a field strength of 1.75 kV/cm (i.e., between E_P and E_e) are shown for various plasmid concentrations in Fig. 7. k_1, the rate constant of the orientation process, is decreased from 1.25 ms^{-1} to 0.5 ms^{-1}. This may reflect either an increase in the buffer viscosity, resulting in an increase in the drag effect, or an absorption of plasmids to the cell envelope, increasing the volume of the body that is submitted to the electric torque. Another explanation can be the change in the induced dipole due to the surface charge neutralization associated with the DNA adsorption. When taking into account the low concentration of plasmids we used in our experiments and the

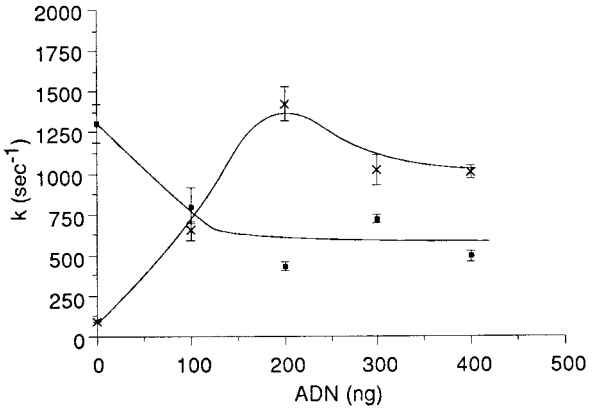

FIGURE 7 Effect of plasmid concentration on the rate constants k_1 (*black*) and k_2 (*cross*). The pulsing parameter was a single pulse of 24 ms duration at 1.75 kV/cm. The plasmid amount was varied from 0 to 400 ng in the turbidity cuvette (600 μl), which corresponds to a maximum concentration of 0.66 μg/ml. The cuvette was filled with a bacterial suspension at a concentration of 2×10^8 cells/ml. As the molecular weight of the plasmid pBR322 was 2.88×10^6 g·mol^{-1}, the ratio DNA/cell changed from 0 to 700.

saturating effect of the lowest DNA concentration on the decrease in k_1, the viscosity effect cannot explain our experimental observation. The sensitivity of k_1 to the addition of DNA is therefore in agreement with the adsorption of DNA to the cell envelope.

k_2, the rate constant of the permeabilization process, is increased from 0.14 ms^{-1} to 1.25 ms^{-1} with the plasmid addition. This is in agreement with our conclusion that a critical step in the plasmid-cell interaction takes place during the pulse (Eynard et al., 1992). But as no increase in ATP leakage is induced (see above), this means that no improvement of permeabilization is triggered. We should mention that under our experimental protocol, most of the ATP leakage occurs after, not during, the pulse (Eynard et al., 1992).

DISCUSSION

The present study shows that two events affect *E. coli* when an electric pulse is applied (i.e., orientation and permeabilization).

Kinetics of orientation and permeabilization

Investigations of kinetics of permeabilization and orientation show that these two phenomena, when present, have characteristic times in the millisecond time range and then may interact. During a single pulse of 24 ms, if the electric field amplitude is smaller than the threshold E_p, only orientation is observed. Between E_p and E_e, orientation and permeabilization events are both observed. Beyond E_e, the extension of permeabilization is very rapid.

From theoretical works on transmembrane potential induction by an external field and as shown by video imaging, the major effect is localized on the part of the cell facing the electrodes (Gabriel and Teissié, 1997). In the case of *E. coli* and elongated particles, the orientation process gives a time-dependent potential at any given position on the cell surface. Except when very strong field intensities are used, when permeabilizing potential differences are reached on a major part of the cell surface but with damaging effects on cell viability, permeabilization is not very efficient during the orientation process. It is only when the rod is parallel to the field lines that effective permeabilization takes place, specifically affecting the extremities facing the electrodes. As a consequence, the pulse duration must be longer than the orientation characteristic time ($\approx$ 1 ms) to trigger an effective permeabilization and its associated events. This observation can explain the biphasic dependence of *E. coli* permeabilization on the pulse duration. Indeed, as previously observed with eukaryotic cells (red blood cells (Riemann et al., 1975) and Chinese hamster ovary cells (Rols et al., 1990), E_p decreases with an increase in the pulse duration T (data not schown). For mammalian cells in suspension (described as spherical cells; Rols et al., 1990), the linear dependence of E_p on the reciprocal of T was observed. In the case of *E. coli*, two linear adjustments were observed, one at short pulse duration (microseconds) and the other at longer pulse duration (milliseconds) (Fig. 8). Extrapolation to infinite values of T for the two adjustments allowed the determination of two "real" threshold values, one for short pulses (E_S = 2.18 kV/cm) and one for long pulses (E_L = 1.4 kV/cm). For bacterial cells, a low efficiency of the cell permeabilization for microsecond pulses as compared to millisecond pulses is clearly present. A similar dependence on the pulse duration of the field-mediated transformation was reported previously when a low yield in transformation was obtained with microsecond pulses (Dower et al., 1988).

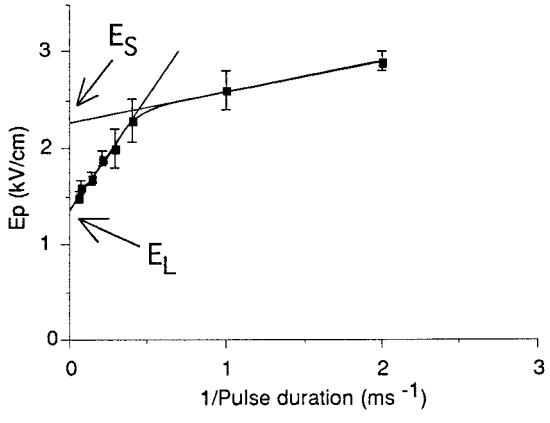

FIGURE 8 Dependence of *Ep* (threshold value for the detection of ATP leakage) on the reciprocal of *T*. Pulsing parameters were a single pulse of various durations. Experiments like the one presented in Fig. 4 *a* were carried out for each pulse duration. The threshold *Ep* was determined for each experiment and reported as a function of the pulse duration. Extrapolation for infinite values of T ($1/T = 0$) gave E_S = 2.18 kV/cm for short (microsecond) pulses, and E_L = 1.4 kV/cm for long (millisecond) pulses.

Origins of orientation and permeabilization processes

Previous works (O'Konski and Haltner, 1957) proposed different hypotheses for the physical orientation of particles in electric fields. The conformational change of molecules like DNA (Porschke et al., 1984; Müller et al., 1989) or biopolymers (Schwarz, 1977) showed that dipole induction and orientation of these dipoles by the field are responsible for numerous effects of field on macromolecules.

The rate constants of such processes depend linearly on the square of the field strength. Results in Fig. 6 show that k_1 and k_2 are functions of the square of field amplitude.

For the orientation process, the theoretical predictions and previous results predicted that orientation of a rod in an electric field was due to the induction of dipoles at the cell surface followed by the orientation of these dipoles. Our results observed in this experiment showed that, for *E. coli*, experiments agree with the theoretical prediction. For fields higher than 1 kV/cm, the plateau value observed for k_1 versus E represents the saturation of the induced dipoles at the bacterial envelope surface (it should be noted that this saturation occurred for fields that induced permeabilization).

The change in turbidity during the permeabilization process can be associated with several phenomena when taking into account the physics of light scattering. This depends on the relative index of the cell envelope versus the buffer and on the shape of the bacteria when the orientation of the rod-shaped bacteria, i.e., *E. coli*, is fixed as it is at the end of the orientation process monitored by IP. The refractive index changes because of the cytoplasmic leakage and the induced change in ionic content of the buffer. The shape may be affected by the electric forces when taking into account the elasticity of the wall (Doyle and Marquis, 1994). The occurrence of these two phenomena is supported by our observation of a loss of contrast under the microscope.

A new result is that the permeabilization process (step 2) is associated with a field-induced dipole effect. We cannot resolve the origin of this process, but we can list several hypotheses:

1. A change in the refractive index of the medium surrounding the cell due to the leakage of cytoplasm content. This was shown in our previous study on the conductance of the suspension during the pulse (Eynard et al., 1992) and in studies with erythrocytes (Kinosita and Tsong, 1979) or liposomes (Neumann and Kakorin, 1996).
2. A change in the refractive index of the cell envelope.
3. A polarization of the counterion atmosphere (O'Konski and Haltner, 1957). Indeed, the cell surface is associated with a thin layer of counterions that gives a reorganization of permanent dipoles or the induction of dipoles. This is known to play a role in the induction of the transmembrane potential.
4. A swelling of the bacteria as described in the case of erythrocytes (Kinosita and Tsong, 1979), even if the presence of the external membrane increases the rigidity of the bacterial cell.

All of these hypotheses have to be taken into account to explain our experimental observation: a field-induced change in light scattering.

Electrotransformation

Rate constants of both orientation and permeabilization were changed when plasmid was present in the sample. The rise time of orientation increased in the presence of DNA, and the permeabilization rate was speeded up by the plasmid. These results suggest a strong interaction between cells and plasmid during the pulse. A critical event between cells and plasmid during the pulse was suggested by our previous works about electrotransformation of *E. coli* (Eynard et al., 1992). As only one rate constant remains associated with each process, this confirms the previous conclusion of Tsong on *E. coli* (Xie et al., 1990) and of our group on yeast (Ganeva et al., 1995) that plasmids were adsorbed to the cell surface before the pulse. The saturation effect on the orientation time reaches its high value (1.7 ms) for a DNA addition as low as 100 ng. A higher concentration is needed to affect the permeabilization process. This further supports our conclusion that two processes are indeed present: orientation and permeabilization. Nevertheless, no effect of plasmid is observed for the long time events of the cell permeabilization such as ATP leakage. ATP leakage is the result of a multiprocess event; the events that occur during the pulse are only the preliminary events of the cell permeabilization. The perturbation of the preliminary events kinetics seems to have no effect on the level of global response of the cell 1 min after the pulse.

CONCLUSION

E. coli permeabilization and transformation are multistep processes: orientation is followed by an envelope alteration, and all steps are affected by plasmid addition. The pulse duration therefore appears to be a critical parameter for gene transfer. This is very important for applications in which high fields cannot be used and where a viscous medium must be used to prevent the osmotic shock but slows down the orientation process.

APPENDIX

E. coli was assumed to belong to three populations:

A: Bacteria not permeabilized and randomly orientated
B: Not permeabilized, but oriented bacteria
C: Oriented and permeabilized bacteria

Therefore, the kinetic scheme for orientation and permeabilization of *E. coli* is given by

$$\underset{\text{Intact bacteria}}{A} \xrightarrow[k_1]{} \underset{\text{Oriented bacteria}}{B} \xrightarrow[k_2]{} \underset{\text{Permeabilized barcteria}}{C}$$

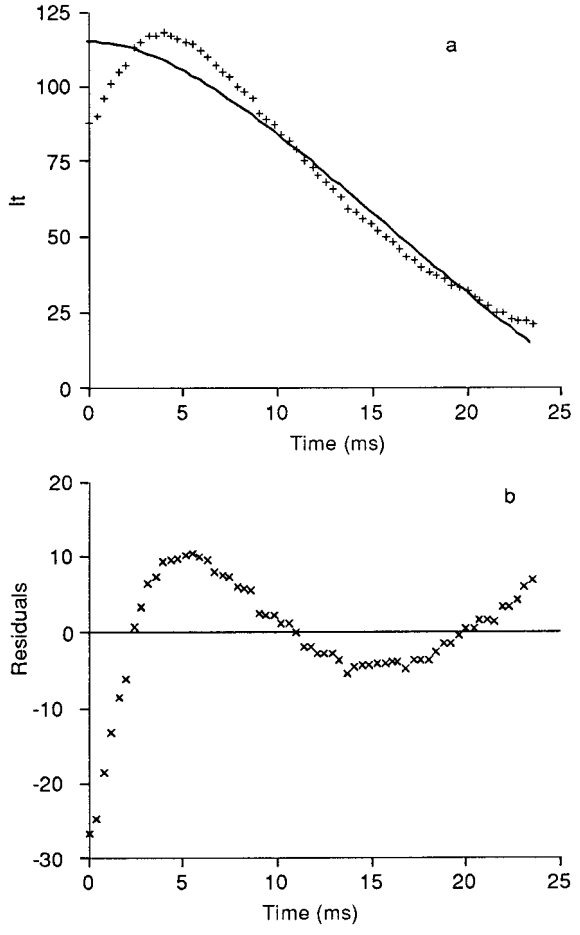

FIGURE 9 Test of the first kinetic model. Pulsing parameters were a single pulse of 24 ms duration at 3 kV/cm. Adjustment (*a*) and residuals (*b*) of the transmitted light data.

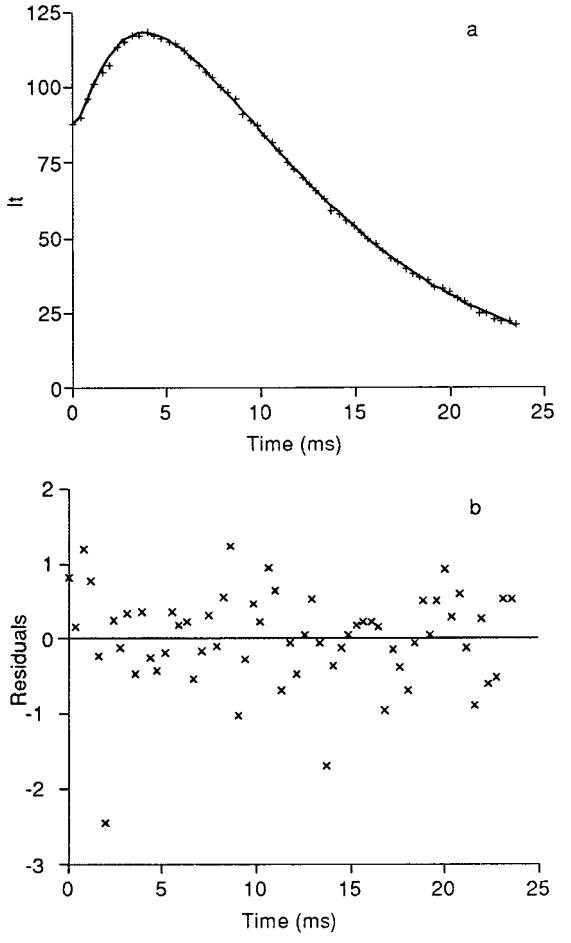

FIGURE 10 Test of the second kinetic model. Pulsing parameters were a single pulse of 24 ms duration at 3 kV/cm. Adjustment (*a*) and residuals (*b*) of the transmitted light data. The hypothesis was that permeabilization induces a change in the scattering properties of the bacteria.

in which k_1 and k_2 are kinetics constants for orientation and permeabilization processes. This compartimental system is analyzed in terms of two successive reactions of the first order. Solutions for the time dependence of populations A, B, and C are well known:

$$A_t = A_0 \cdot e^{(-k_1 t)} \tag{1}$$

$$B_t = A_0 \cdot \frac{k_1}{k_2 - k_1} \cdot [e^{(-k_1 t)} - e^{(-k_2 t)}] \tag{2}$$

$$C_t = A_0 \cdot \left[1 - \frac{k_2}{k_2 - k_1} \cdot e^{(-k_1 t)} + \frac{k_1}{k_2 - k_1} \cdot e^{(-k_2 t)}\right] \tag{3}$$

in which A_0 is relative to the bacterial concentration.

We assumed that the transmitted light Y_t is a function of A_t, B_t, and C_t:

$$I_t = f(A_t, B_t, C_t) + I_0 \tag{4}$$

in which I_0 is a continuous component equivalent to the transmittance of the medium without electric pulse.

The transmitted light data were fitted by the following kinetics models, after correction of wave deformation, using a nonlinear least-squares package we developed (Rodriguez et al., 1994). The merit function is expressed as a nonweighted sum of squares of residues between experimental and simulated data. Assuming the noise as Gaussian, the parametric confidence intervals at 5% of error risk were calculated by a modified boundary method (Hamilton, 1984).

First model: permeabilization induces a loss in orientation

This hypothesis is supported by the observations of a loss in orientation after the pulse and by the two phases observed in turbidity when permeabilization occurs. Population B is therefore affected and is the only contribution to the change in transmitted light. The orientation of population C is assumed to be randomly distributed, as described for population A. Populations A and C make a similar contribution to the transmitted light.

By this hypothesis, the rate equations 1–3 can be resolved to obtain transmitted light (I_t) as a function of time:

$$I_t = B_t = t = A_0\left[\frac{k_1}{k_2 - k_1}\right] \cdot e^{-k_1 t} - A_0\left[\frac{k_1}{k_2 - k_1}\right] \cdot e^{-k_2 t} + I_0 \tag{5}$$

A typical fit is shown in Fig. 9; the fit is poor. This hypothesis is not relevant to the experimental observation.

Second model: permeabilization induces a change in the scattering properties of the bacteria

Although the orientation of the rods results in an increase in transmitted light, permeabilization results in a decrease in transmitted light. The

contribution to the change in transmitted light by a single bacterium cannot be assumed to be the same for both processes. Let α be the ratio of both contributions; the change in transmitted light is therefore

$$I_t = B_t - \alpha \cdot C_t = A_0\left[\frac{k_1 + \alpha k_2}{k_2 - k_1}\right] \cdot e^{-k_1 t} - A_0\left[\frac{k_1(1+\alpha)}{k_2 - k_1}\right] \cdot e^{-k_2 t} - \alpha A_0 + I_0 \quad (6)$$

Therefore, parameters of the following model to be optimized are A_0, k_1, k_2, α, and I_0. As shown in Fig. 10, a very good fit was then obtained with this model. The conclusion is therefore that permeabilization only occurs in bacteria that have oriented parallel to the field lines.

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