Role of Proton Motive Force in Genetic Transformation of
Bacillus subtilis

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This study explored the role of the proton motive force in the processes of DNA binding and DNA transport of genetic transformation of Bacillus subtilis 168 strain 8G-5 (trpC2). Transformation was severely inhibited by the ionophores valinomycin, nigericin, and 3,5-di-tert-4-hydroxybenzylidene malononitrite (SF-6847) and by tetraphenylphosphonium. The ionophores valinomycin and nigericin also severely inhibited binding of transforming DNA to the cell envelope, whereas SF-6847 and carbonylcyanide-p-trifluoromethoxyphenylhydrazone hardly affected binding. The proton motive force, therefore, does not contribute to the process of DNA binding, and valinomycin and nigericin interact directly with the DNA binding sites at the cell envelope. The effects of ionophores, weak acids, and tetraphenylphosphonium on the components of the proton motive force and on the entry of transforming DNA after binding to the cell envelope was investigated. DNA entry, as measured by the amount of DNase I-resistant cell-associated [3H]DNA and by the formation of DNA breakdown products, was severely inhibited under conditions of a small proton motive force and also under conditions of a small ΔpH and a high electrical potential. These results suggest that the proton motive force and especially the ΔpH component functions as a driving force for DNA uptake in transformation.

In genetic transformation of Bacillus subtilis, the following steps can be distinguished: (i) binding of DNA to the cell envelope, quickly followed by the formation of double-stranded donor DNA fragments (4, 6, 7, 14), (ii) entry of single-stranded donor segments (5, 7, 20) with concomitant production of acid-soluble donor DNA-derived breakdown products (6, 14), (iii) integration of the single-stranded donor DNA in the recipient chromosome to form a heteroduplex molecule (1, 8, 9; C. A. Vermeulen, Ph.D. thesis, University of Groningen, The Netherlands, 1972), and (iv) resolution of the heteroduplex by segregation (25) and expression of the transformed marker. In transformation, DNA has to be translocated across the cytoplasmic membrane. Since this is an energy-requiring process, the question about the nature of the driving force for this process has been put forward. As a result of the activity of the primary proton pumps, the respiratory chain, and the Ca²⁺, Mg²⁺-stimulated ATPase complex, a proton motive force is generated across the cytoplasmic membrane. This proton motive force which is composed of an electrical potential (ΔΨ) and a chemical gradient of protons (ΔpH) has been shown to be the driving force for the translocation of many solutes across the cytoplasmic membrane (17). Possibly, this proton motive force is also the driving force for DNA uptake. This aspect has been investigated by Grinius et al. (3, 10, 11). These authors have reported on the effects of ionophores, which dissipate one or both components of the proton motive force and metabolic inhibitors on genetic transformation in B. subtilis. They conclude that the effects of ionophores have to be explained at the level of a proton motive force dependence of the transmembrane transport of DNA. From the effects of potassium-specific ionophores (valinomycin and nigericin, added to vary the composition of the proton motive force), they concluded that both the ΔpH and ΔΨ component of the proton motive force are effective in energizing DNA transport. The latter observation led Grinius (10) to postulate the existence of a net positively charged cation-DNA complex, which is transferred across the cytoplasmic membrane during transformation.

Since it is possible to measure DNA binding and DNA transport separately, we decided to investigate the proton motive force dependence of these processes. In addition, a series of proton motive force measurements was carried out with competent cultures of B. subtilis. As artefactual effects of the potassium-specific ion-
ophores were observed, the composition of the \( \Delta \mu \) was independently varied by changing the medium composition (18).

**MATERIALS AND METHODS**

**Bacterial strains.** *Bacillus subtilis* 168 strain 8G-5 (trpC2) was used as recipient. trp+ DNA was isolated from *B. subtilis* W23 and \(^3\)H-labeled trp+ DNA (10\(^5\) to 10\(^6\) cpm/mg of DNA) was isolated from *B. subtilis* 168-2G-8 (thy) by the methods of Venema et al. (24) and Buitenwerf and Venema (2), respectively.

**Transformation.** Competent cultures of *B. subtilis* 8G-5 were prepared as described (2), except that starvation was prolonged to 2 h at 37°C. trp+ DNA was added to a final concentration of 1 to 2 \( \mu \)g/ml, and transformation was terminated by the addition of 100 \( \mu \)g of DNAse I per ml. Transformants were scored on appropriately supplemented minimal agar plates.

**Proton motive force measurements.** The magnitude of the two components of the \( \Delta \mu_H \), \( \Delta \mu_P \) and \( \Delta \psi \), was calculated from the distribution of 16 M \(^{14}\)Cbenzoic acid and 0.18 \( \mu \)M \(^{3}\)Htetraphenylphosphonium (TPP+) respectively, as measured with the silicon oil centrifugation technique (12). Competent cells were incubated with one of the radioactively labeled probes at 37°C in a New Brunswick Scientific shaking water bath at 200 rpm. After 15 min of incubation, the cells were separated from the medium by centrifugation at 37°C. If necessary, the cells were preincubated for 3 min in the presence of salts or ionophores. \( \mathrm{H}_2\mathrm{O} \) (4 \( \mu \)l/ml) and \( [\text{carboxy-}^{14}\text{C}] \) dextran (0.36 mg/ml) were used to assay the amount of extracellular water that cosedimented with the cells. Incubations with tritiated water were terminated after 1 min by centrifugation. Also, in *B. subtilis*, a considerable amount of binding of TPP+ to deenergized cells was observed (23, 24, 26; J. S. Lolkema, K. J. Hellingwerf, and W. N. Konings, Biochim. Biophys. Acta, in press). Since at this stage of the investigation a quantitative determination of this binding under energized and deenergized conditions was not performed, the \( \Delta \psi \) values were estimated by subtracting the amount of TPP+ bound under deenergized conditions from the amount of accumulated TPP+ (18, 26). The given \( \Delta \psi \) values therefore could be slightly different from the actual \( \Delta \psi \) values.

**DNA binding to competent cells.** \(^{1}\)HJDNA (trp+); 1.8 \( \mu \)g/ml was added to a culture of competent cells after a preincubation of the cells for 10 min with 40 mM potassium-EDTA at 37°C in a New Brunswick Scientific shaking water bath (200 rpm). After 15 min of incubation, DNA binding was terminated by diluting the culture (1:1) with ice-cold standard saline citrate (150 mM sodium chloride plus 15 mM sodium citrate), containing 1 mg of highly polymerized calf thymus DNA per ml and 40 mM potassium-EDTA. Samples of 1 ml were layered on top of ice-cold stepwise sucrose gradients (6 ml of 10% sucrose and 6 ml of 15% sucrose in starvation medium [2] plus 40 mM potassium-EDTA) at 4°C (5,000 \( \times \) g, 30 min). The pellets from these centrifuge tubes were frozen in ethanol (\(-80^\circ\)C) and washed with starvation medium. After the pellets were thawed, they were resuspended and filtered on 0.15-\( \mu \)M Millipore filters. The amount of radioactivity on dried filters was subsequently determined after addition of 5 ml of Hydroolum (J. T. Baker Chemicals, Deventer, The Netherlands), with the use of a Nuclear Chicago Mark I liquid scintillation counter. If necessary, the cells were preincubated with ionophores for 3 min before adding the radioactive trp+ DNA.

Correction for the amount of DNA transported into the cells, was made by performing the dilution step with starvation medium (37°C) containing 120 \( \mu \)g of DNAse I plus 70 mM MgSO\(_4\) followed by an additional 10-min incubation at 37°C.

**Measurement of DNA entry.** \(^3\)H-labeled trp+ DNA was bound to competent cells as described above, except that 15 mM potassium-EDTA was used, which is sufficient to prohibit DNA entry into the cells. After a 1:1 dilution with standard saline citrate, containing 1 mg of highly polymerized calf thymus DNA per ml plus 15 mM potassium-EDTA at 37°C, the various agents to manipulate the composition of the proton motive force, were added and the incubation was continued for 3 to 5 min. DNA transport was initiated by the addition of excess MgSO\(_4\) (21 mM) and terminated after various intervals by the addition of excess potassium-EDTA (30 mM).

The intracellular DNA was separated from the extracellular DNA by centrifugation of the cells as described above after the addition of 80 \( \mu \)M thymidine and 160 \( \mu \)M thymine to prevent the incorporation of DNA breakdown products during chromosomal DNA synthesis. DNA, bound to the cell envelope, was removed by suspending the pellet from this centrifugation in starvation medium plus 80 \( \mu \)M thymidine and 160 \( \mu \)M thymine at 37°C. Subsequently, DNase I (100 \( \mu \)g/ml) and MgSO\(_4\) (30 mM) were added, and after 5 min of incubation, the cells were washed three times with ice-cold starvation medium plus 15 mM potassium-EDTA. The amount of radioactivity inside the cells was determined by suspending the pellet obtained after the third wash step in 0.5 ml of buffer and by mixing 0.45 ml of this suspension with 5 ml of Hydroolum and 100 \( \mu \)l of 15% (wt/wt) perchloric acid.

Since a strong correlation exists between DNA entry and the amount of acid-soluble products produced (14), the appearance of acid-soluble DNA fragments in the cell supernatant was determined. A sample of the supernatant of the stepwise sucrose gradient was mixed with an equal volume of 6% perchloric acid and left on ice for 1 h. After 7 min of centrifugation at 12,000 \( \times \) g and 4°C, 0.5 ml of the supernatant was mixed with 5 ml of Hydroolum, and the radioactivity determined as described above.

**Materials.** \(^{1}\)Cbenzoic acid (108 Ci/mmol), \( \mathrm{H}_2\mathrm{O} \) (91 Ci/mmol), and \([\text{methyl-}^{3}\text{H}]\)thymidine (20.7 Ci/g) were obtained from the Radiochemical Centre, Amersham Corp., Bucks, Great Britain, and \([\text{carboxyl-}^{14}\text{C}]\)dextran (2.8 mCi/g) from New England Nuclear Corp., Boston, Mass. \(^{3}\)Htetraphenylphosphonium bromide (2.5 Ci/mmol) was purchased from the Nuclear Research Centre, Negev, Beer-Sheva, Israel.

DNase I was obtained from Miles Laboratories, Stoke Poges, Slough, Great Britain, and valinomycin from Sigma Chemical Co., St. Louis, Mo. Nigericin, monensin, and 3,5-di-N-methylbutyl-4-4'-di-aminostyril-denemalononitrite (SF-6847) were kind gifts of W. C. Pettinga (Eli Lilly & Co., Indianapolis, Ind.), J. C. Arents (University of Amsterdam, The Netherlands) and Y. Nishizawa (Sumitomo Chemical Industry, Osa-ka, Japan). All other chemicals were reagent grade and obtained from commercial sources.
RESULTS

Relation between $\Delta \mu_{\text{H}^+}$ and transformation.

The effects of ionophores and membrane-permeant cations and weak acids on the composition and magnitude of the proton motive force and on genetic transformation was investigated (Table 1 and Fig. 1). All ionophores tested (10 $\mu$M valinomycin, 1 $\mu$M nigericin, and 10 $\mu$M SF-6847) severely inhibited transformation. The inhibition was most complete with those ionophores which completely dissipated the $\Delta \psi$ (nigericin and SF-6847). Furthermore, complete inhibition of transformation was observed in the presence of 1 mM TPP$,^+$, whereas a high concentration (40 mM) of the weak acid formate inhibited transformation by only 30 to 50%. The magnitude and composition of the $\Delta \mu_{\text{H}^+}$ under these experimental conditions is given in Table 1. Without further additions, the $\Delta \mu_{\text{H}^+}$ is mainly composed of a $\Delta \psi$, as a result of the relatively high pH of the transformation medium (pH 7.1) and its significant NH$_4$ concentration. As expected, in the presence of the uncoupler SF-6847, the total $\Delta \mu_{\text{H}^+}$ is dissipated, whereas nigericin resulted in a dissipation of the $\Delta \psi$ component. In the latter case, a small interconversion of $\Delta \psi$ into $\Delta \psi$ occurred. Also, formate and acetate affected the components of the $\Delta \mu_{\text{H}^+}$ in a predictable way: dissipation of the $\Delta \psi$ with some simultaneous increase in $\Delta \psi$. Here, 100 mM acetate is much more effective than 40 mM formate in dissipating the $\Delta \psi$. Valinomycin and TPP$^+$ both decreased the $\Delta \psi$ dramatically, as expected, although with valinomycin this dissipation was not complete. Unexpectedly, both compounds decreased to some extent the $\Delta \psi$ component of the proton motive force.

Effect on binding of transforming DNA to the cell envelope.

Table 2 shows the result of an experiment in which the effect of various compounds on the binding of transforming DNA to the cell envelope, was investigated. The ionophores and

| TABLE 1. Magnitude and composition of the proton motive force in cells of competent cultures$^a$ |
|---------------------------------|-----------------|-----------------|-----------------|
| Addition                        | $\Delta \psi$ (mV) | $\Delta \psi$ (mV) | $\Delta \mu_{\text{H}^+}$ (mV) |
| Control                         | 89               | 33              | 122             |
| 10 $\mu$M SF-6847               | 0                | 0               | 0               |
| 10 $\mu$M Valinomycin           | 10               | 27              | 37              |
| 1 $\mu$M Nigericin             | 108              | 0               | 108             |
| 0.45 mM TPP$^+$                | 0                | 15              | 15              |
| 40 mM Tris-formate             | ND$^b$           | 25              | 25              |
| 100 mM Potassium acetate       | 96               | 10              | 106             |

$^a$ For the intracellular compartment of B. subtilis 8G-5, a volume of 4.04 $\mu$g/mg of protein per ml was obtained. For further experimental details, see the text.

$^b$ ND, Not determined.

FIG. 1. Effects of ionophores, protonophores, and changes in the medium composition on genetic transformation in B. subtilis 8G-5. The time course of transformation of the trpC2-gene in competent cells of B. subtilis 8G-5, exposed to 1.8 $\mu$g of trp$^+$ DNA per ml, was measured as described in the text. The transformation frequency in the control experiments varied between 1.0 and 2.6%. The various additions were made 5 min before the addition of the transforming DNA. Symbols: $\square$, no additions or addition of solvent for ionophores only; $\triangle$, plus 40 mM Tris-formate; $\bigcirc$, plus 10 $\mu$M valinomycin; and $\times$, plus 1 $\mu$M nigericin, 1 mM TPP$^+$, or 10 $\mu$M SF-6847.

| TABLE 2. Effects of various ionophores and compounds on the binding of transforming DNA to competent cells |
|---------------------------------|-----------------|-----------------|
| Addition                        | Relative amount of bound DNA (%) |
| Control                         | 100             |
| 10 $\mu$M Valinomycin           | 11              |
| 1 $\mu$M Nigericin             | 10              |
| 10 $\mu$M FCCP$^a$             | 107             |
| 10 $\mu$M SF-6847              | 96              |
| 20 mM Tris-formate             | 98              |
| 2 mM TPP$^+$                    | 77              |
| 2 mM TPP$^+$ plus 20 mM Tris-formate | 77 |

$^a$ In the control experiments, 0.76 to 0.44 $\mu$g of DNA was bound to the cells per milligram of protein, depending on the degree of competence of the culture.

$^b$ FCCP, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.
phores valinomycin and nigericin gave 90% inhibition of the DNA binding, whereas protonophores (either SF-6847 or carbonylcyanide-p-trifluoromethoxyphenylhydrazone, both at 10 μM) were not significantly inhibitory. In the control experiment, it was demonstrated that more than 98% of the DNA was sensitive to DNase I and therefore bound to the cell envelope (i.e., not inside the cells).

Changes in the medium composition have only minor effects on the amount of bound DNA. Formate (20 mM) had no effect, and TPP⁺ hardly inhibited DNA binding. An inhibition of 23% is obtained with 2 mM of this lipophilic cation.

The results of Table 2 show that DNA, once bound to the cell envelope, is not released by the addition of ionophores. The results of Table 3 demonstrate that in the presence of valinomycin and nigericin the amount of cell-associated DNA remains essentially constant. Even the time-dependent decrease in the amount of bound DNA in the control incubation is absent in the presence of valinomycin and nigericin. The decrease is caused by an endogenous nuclease activity (14). Possibly this nuclease is inhibited by the ionophores.

Proton motive force dependence of DNA entry. The effect(s) of ionophores (valinomycin, nigericin, and monensin) on the entry of transforming DNA was studied by adding these ionophores to cells to which DNA had been bound in the absence of Mg²⁺ (2). Subsequently, DNA uptake was initiated by the addition of excess Mg²⁺. The entry of DNA was measured by determining the amount of DNase I-resistant cell-associated [³H]DNA (Fig. 2). Only a small inhibition of DNA entry occurred in the presence of valinomycin, which decreases Δψ dramatically and ΔpH slightly (Table 1). The ionophores that dissipate ΔpH with a small increase of Δψ (nigericin and monensin) markedly inhibited the entry of cell envelope-bound DNA.

**DISCUSSION**

The data presented in this report clearly demonstrate that the inhibition of transformation by various agents (Fig. 1) cannot be explained by their effect on the proton motive force only. From the results of DNA binding measurements (Table 2), one is led to conclude that the proton motive force does not contribute to this process and that valinomycin, nigericin, and, to a lesser extent, TPP⁺ inhibit binding of DNA via a direct interaction with the DNA binding sites at the cell envelope. Since the amount of cell-bound DNA is not affected by the addition of ionophores (Table 3), their effect on DNA entry can be determined. The results of Fig. 2 and 3 and Table 1 indicate that ΔpH is and Δψ is not involved as a driving force for DNA entry in transformation. However, it should be noted that one would expect TPP⁺ to be about as inhibitory as valinomycin (Table 1), which is not the case. Possibly, in addition to its effect via the pH, TPP⁺ also interferes directly with DNA transport. The conclusion reached by Grinius

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**TABLE 3. Effect of ionophores on the release of transforming DNA from competent cells**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Transforming DNA (μg) per mg of protein at following time:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Control</td>
<td>0.75</td>
</tr>
<tr>
<td>10 μM Valinomycin</td>
<td>0.76</td>
</tr>
<tr>
<td>1 μM Nigericin</td>
<td>0.73</td>
</tr>
</tbody>
</table>

* In the standard procedure for the determination of the amount of transforming DNA bound to competent cells of *B. subtilis* 8G-5 (see the text), the incubation in the starvation medium was continued for an additional period of 30 min after proper additions had been made to the three parallel incubations.
transformation as the addition of salts or ionophores does not always show the effect that one would expect. The deviating effect of the addition of TPP⁺ is difficult to explain, although a similar effect of this cation on the ΔpH in Rhodopseudomonas sphaeroides has been noted (18).

DNA transport during transformation itself

![Graph](http://jb.asm.org/)

**FIG. 3.** Effect of ionophores and acetate on the production of acid-soluble donor DNA breakdown products. The results of panels A and B are obtained with two different batches of competent cells. For experimental details, see the text. The ionophores were added after DNA binding was established. Symbols: ○, control; ×, plus 10 μM valinomycin; Δ, plus 10 μM monensin; □, plus 1 μM nigericin; ▲, plus 100 mM potassium acetate; ■, plus 0.18 mM TPP⁺; and ●, plus 10 μM SF-6847.

(10), i.e., the involvement of both the ΔpH and Δψ components of the proton motive force in the transport of the transforming DNA across the cellular membrane, conflicts with our conclusion that mainly the ΔpH component is involved. The reason for this discrepancy is rather straightforward. Grinius et al. (3, 10, 11) have measured the effect of the ionophores on the end result of genetic transformation (i.e., numbers of transformed cells on agar plates), we studied their effect on both the binding and the transport of DNA. The dissipator of the Δψ component of the proton motive force, valinomycin, strongly inhibits the binding of DNA, not because of its Δψ dissipating activity but rather because of its interaction with the DNA receptor sites (Table 2). Therefore, the frequency of transformation will be, of course, strongly reduced. Thus, erroneous conclusions were reached by not taking into account that binding must occur before transport can proceed.

The results of the measurements of the proton motive force in *B. subtilis* compare well with values reported in the literature. At this stage only qualitative data of the Δψ can be presented. A quantitative determination of the Δψ would require a detailed investigation of the concentration dependent TPP⁺ binding to the cellular components (cell wall, cytoplasmic membrane, cytoplasm) (26; Loikima et al., in press). Hosoi et al. (13) report a value of 80 to 120 mV for Δψ at pH 7. Shioi et al. (22) report values of about 130 mV for the proton motive force in the pH range from 5 to 8, consisting of a Δψ of 105 mV and a ΔpH of 25 mV at pH 7. Somewhat lower values (ΔμH⁺ at approximately −80 mV from pH 5.5 to 7.5) were obtained by Khan and Macnab (15). However, the same authors report a value as high as 105 mV for Δψ at pH 7.5 (15) and furthermore, these values were determined by a filtration technique, which might lead to some underestimation. No significant differences were observed when the magnitude of the proton motive force was compared in *B. subtilis* W23 and *B. subtilis* 8G-5. In the experiments reported by Grinius et al., only a few values for the Δψ component of the proton motive force were given. Griniuviene et al. (11) reported values of 70 to 100 mV, whereas Chaustova et al. (3) mentioned a range of 100 to 140 mV, measured at pH 7.5 and 7.0, respectively. However, in both reports, these measurements were performed in a medium which differed completely from the starvation medium used for the transformation assay. Also, no measurements of the ΔpH component of the proton motive force were reported by Grinius. The results of the experiment described in Table 1 demonstrate the necessity of proton motive force determinations under the exact experimental conditions of transformation.
does not give rise to a depolarization of the proton motive force (data not shown). To a large extent, this is caused by the fact that only a fraction of the total number of cells is competent (26) so that a significant effect of DNA transport in the competent cells is obscured by cells in which no changes take place.

It is interesting to note that recently conflicting results have been reported about the role of the proton motive force in transformation in Escherichia coli. For this organism, Santos and Kaback (20) reported the involvement of the proton motive force in transport of plasmid DNA during Ca\(^{2+}\)- and heat shock-induced transformation. However, Sabelnikov and Domaradsky (21) concluded that "the proton motive force does not play any significant role in DNA entry into Ca\(^{2+}\)-treated Escherichia coli cells."

This investigation demonstrates that the proton motive force is involved in genetic transformation of Bacillus subtilis via an effect of the \(\Delta pH\) on the transport of DNA across the cytoplasmic membrane and not via modulation of DNA binding to the cell envelope. The \(\Delta pH\) dependence suggests that the DNA is transported edelectroneutrally, in symport with protons.

Grinius (10) concluded, on basis of the effects of potassium ionophores, that DNA uptake in \(B.\) subtilis transformation is an electronegenic process. However, as we have shown in this report, the potassium ionophores valinomycin and nigericin not only affect the composition of the proton motive force but also interact with the sites for DNA binding. Thus a straightforward interpretation of the effects of these potassium ionophores on transformation in \(B.\) subtilis is no longer possible.

**LITERATURE CITED**