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DNA Translocation Across Planar Bilayers Containing *Bacillus subtilis* Ion Channels*

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Ildikò Szabò‡, György Bàthori§, Francesco Tombola, Marisa Brini, Angelina Coppola¶, and Mario Zoratti‡*

From the CNR Unit for the Study of Biomembranes, Department of Biomedical Sciences, University of Padova, Viale G. Colombo 3, 35121 Padova, Italy

The mechanisms by which genetic material crosses prokaryotic membranes are incompletely understood. We have developed a new methodology to study the translocation of genetic material via pores in a reconstituted system, using techniques from electrophysiology and molecular biology. We report here that planar bilayer membranes become permeable to double-stranded DNA (kilobase range) if *Bacillus subtilis* membrane vesicles containing high conductance channels have been fused into them. The translocation is an electrophoretic process, since it does not occur if a transmembrane electrical field opposing the movement of DNA, a polyanion, is applied. It is not an aspecific permeation through the phospholipid bilayer, since it does not take place if no proteins have been incorporated into the membrane. The transport is also not due simply to the presence of polypeptides in the membrane, since it does not occur if the latter contains gramicidin A or a eukaryotic, multiprotein vesicle fraction exhibiting 30-picosiemens anion-selective channel activity. The presence of DNA alters the behavior of the bacterial channels, indicating that it interacts with the pores and may travel through their lumen. These results support the idea that DNA translocation may take place through proteic pores and suggest that some of the high conductance bacterial channels observed in electrophysiological experiments may be constituents of the DNA translocating machinery in these organisms.

Current understanding of the transport of proteins and nucleic acids across biomembranes lags behind the knowledge accumulated on transport of smaller molecules and ions. In these latter fields, a crucial step is the reconstitution of the transport activity as membrane fractions, crude detergent extracts, and finally as isolated molecular species in artificial membrane systems. For prokaryotes, important advances along these lines have been made in the case of protein transport (1–3), but less progress has been achieved in the case of nucleic acids. We have now devised an experimental strategy that might prove fruitful in this respect. We assayed DNA translocation through a planar bilayer separating two chambers, taking advantage of the powerful techniques of PCR[1] amplification and Southern blotting to detect the presence of DNA, initially added in one (*cis*) chamber, in the opposite (*trans*) one. Since the bilayer is built in the context of a standard electrophysiological setup, the procedure also allows the simultaneous monitoring of channel activity in (or, in general, current conduction by) the membrane, and it is thus particularly suitable to study pore-mediated translocation of genetic material.

The evidence is growing that large channels, or channel-like structures, are involved in macromolecule transport. Signal peptides have been reported to open protein-conducting channels in *Escherichia coli* (4). Three proteins of the outer membrane of Gram-negative bacteria, namely FepA (5, 6) and FhuA (7), two siderophore receptors, and TolC (8), a component of macromolecule transport systems, have been shown to form channels when part of their sequence (the "gate") is deleted. The evidence pointing to the involvement of proteic DNA-conducting channels in bacterial conjugation and transformation and in phage infection has been reviewed recently (9). Some of the most interesting examples concern phage DNA injection into the parasitized cells. For example, FhuA, which is also a phage receptor, gives rise to large ion- and possibly DNA-conducting channels upon binding bacteriophage T5 (10). Phage lambda can inject its DNA into liposomes bearing only its receptor, LamB, in a process involving the formation of long-lived open channels (11). LamB is a porin of known structure (12), but whether LamB itself or phage proteins form the conduit for DNA is still unclear. Channels formed by filamentous phage proteins Pb2 (13–16), G3p (17), and Pf3 coat protein (18) have been proposed to be involved in the injection of DNA into the infected cells. In the laboratory, uptake of DNA by bacteria is often obtained by exposing the cells to the genetic material under specific conditions of temperature and Ca^{2+} concentration (19). The mechanism of the process is obscure. Much also remains to be learned on the naturally occurring transformation processes (9, 20–23). *Bacillus subtilis*, the bacterium used in most of our experiments, as well as other cells, also spontaneously releases DNA, along with RNA and proteins, by an unknown mechanism. "Horizontal gene transfer" is a process of relevance for bacterial physiology (24) and is believed to play an important role in the development of drug resistance by prokaryotes (22).

Over the past decade, the application of patch clamp has led

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‡ To whom correspondence should be addressed. Tel.: 39 49 8276054; Fax: 39 49 8276049; E-mail: zoratti@civ.bio.unipd.it.

§ Supported in part by an EMBO short term fellowship and by a Telethon fellowship. Present address: Dept. of Physiology, Semmelweis Medical University, H-1088 Budapest, Hungary.

¶ Trainee supported by a CNR fellowship.

[1] The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); pS, picosiemens; nS, nanosiemens.

to the discovery and characterization of non-porin, stretch-activated, voltage-dependent channels in the cytoplasmic membrane of Gram-negative (25–36) and Gram-positive (37–41) bacteria. No information is yet available on the proteins forming these pores, with the exception of one *E. coli* channel that has been sequenced and cloned (35). At least in Gram-positive bacteria, the various conductances, ranging from several pS to a few nS, have similar voltage dependence, selectivity, and sensitivity to stretch (39, 40), suggesting that they may be due to a small number of proteins, possibly acting as oligomeric complexes (42). We have presented evidence supporting a role of these channels in osmoprotection (30). When the cells are subjected to an osmotic downshock the pores open, allowing the efflux of cytoplasmic solutes, including glutamate, lactose, and ATP, at high rates. The ease with which such relatively high molecular weight species flow through the pores suggested to us that they might be capable of conducting biopolymers, in particular DNA. The results presented here support this notion, suggesting that some of the pores might be constituents of the machinery responsible for DNA translocation *in vivo*.

EXPERIMENTAL PROCEDURES

Electrophysiology—For planar bilayer experiments, a homemade apparatus was used, following established techniques (43). Phosphatidylethanolamine (Avanti) or sometimes purified soybean azolectin (Sigma) planar bilayers with a capacity of approximately 300 picofarads were prepared by painting a decane solution (25–40 mg/ml) across a smoothed hole in a Teflon film (25-μm thickness, Goodfellow) separating two chambers carved in a Teflon block. The standard experimental medium was 100 mM KCl, 0.1 mM $CaCl_2$, 20 mM Hepes/K^+, pH 7.2. Connection to the electrodes was provided by agar bridges. The contents of the chambers (3 ml) could be stirred by magnetic bars. The output of the amplifier was recorded on tape and analyzed off-line.

Patch-clamp experiments were conducted essentially as described previously (39, 40). The chamber contained approximately 1 ml of medium (350 mM KCl, 10 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes/K^+, pH 7.2). To test the effect of DNA, an excised inside-out patch was established, 5–30 μg of total *E. coli* DNA (Sigma) was added to the chamber with a pipette, and the contents of the chamber were mixed manually by withdrawing and re-adding approximately 0.7 ml of the contents three times.

For bilayer experiments, the *cis* side voltage is reported. For patch-clamp experiments, the voltage reported is that of the bath side of the patch (opposite of the pipette voltage). Current (cations) flowing from the *cis* to the *trans* compartments in bilayer experiments or into the pipette in patch-clamp experiments are considered positive and plotted upward. Current was filtered at 1–3-kHz and sampled at 5–10-kHz for computer analysis.

DNA Translocation—In a typical experiment, the planar bilayer was monitored for a few minutes to check for stability and possible leak current. 300 μl were withdrawn from the *cis* chamber and replaced with 300 μl of 3 M KCl to favor fusion and to establish a transmembrane salt concentration gradient. Mostly inside-out *B. subtilis* membrane vesicles, prepared as described (41), containing approximately 5–20 μg of protein were then added to the *cis* side, and the chambers were stirred at intervals until incorporation occurred. Activity was monitored at a few voltages for a short time. The *cis* chamber was then perfused with 20 ml of the experimental medium to avoid further incorporation, and activity was again monitored at a few voltage values. In most cases a sample of medium, to be used as a control against contaminations, was then taken from the *trans* side. A solution of plasmid containing the aequorin cDNA (see below) was then added to the *cis* compartment. The final concentration was in the range 0.8–8 μg of DNA/ml. Negative voltages in the −40 to −80 mV range were applied to the *cis* compartment, to provide a driving force for DNA translocation. 100- or 200-μl samples were taken at regular intervals from the *trans* compartment and replaced with the same volume of fresh experimental medium. The contents of both compartments were stirred for several seconds before each sampling and after additions. Samples from both compartments ("final cis" and "final trans") were taken 10–30 min after the eventual membrane collapse, for use as positive controls in the subsequent PCR amplifications. The samples collected were stored in Eppendorf tubes at −20 °C until needed. Stringent precautions were necessary to avoid contamination artifacts. For example, the agar bridges were discarded, and the Teflon chambers were left immersed in sulfochromic mixture overnight after each experiment. Variable-volume pipettes were kept segregated according to their use and were washed (HCl/NaOH/UV light) every few days. Only filter-equipped pipette tips were employed throughout. All refuse containing (or suspected to contain) plasmid DNA was immediately eliminated in closed containers.

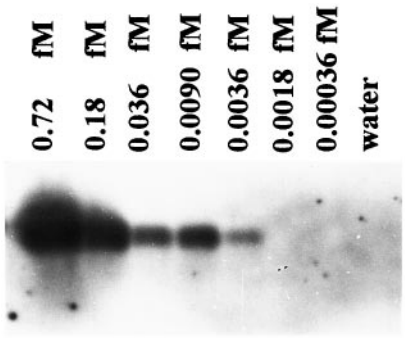

FIG. 1. **Sensitivity of the DNA detection protocol.** Southern blot of the mixtures produced by standard PCR amplification (35 cycles) of plasmid mtAEQ-pcDNAI solutions having the indicated concentrations is shown. 20 μl of PCR mixture were loaded into each well. See "Experimental Procedures" for details.

DNA—To test DNA translocation we used either the recombinant plasmids mtAEQ-pcDNAI (44) or cytAEQ-pcDNAI (45), *i.e.* 4.2-kilobase pair double-stranded plasmids containing a construct comprising the cDNA of aequorin and addressing sequences or the 633-bp aequorin cDNA itself. The choice was dictated mainly by the easy availability of the material in our department. The plasmid solutions used contained linearized and/or fragmented species as well, produced by normal pipetting and handling.

DNA Detection—Aliquots of the samples withdrawn during each experiment were subjected to 35- or sometimes 40-cycle (95/57/72 °C) PCR amplification. The primers were chosen so as to lead to the amplification of the 633-base fragment encoding the aequorin gene. Premixed PCR reaction medium, including *Taq* polymerase, and sample were used in a 4:1 ratio. Stringent precautions were required to avoid contamination artifacts. For example sample-loading operations were conducted under a "clean" hood in a room where the aequorin DNA was never otherwise introduced, strict measures were taken to avoid the accidental transfer of DNA from one sample to another during handling, and water samples were included in every amplification series as a contamination control. Unless otherwise specified, 20-μl aliquots of the amplified mixtures were electrophoresed on 1% agarose gels and Southern blotted onto positively charged nylon sheets (Boehringer Mannheim). The blots were then processed using the Boehringer Mannheim digoxigenin non-radioactive DNA labeling and detection reagents, using CSPD® as a substrate for chemiluminescent detection. Kodak Biomax film was used to obtain images of the blot. The amplification product migrated on gels slightly more slowly than the 564-bp mass marker.

The translocation of only a relatively small number of DNA species was expected to take place in our experiments, and an estimate of the sensitivity of the method was desirable. Routine amplification and processing of solutions of the plasmid used in the translocation experiments, prepared by dilution in standard experimental medium, showed that we could reproducibly detect the presence of 10 (or, often, less, as in Fig. 1) template molecules in the amplification sample, leading to a conservative estimate of our lower detection limit as 6000 translocated molecules (Fig. 1).

RESULTS

Channel Activity in Planar Bilayers—High conductance, low selectivity channels were routinely observed upon fusion of *B. subtilis* vesicles with planar bilayers, in agreement with the observations by Alcayaga *et al.* (41). Incorporation of a vesicle invariably led to the appearance of several conductances, ranging from about 0.1 to 1.5 nS (100 mM KCl), some of which clearly represented substates of others. The abundance of conductances and substates observed both in patch clamp and in bilayer experiments suggests the presence of interacting and cooperating components forming a channel system (see below).

Channels of various conductances can be activated in patch-clamp experiments by applying a pressure difference across the membrane. Similarly, in bilayer experiments the activity often displayed a characteristic dependence on the presence of an osmotic pressure difference between the two compartments separated by the planar membrane. The open probability of the channels was higher in the presence of the gradient. Fig. 2 illustrates this behavior that had not been previously reported for planar bilayer experiments. A transmembrane osmotic pressure gradient was routinely present at the beginning of our experiments, due to the higher [KCl] in the *cis* compartment (see "Experimental Procedures"). Channel activity arose under these conditions, but in many experiments (not always) continued also after the medium of the *cis* compartment had been replaced with the same solution of the *trans* side. This behavior is analogous to the patch-clamp experiments where "aging" or the repeated application of suction often led to stretch-independent activity (38–40). The channels reconstituted in planar bilayers presumably underwent the same modification of their properties. Sub-millimolar concentrations of gadolinium have been shown to inhibit various stretch-activated channels, including those of bacteria (30). Fig. 3 shows the inhibitory effect of 500 μM Gd^{3+} on a channel studied in the presence of transmembrane osmotic gradient ($n = 4$). The effect is well observable both from the current traces (Fig. 3, *A* and *B*) showing a marked decrease of the open probability and by comparison of

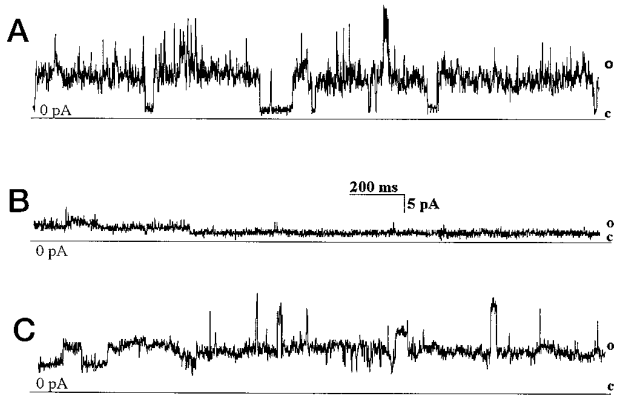

FIG. 2. **Activation of bacterial channels in a planar bilayer by a transmembrane osmotic pressure gradient.** Representative current traces are shown. *o*, open; *c*, closed. $[KCl]_{trans}$, 100 mM. V_{cis}, +60 mV. *A*, $[KCl]_{cis}$, 650 mM. *B*, after perfusing the *cis* chamber with 20 ml of 100 mM KCl (no gradient). *C*, after re-establishing a gradient by increasing $[KCl]_{cis}$ to 390 mM.

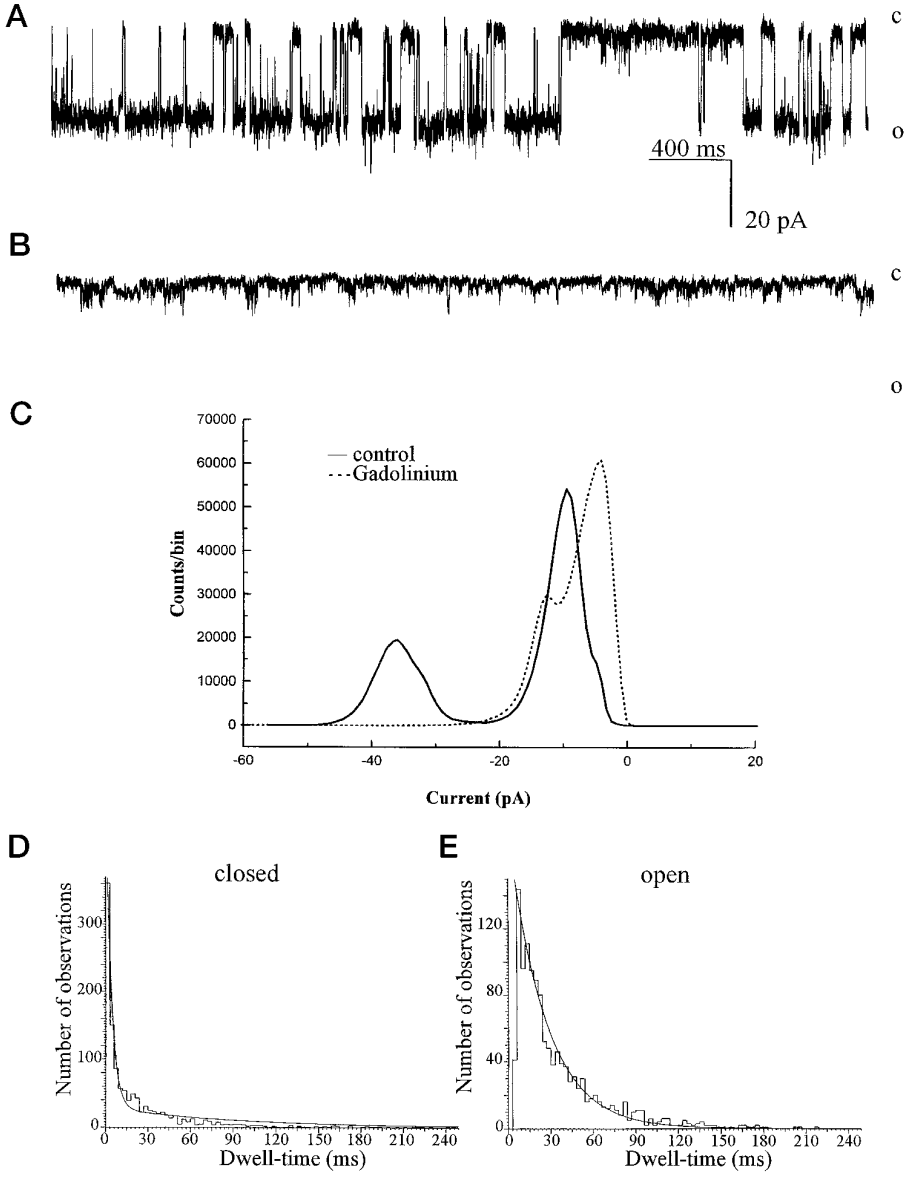

FIG. 3. **Bacterial channels in planar bilayer experiments are inhibited by gadolinium.** Representative current traces were recorded at V_{cis} −40 mV in the presence of salt gradient (600 mM KCl *cis*, 100 mM KCl *trans*) before (*A*) and after (*B*) the addition of 500 μM Gd^{3+}. The 530-pS conductance channel was slightly anion-selective ($P_{Cl^-}/P_{K^+} = 1.75$) and had an open probability of 0.312 before the addition of Gd^{3+}. After the addition of the inhibitor the channel was closed as it is also observable from the shift of the peak toward 0 pA in the current amplitude histogram (*C*). *D* and *E*, show the probability density distributions for the closed and open states, respectively, from the same experiment prior to the addition of Gd^{3+}. The histogram for the closed state was adequately fitted by the sum of two exponentials (time constants: $\tau_1 = 3.8$ ms; $\tau_2 = 91.8$ ms), and the open dwell-time histogram was fitted by one exponential with $\tau = 25.6$ ms.

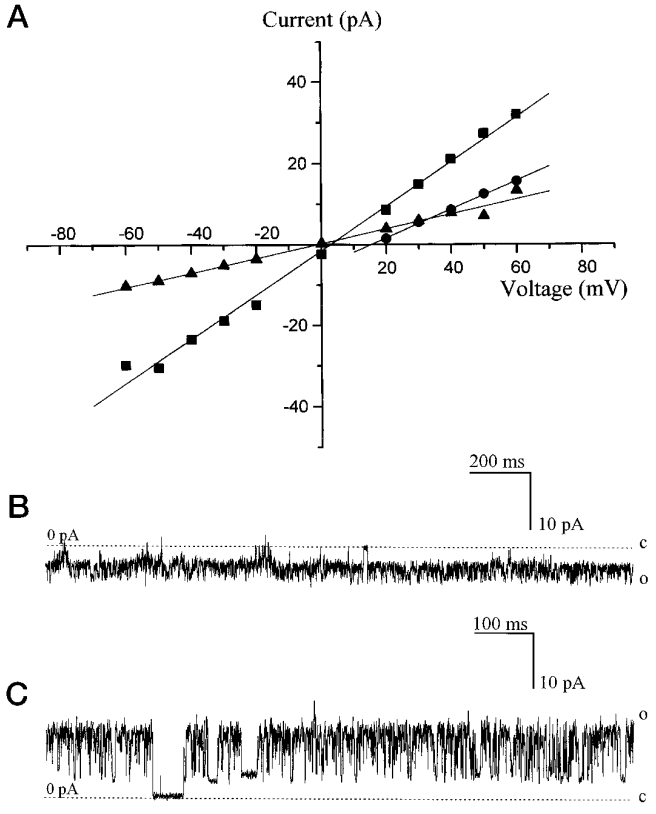

FIG. 4. **Bacterial high conductance channels exhibit low selectivity.** *A*, current-voltage relationship of a 180-pS channel in symmetrical 100 mM KCl (▲) and in salt gradient (535 mM KCl *cis* side, 100 mM KCl *trans* side) (■). The conductance of the main conductance level of the channel in this latter medium was 547 pS, and it was slightly selective for anions ($P_{Cl^-}/P_{K^+} = 1.22$). *B* shows a representative current trace recorded from the same experiment at 0 mV in the presence of salt gradient. Beside the main conductance level a lower conductance substate (350 pS in gradient) was also evident in this experiment at positive voltages (●) (*A*). This substate exhibited a permeability ratio for Cl^- over K^+ of 2.46. The presence of the substate is observable also in trace *C* recorded at V_{cis} +30 mV in the presence of gradient. The 0 current levels are indicated by *dashed lines*.

the current amplitude histograms (Fig. 3*C*). Fig. 3 shows also the probability density histograms for the closed (Fig. 3*D*) and open (Fig. 3*E*) levels obtained from this representative experiment. The time constants obtained by fitting the histograms had values similar to the ones obtained for bacterial channels in patch-clamp experiments (40). However, in general the kinetic parameters of the channels varied from experiment to experiment in both the bilayer and the patch-clamp systems.

Concerning the selectivity, we have reported (39, 40) that the various conductances observed in patch-clamp experiments discriminated poorly between cations and anions. This is also the case for the channels observed in planar bilayers. Fig. 4 shows current-voltage relationships in the absence and in the presence of salt gradient from a representative experiment. The ohmic relationship is a further similarity to the channels observed in patch-clamp experiments. In the case presented here, the channel exhibited a slight anion selectivity (Fig. 4, *A* and *B*), whereas slightly cationic conductances were also observable in other bilayer experiments (not shown). As mentioned above, one of the characteristics of the bacterial channels studied using patch clamp is the presence of substates, *i.e.* of different current levels within a multi-level event, which are not due to the operation of independent channels. Substates were often present in bilayer experiments as well; one example is shown on Fig. 4*C*. The lower current substate was visited by the channel only at positive *cis* side potentials (see Fig. 4*A*) and exhibited a slightly more anionic selectivity with respect to the main conductance level.

In most cases the channels exhibited voltage-dependence, although less markedly than in patch-clamp experiments; negative *cis* potentials favored closed or lower conductance states, as expected from the fusion of inside-out vesicles. Fig. 5 shows representative current traces at various potentials and current amplitude histograms, demonstrating that lower conductance states were predominant at negative voltages. In some cases, however, an opposite voltage dependence was observed, probably due to the opposite orientation of the vesicle incorporated, and in a few cases the channels seemed to have lost their voltage dependence.

In summary, the dependence on stretch and voltage, the weak selectivity, the sensitivity to Gd^{3+}, and the size of the observed conductances in bilayer experiments were analogous to the properties of the channels observed in patch-clamp recordings and strongly suggested that the same molecular species were involved.

DNA Translocation—We investigated whether DNA could be translocated across a planar bilayer membrane containing *B. subtilis* channels. While pore activity was continuously monitored, DNA (see "Experimental Procedures") was added on one side, and samples were taken at regular intervals from the other. The samples were then subjected to PCR amplification and Southern blotting to verify DNA content. Most of our DNA-translocation experiments were conducted in the absence of a transmembrane salt gradient. To better observe the channels' behavior and to exclude the presence of membrane "leaks," the experiments were conducted with only a few active channels in the membrane. For an overall evaluation of the results, we arbitrarily considered only experiments lasting at least 30 min and yielding at least two samples. In 21 experiments, lasting 30 min to 2 h, *cis* potentials in the −40 to −80 mV range were applied. In 12 experiments there was clear evidence of translocation (*i.e.* DNA in all samples), while in 2 cases we could not detect translocated DNA and in 7 experiments the outcome was dubious, since bands appeared only in part of the blots. In other attempts the membrane lifetime was too short for the performance of a meaningful translocation experiment.

The translocation of any specie through pores is driven by the electrochemical gradient of the specie itself. In the case of DNA, a polyanion, the voltage gradient is thus expected to contribute importantly to the driving force. Furthermore, its polarity and magnitude may be expected to have a strong influence on the kinetics of the translocation. We therefore verified whether translocation would take place in the presence of a field opposing the electrophoretic movement of the polyanion, *i.e.* at *cis* side positive voltages. We could not detect any DNA in the samples from the *trans* side if the V_{cis} was held at positive values (four experiments, lasting 40–100 min). In three further experiments we applied first a *cis* side positive voltage (20–40 min) followed by a period at V_{cis} negative (40–80 min). The Southern blot from one of these experiments is shown in Fig. 6*A*. Translocation of DNA was not observed in the presence of *cis*-positive voltage, although it occurred upon application of *cis*-negative voltage in the same experiment.

Control experiments were conducted following the procedures described above, except that the addition of membrane vesicles was omitted, and therefore the planar membrane contained no proteins. Fig. 6*B* shows the Southern blot resulting from one such experiment. No DNA permeation was detected. The same result was obtained in nine other similar experiments, lasting 40–240 min. The addition of plasmid had little or no effect on the membrane conductance, and no channel-like or "noisy" events were induced by it (not shown).

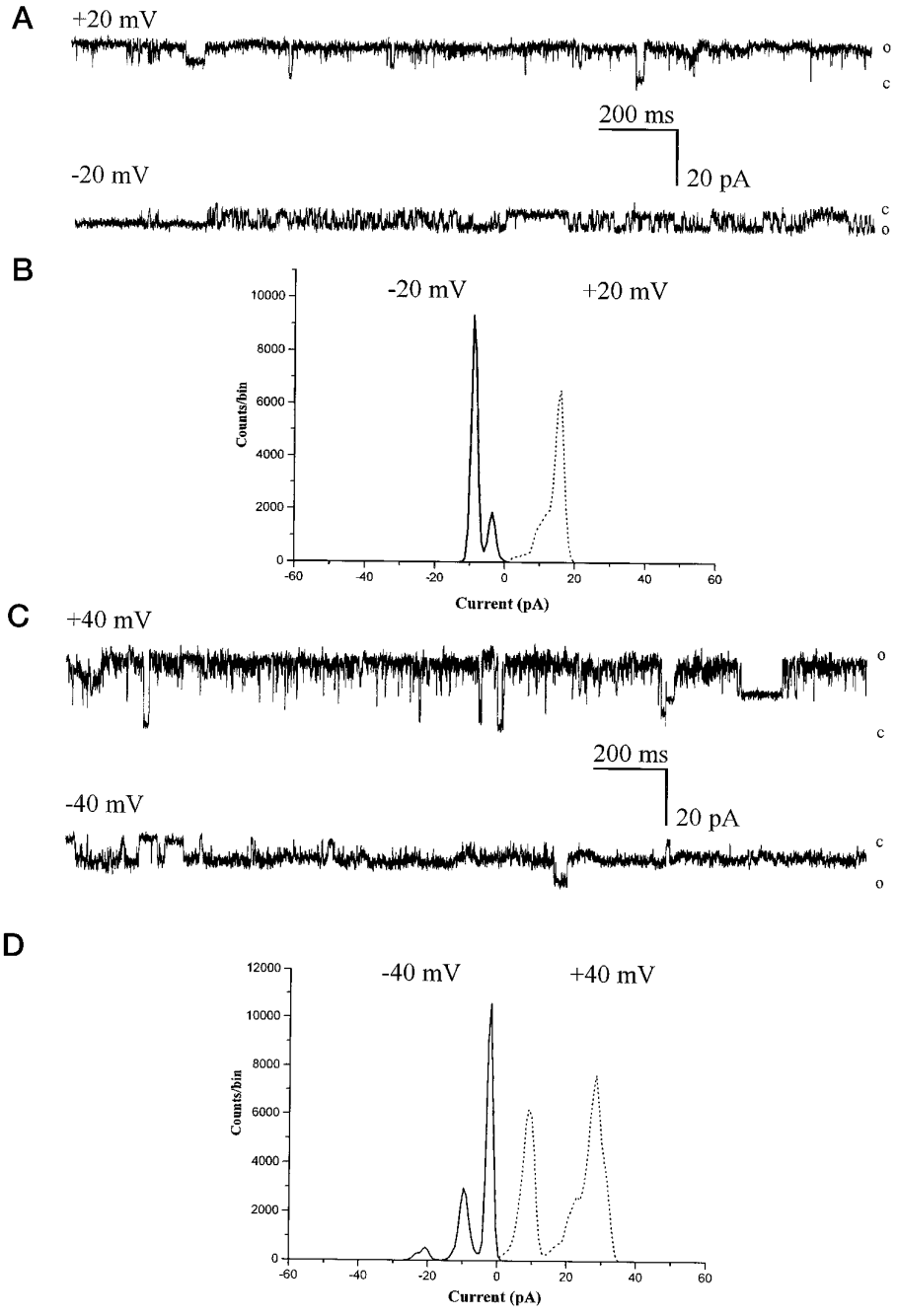

FIG. 5. **Voltage dependence of bacterial channels in bilayer experiments.** The figure shows representative activity recorded at plus and minus 20 and 40 mV V_{cis}, respectively (*A* and *C*) in symmetrical 100 mM KCl. More current is conducted at positive voltages, as it is seen from the current amplitude histograms as well (*B* and *D*), whereas negative potentials favor the function of the channel in lower than maximal conductance substates.

To check whether the translocation might be an aspecific consequence of the presence of proteins in the bilayer, we performed two types of experiments. In the first, gramicidin A was incorporated into the membrane. This well-known (46) peptide forms small, single-file cation channels via the apposition of two single β-strands residing in the two leaflets of the bilayer (47). As illustrated in Fig. 7*A*, no translocation could be detected in the presence of a few such channels in the bilayer ($n = 4$).

In the second type of experiments ($n = 2$), we fused into the membrane vesicles obtained from dog respiratory epithelia containing a variety of membrane proteins (courtesy of Dr. M. Sargiacomo). In these experiments the only channel activity displayed was due to a 30-pS strongly anionic channel. Again, no DNA translocation was observed (Fig. 7*B*).

The only known inhibitors for the stretch-activated bacterial channels are lanthanides (gadolinium has been used most often), which are known to affect many proteins (48). As shown in Fig. 3, gadolinium (sub-millimolar) inhibited channel activity in the bilayer system, in analogy to the observations in patch-clamp experiments (30). We therefore planned control translocation experiments with Gd^{3+}-inhibited channels but could not perform them because experiments showed that Gd^{3+} inhibited PCR amplification, even in the presence of a 10-fold excess of EDTA (at the appropriate free $[Mg^{2+}]$)).

Electrophysiological Effects of the Presence of DNA—The presence of DNA had complex effects on channel activity. Part of the complexity arose from the presence of various different conductances, as well as, in some cases, of what may be termed "silent" channels, *i.e.* pores that only manifested themselves after the addition of DNA. In the translocation experiments, channels ranging in size from 0.1 to 1.5 nS (100 mM KCl) were observed to be affected by plasmid addition. The post-DNA addition activity was generally characterized by a high gating frequency, often resulting in a noisy appearance of the current recordings. Along with this type of "blocking" effect, in part (9

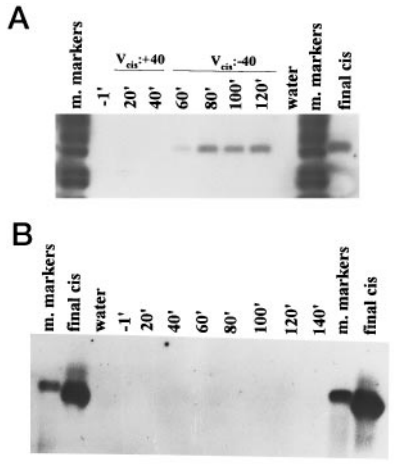

FIG. 6. **DNA translocation through planar membranes incorporating *B. subtilis* membrane vesicles and channels is an electrophoretic process (*A*); DNA does not permeate phospholipid bilayers (*B*).** *A*, Southern blot of the PCR amplification mixtures from the samples taken during a representative experiment. The time of sampling, relative to plasmid addition, is indicated *above* the lanes. In this experiment V_{cis} was held at +40 mV for 40 min and then changed to −40 mV for a further 80 min. *Water* indicates a water sample subjected to PCR amplification (control). The *final cis* sample was diluted 1 million-fold with medium prior to PCR amplification. Mass markers: Boehringer Mannheim MW Marker VI, digoxigenin-labeled. The standard medium was present in both chambers. $[DNA]_{cis}$, 0.8 μg/ml. *B*, Southern blot of the PCR amplification mixtures from a representative control experiment (no addition of membrane vesicles or proteins). The equivalent of 0.04 μl of amplified *final cis* sample was loaded in the corresponding wells. Mass marker: Boehringer Mannheim MW Marker II, digoxigenin-labeled (564-bp band). V_{cis}, −50 mV. $[DNA]_{cis}$, 7.5 μg/ml. See Fig. 6*A* and text for further details.

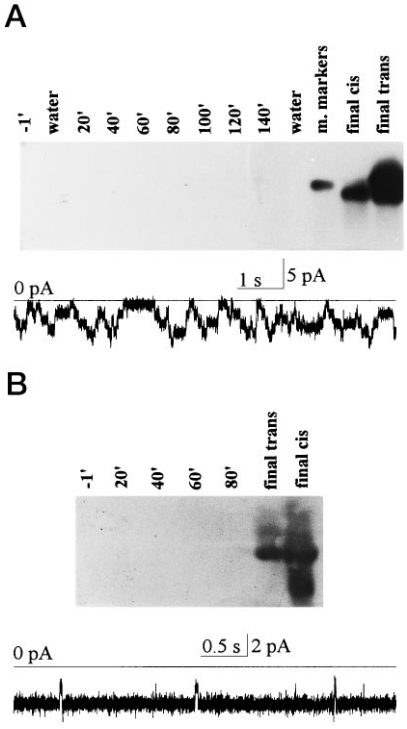

FIG. 7. **DNA is not translocated when the the planar membrane contains gramicidin A (*A*) or an eukaryotic multi-protein membrane fraction exhibiting activity by a 30-pS channel only (*B*).** Southern blots and representative current traces in the presence of DNA are shown. Highly diluted amplification mixtures derived from the *final trans* and *final cis* control samples were loaded into the corresponding wells. *A*, a representative experiment with gramicidin. V_{cis}, −80 mV. $[DNA]_{cis}$, 0.8 μg/ml. Mass markers, Boehringer Mannheim MW marker II, digoxigenin-labeled (564-bp band). *B*, a representative experiment with a plasma membrane fraction. V_{cis}, −50 mV. $[DNA]_{cis}$, 1.6 μg/ml.

out of 21) of the experiments we could detect an "activating" action as well, meaning that new conductances, not observed before the addition of DNA, appeared, and/or already active channels became more prominent. In general, the "activated" conductances displayed fast kinetics and/or a high level of "open channel noise." Because of the presence of various conductances and substates in the various experiments, it is difficult to characterize the effect of DNA in terms of changes in kinetic parameters. Analogous effects were observed in experiments employing *E. coli* genomic DNA (Sigma) at higher concentrations (20 μg/ml) ($n = 6$) (not shown).

This double effect of plasmid is best illustrated by an example (Fig. 8). In this experiment before the addition of plasmid the observed activity was due to a nearly always open 160-pS channel and to well behaved approximately 100, 200, and 500-pS conductances (100 mM KCl) displaying low open probability (at $V_{cis} = -40$ mV, the voltage subsequently applied throughout the translocation period) (Fig. 8*A*). Immediately after the addition of plasmid, and for the rest of the experiment (60 min), the 160-pS conductance was no longer identifiable, while overall there was a marked increase in channel activity and gating frequency ("flickering") (Fig. 8, *C* and *D*). These changes are reflected in the current amplitude histograms (Fig. 8*E*). DNA was translocated in this experiment as assessed by PCR followed by Southern blot (not shown).

Experiments with relatively high concentrations of uncharacterized DNA, conducted on the "native" membrane of *B. subtilis* protoplasts using the patch-clamp technique, also provided evidence for an activating effect of the genetic material. In patch-clamp experiments the application of stretch and of positive (cytoplasmic side) voltages synergistically increase the open probability of the channels (25, 39, 40). After the addition of DNA, patches subjected to a constant suction displayed a marked increase of channel activity in the negative voltage range (not shown). Furthermore, DNA could also elicit channel activity in patches not subjected to stretch, a situation similar to the circumstances of bilayer experiments. Fig. 9 presents such an experiment. This excised inside-out patch exhibited stretch-dependent activity by channels in the 1–4.4 nS range (350 mM KCl) (not shown). In the absence of stretch and DNA the channels remained closed. When DNA was added, without applying suction, channel activity developed while the patch was held at negative potentials, displaying an "inverse" voltage dependence and fast kinetics (Fig. 9, *A* and *B*). The DNA-activated channels also had conductances in the 1–4.4 nS range, with the substates typical of bacterial stretch-activated channels (33, 38–40) (Fig. 9*C*). In general, DNA-activated channels had high open channel noise and longer open times than the control activity.

These effects of DNA were observed in 12 patch-clamp experiments out of 27 and were often accompanied by an increase in the leak current. In the other 15 cases, either no evident effect could be detected (6 cases), or the addition of DNA appeared to lead only to an increase in leak current (9 cases).

DISCUSSION

The translocation experiments summarized above show that DNA can cross a planar bilayer membrane into which vesicles of *B. subtilis* plasma membrane containing high conductance channels have been fused. The properties of these channels strongly resemble those of the stretch-activated channels observed by patch-clamping protoplasts, indicating that they are the same. The driving force for DNA translocation was largely provided by the applied potential, negative on the DNA side. Control experiments with "empty" membranes, and with bilay-

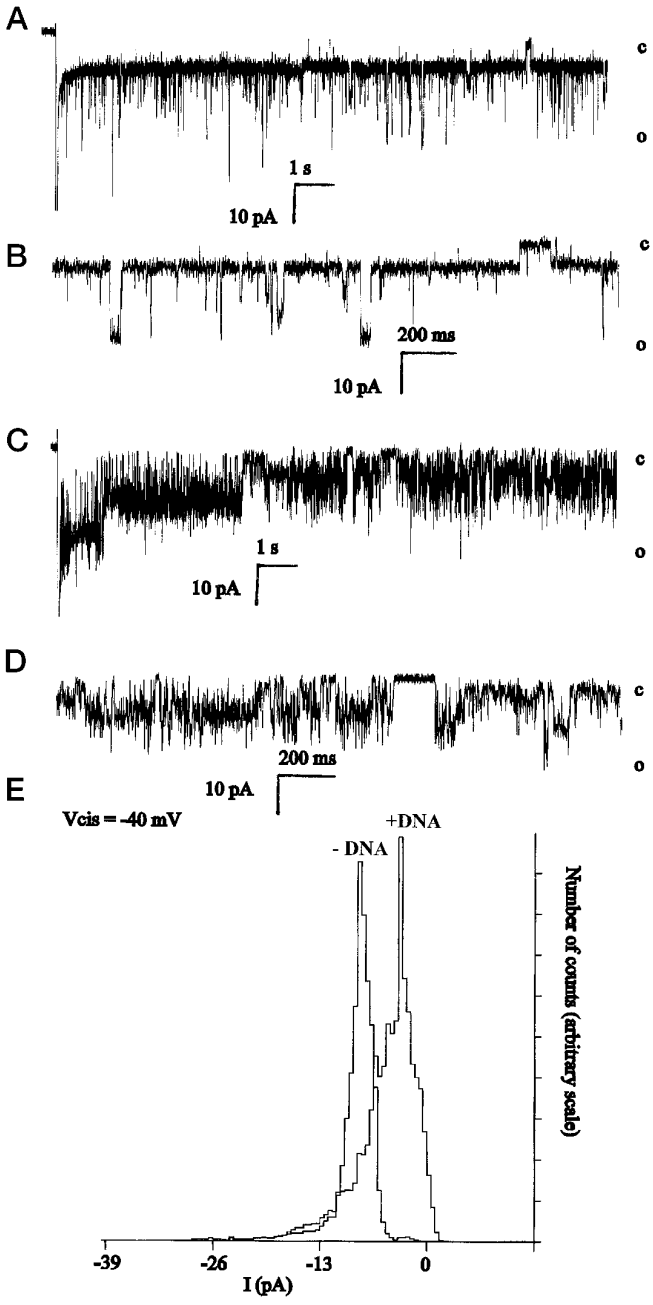

FIG. 8. **Effects of DNA on channel activity.** A representative experiment is shown, see text. V_{cis}, −40 mV. *A*, current trace exemplifying the activity observed before the addition of plasmid. The voltage is switched from 0 to −40 at the beginning of the trace. *B*, a pre-plasmid segment plotted with an amplified time scale. *C*, current trace recorded a few seconds after the addition of plasmid (1.6 μg/ml). Compare with *A* (same conditions). *D*, a post-plasmid segment plotted with an amplified time scale. Compare with *B* (same conditions). *E*, current amplitude histograms obtained before (ordinate unit = 1400N/division) and after plasmid addition (ordinate unit = 7100N/division), as indicated.

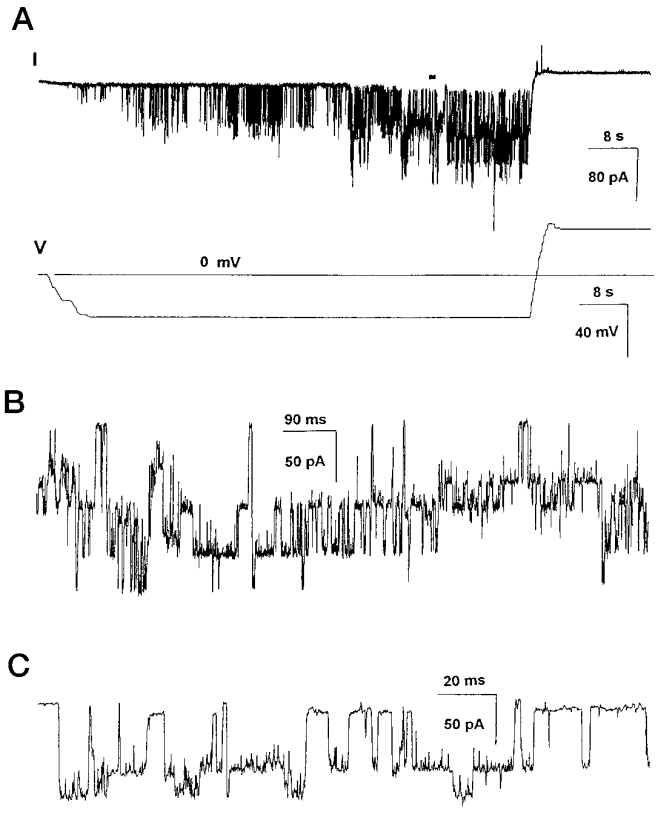

FIG. 9. **DNA elicits channel activity in a resting *B. subtilis* excised patch.** See text. *A*, current (*I*) and voltage (*V*) traces. The voltage was varied between −38 and +40 mV. *B*, the *underlined* segment of the current trace in *A*, plotted with an expanded time scale. *V*, −38 mV. The four main current levels correspond to chord conductances of about 1.55, 2.2, 3.5, and 4.4 nS. *C*, conductance substates (a segment from the current trace in *A*). *V*, −38 mV. The three main current levels correspond to chord conductances of 0.25, 1.8, and 2.45 nS. Parameters as in *B*.

ers containing gramicidin A, or containing many eukaryotic proteins but displaying activity by a small anion-selective channel only, showed that the translocation was not an unspecific phenomenon due to the properties of the bilayer itself or to the presence of peptides in it.

The question arises of whether the channels themselves are directly involved in the translocation of DNA, as is strongly suggested by the complex of our observations. The lack of specific inhibitors and technical problems preventing the use of lanthanides, the only known inhibitors of these channels, hampered a direct assessment of their role in the translocation. DNA transport might thus conceivably have been mediated by other components of the incorporated vesicles. This hypothesis seems, however, unlikely since translocation proceeded rapidly in the absence of ATP and of ion gradients, and it was electrophoretic. A definitive answer will presumably come from experiments employing pure channel protein(s). Unfortunately, no such preparation is currently available for the channels of Gram-positive bacteria. The electrophysiological data strengthen the idea that the high conductance pores were indeed involved. DNA apparently had a double effect; on one hand it activated the pores and on the other hand it altered the properties of the conductances in a manner that may be interpreted as a blockade by transiting macromolecules. *A priori*, channel activation might originate either from specific interactions between DNA and the channel proteins or from aspecific effects of the DNA, presumably on the membrane surface potential. Since the DNA is a polyanion, its binding should make the surface potential of the membrane more negative. At least in patch-clamp experiments, this would be expected to favor the closed state of the channels, whereas the opposite effect has been observed. In any case, it seems difficult to attribute both the activating and the blocking effects to aspecific interactions. An interpretation of the latter as due to the transit of macromolecules is instead plausible. Although precisely what effect should be exerted on current conductance by these pores by a transiting DNA molecule is unknown, it is reasonable to assume that it should decrease it and induce changes in the apparent gating kinetics of the pore. The blocking effect of transiting macromolecules has precedents in the literature. For

example, in the case of protein translocation across the endoplasmic reticulum (49) and the nuclear (50, 51) membranes, evidence has been presented pointing to a block of pores by the transiting peptides. Translocation of leader peptides results in a fast block of a mitochondrial channel (52–54). Polyethylene glycol molecules passing through alamethicin pores also induced a conductance decrease and an increase in open channel noise (55).

Other considerations support the idea that DNA may travel through the lumen of channels. Recently Kasianowicz *et al.* (56), using an experimental approach similar to ours, found that single-stranded DNA can move through the *Staphylococcus aureus* α-hemolysin pore. The diameter of this pore at its narrowest point is 1.4 nm (57), and translocation was associated with a nearly complete, fast block. The conductances of the bacterial channels are comparable to (or higher than) those of other pores believed to be involved in macromolecule transport, such as the endoplasmic reticulum and nuclear pores referred to above, those activated by leader peptides in the *E. coli* plasma membrane (4), and the possibly DNA-conducing bacterial and phage proteins mentioned in the Introduction. It should at any rate be considered that the conductance of a channel may be only marginally related to the size of the species that can move through it (58). The mitochondrial porin, VDAC, is also able to conduct genetic material.[2] The fact that FhuA, LamB, VDAC, and α-hemolysin are all believed to have a β-barrel conformation suggests that pores having this structure might be especially suitable for DNA transport. It has been proposed that pores involved in macromolecule transport might possess an elastic structure, capable of adapting itself to the macromolecule being transported (59).

It is difficult at present to assess whether DNA specifically interacted with some channels and not with others. Various different conductances were affected, but how many molecular species contribute to the activity observed in these membranes is not known (see Introduction). The phenomena described in this paper probably do not correspond precisely to those occurring *in vivo*, since any soluble factors had been presumably lost in our experiments. Nonetheless, if channels can provide a conduit for DNA under our experimental conditions, they may be supposed to perform the same task in the cell, probably as part of a complex machinery.

Finally, we would like to emphasize that this work demonstrates the feasibility of studying DNA transport in a reconstituted system, using PCR amplification plus Southern blotting as the detection system. The possibility to simultaneously clamp the transmembrane voltage, monitor transmembrane currents, and change the medium composition at will makes this a powerful new tool for the study of this important biological process.

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DNA Translocation Across Planar Bilayers Containing *Bacillus subtilis* Ion Channels

Ildikò Szabò, György Bàthori, Francesco Tombola, Marisa Brini, Angelina Coppola and Mario Zoratti

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