Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*

R. Palmén, B. Vosman, P. Buikman, C. K. D. Breek and K. J. Hellingwerf*

Department of Microbiology & Biotechnology Centre, University of Amsterdam, Nwe. Achtergracht 127, 1018 WS Amsterdam, The Netherlands

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*Acinetobacter calcoaceticus* BD413 develops competence for natural transformation immediately after the start of the exponential growth-phase and remains competent up to a few hours into the stationary phase, after which competence gradually declines. The transformation frequencies obtained strongly depend on the kind of transforming DNA and the incubation time with DNA. Up to 25% of the cells in a culture can be transformed. DNA uptake in *Acinetobacter* does not display sequence specificity, is Mg²⁺-, Mn²⁺- or Ca²⁺-dependent and is uncoupler sensitive. The transforming DNA enters the cells in single-stranded form. These properties constitute a unique combination, not previously observed in other bacteria, and make *A. calcoaceticus* ideally suited for detailed studies of the bioenergetics of DNA translocation.

Introduction

Natural transformation is a feature that allows (prokaryotic) cells to take up naked DNA from the environment and exploit the coding capacity of the internalized nucleic acid. This transformation system is encoded by the genome of the recipient, and competence for natural transformation is induced under physiological conditions without the use of chemical or (bio)physical treatment of the cells as in artificial transformation. An understanding of this process is of importance for such widely diverse topics as safety of the release of genetically engineered organisms and estimation of the importance of sexual interactions between bacteria for prokaryotic evolution.

Natural transformation is observed in a wide range of organisms. Among these are representatives of both Gram-positive (e.g. *Bacillus subtilis*, Dubnau, 1991; *Streptococcus pneumoniae*, Avery et al., 1944), and Gram-negative bacteria (e.g. *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Azotobacter vinelandii*, *Pseudomonas stutzeri*; for a review see Stewart & Carlson, 1986). As more and more details of natural transformation in a number of these bacteria have been elucidated, it is becoming clear that distinct differences exist in this process between these organisms. Well-known examples are the sequence specificity of DNA uptake in *Haemophilus* (Goodgal, 1982) and *Neisseria* (Goodman & Scocca, 1991), which is not seen in some of the other bacteria, and the very pronounced and complex regulation of competence development in *Bacillus subtilis* (Dubnau, 1991). These differences suggest that not only the mechanism of DNA uptake in natural transformation differs among these bacteria, but that possibly its function varies also in different species.

*Acinetobacter calcoaceticus*, a Gram-negative, naturally transformable and metabolically versatile organism, has been the subject of much study over the last 30 years. These studies predominantly concerned the physiology of the organism, most often inspired by its very broad degradative capacity (Juni, 1978).

Our interest in *A. calcoaceticus* is to use it as a model to study natural transformation in Gram-negative bacteria, because high transformation frequencies can be obtained and it is easily grown in a competent state (Juni & Janik, 1969; Juni, 1972; Sawula & Crawford, 1972; Ahlquist et al., 1980). This is important because many basic questions remain unsolved. Three important questions follow. (i) What mechanism allows passage of a large nucleic acid molecule through the cell envelope of...
a (Gram-negative) bacterial cell? (ii) How is the investment of free energy coupled to this translocation process? (iii) What is the biological function of natural transformation in a particular organism? We think that, to tackle these questions successfully, a genetic approach must be used. Elsewhere, we describe the identification and characterization of the first set of mutants of *A. calcoaceticus*, which have an altered capacity to undergo natural transformation (Palmen *et al.*, 1992).

In this study, we present data on the physiological characterization of natural transformation in *A. calcoaceticus*. Using transformation frequencies, the optimal conditions for transformation have been determined. Additionally, we achieved a qualitative description of DNA uptake during natural transformation of *A. calcoaceticus*.

**Methods**

*Bacteria, media and chemicals.* The strains and plasmids used in this study are listed in Table 1. Restriction enzymes were used as recommended by the manufacturer (Pharmacia LKB). Luria–Bertani medium (LB) and LB-agar were prepared as described previously (Vosman & Hellingwerf, 1991). Minimal medium and minimal agar were prepared according to Juni (1974). They contained 60 mM-lactic acid, 11 mM-KH₂PO₄, 95 mM-Na₂HPO₄, 0.81 mM-MgSO₄, 37 mM-NH₄Cl, 0.068 mM-CaCl₂ and 1.8 µM-FeSO₄.

**DNA isolation.** Chromosomal DNA was isolated as described by Vosman & Hellingwerf (1991). Plasmid DNA was isolated according to the method of Ish-Horowicz & Burke (1981). Single-stranded DNA was obtained by heating double-stranded DNA at 95 °C for 10 min and subsequent rapid chilling on ice. Additional molecular genetic techniques were applied according to Maniatis *et al.* (1982).

**Transformation.** *A. calcoaceticus* was grown to competence as follows. An overnight culture in LB or minimal medium (1 ml) was diluted into 25 ml of fresh medium and cultured for an additional 2 h at 30 °C. At this stage the culture is competent for genetic transformation. For transformation, 0.5 ml of culture was incubated with approximately 2 µg DNA (unless stated otherwise) for 60 min at 30 °C. After incubation, 50 µg DNAase I (stock solution: 5 mg ml⁻¹) was added to prevent further DNA uptake. When prototrophy for isoleucine, valine and leucine (IVL) auxotrophy was selected, the transformation mixture was directly plated on minimal-agar plates without IVL. In case of selection for antibiotic resistance, the transformation mixture was incubated for an additional hour to allow expression of the marker. Antibiotic-resistant transformants were selected on media containing 15 µg kanamycin ml⁻¹, 100 µg rifampicin ml⁻¹ or 100 µg ampicillin ml⁻¹, depending on the resistance marker used. The viable count was determined on LB-plates. Colonies were counted after incubation for 2 d at 30 °C. The transformation frequency was calculated as the number of transformed cells, divided by the total viable count.

**Effect of pH on natural transformation.** *A. calcoaceticus* BD413 was grown in a minimal medium that consisted of 60 mM-lactic acid, 0.81 mM-MgSO₄, 37 mM-NH₄Cl, 0.068 mM-CaCl₂, 1.8 µM-FeSO₄ and 40 mM-potassium phosphate buffer of the required pH (in the range between 5 and 8). Cells were transformed either in the growth medium or, after transfer into fresh medium, at the indicated pH.

**EDTA treatment.** Cells grown to the competent state, as described above, were collected by centrifugation, washed in 25 mM-MOPS/NaOH buffer, pH 7.0, and transferred into an equal volume of the above buffer containing 0.5 mM-EDTA. The cells were incubated for 30 min at 30 °C, collected, washed and transferred into the buffers indicated. The transformation efficiency was calculated using the transformation frequency of an untreated sample as a reference.

**Inhibitor studies.** The effect of dinitrophenol (DNP) on the transformation efficiency was assayed by growing cells to the competent state on LB-medium containing an additional 20 mM-KCl, using the standard procedure (see section on transformation). DNP was added to 0.5 ml of cells, 10 min before addition of 2-4 µg pAVA213-8 DNA. The cells were incubated with DNA for 60 min at 30 °C, after which 50 µg DNAase I (5 mg ml⁻¹) was added and an expression time of 1 h was allowed before plating on selective media. The effect of DNP on the growth rate of *Acinetobacter* was determined by diluting an overnight culture 1:20 into LB-medium, supplemented with 0, 0.5, 1.0, 2.5 or 50 mM-DNP. Growth was determined by measuring the optical density at 540 nm.

**Identification of single-stranded DNA.** Competent AAC400 cells (20 ml) were incubated for 1.5 h with 8 µg pAVA213-8 DNA at 30 °C. The bacteria were collected by centrifugation for 10 min at 12000 g and lysed in 8 ml TrisEDTA (50 mM-Tris/HC1, 20 mM-EDTA, pH 7.6), 350 µl 30% (w/v) Sarkosyl and 300 µl proteinase K (10 mg ml⁻¹) for 30 min at 45 °C. The lysate was extracted three times with phenol/chloroform.

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype/phenotype</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. calcoaceticus</em> BD413</td>
<td>Wild-type</td>
<td>Juni (1972)</td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> BD413-ivl10</td>
<td>Auxotrophic for IVL</td>
<td>Juni (1972)</td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> AAC1</td>
<td>Rif²</td>
<td>This study*</td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> BD413-ivl10::pAVA213-8</td>
<td>Auxotrophic for IVL, Kan¹</td>
<td>This study*</td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> AAC400</td>
<td>recA::nptII</td>
<td>Palmen <em>et al.</em> (1992)</td>
</tr>
<tr>
<td><em>A. calcoaceticus</em> AAC400-ivl10</td>
<td>Auxotrophic for IVL, recA::nptII</td>
<td>Palmen <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>pGV1</td>
<td>Kan¹</td>
<td>Vosman <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>pWH11274</td>
<td>Amp² Tet²</td>
<td>Hunger <em>et al.</em> (1990)</td>
</tr>
<tr>
<td>pAVA213-8</td>
<td>Amp² Kan¹</td>
<td>Palmen <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>pAVA213-41</td>
<td>Amp² Kan¹</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Spontaneous rifampicin resistant mutant of *A. calcoaceticus* BD413. † Obtained after transforming *A. calcoaceticus* BD413-ivl10 with pAVA213-8 and selection for kanamycin resistance. ‡ Obtained via transfer of the *A. calcoaceticus* AAC400-ivl10 RecA mutation to *A. calcoaceticus* BD413.
Natural transformation in Acinetobacter calcoaceticus

(1:1, v/v) and once with chloroform/isoamylalcohol (24:1, v/v). Subsequently, the DNA was precipitated with an ethanol/acetate mixture (96% ethanol and 3 M-potassium acetate; 25:1, v/v) and dissolved in 200 µl T<sub>2</sub>E<sub>20</sub>. All DNA in this sample was purified by electrophoresis on a 0.8% agarose gel with single-stranded pAVA213-8 DNA as a marker. To concentrate and to get rid of RNA and some of the chromosomal DNA, fragments, between 2.5 and 11 kb in size, were isolated via electro-elution and after ethanol precipitation, resuspended in 200 µl T<sub>2</sub>E<sub>20</sub> (single- and double-stranded pAVA213-8 runs in this size range). This fraction was re-run on a 0.8% agarose gel and transferred to nitrocellulose in 10 x SSC, backed for 1 h at 80 °C and analysed by Southern hybridization according to the protocol supplied by Boehringer Mannheim. pAVA213-8 DNA was used as a probe, after labelling with digoxigenin-dUTP, according to the manufacturer (Boehringer Mannheim).

Reproducibility. The quantitative reproducibility of the transformation data is affected by (i) the level of competence reached in the separate batches of cells used in the experiments, and (ii) the deviation between equally treated samples from the same competent culture. To provide some insight in the variation that exists between experiments, we calculated the mean and standard deviation of eleven transformations of A. calcoaceticus with pAVA213-8 DNA. The observed transformation frequencies ranged between 1-6 x 10<sup>-5</sup> and 9.9 x 10<sup>-4</sup>, with a mean of 5.6 x 10<sup>-5</sup> and a standard deviation of 2.9 x 10<sup>-4</sup>. The variation between transformation frequencies within one competent culture was much smaller. To demonstrate this, we analysed the transformation frequencies from five datapoints from one experiment (Fig. 4; pAVA213-8 DNA, from 2 to 20 µg ml<sup>-1</sup>). The transformation frequencies ranged between 4.3 x 10<sup>-3</sup> and 5.6 x 10<sup>-3</sup>, with a mean of 5.1 x 10<sup>-3</sup> and a standard deviation of 5.4 x 10<sup>-4</sup>. Thus, the variability between experiments was much larger than within one experiment. For this reason we have chosen to present most of our data from single experiments, rather than determining mean values and standard deviations from multiple experiments.

Results

Effect of DNA type and selection on transformation efficiency

To investigate the effect of different types of DNA and selection markers on the transformation efficiency of A. calcoaceticus, transformation experiments were performed using different types of DNA as listed in Table 2. From these results, it can be seen that when chromosomal DNA was used as transforming DNA and transformants were selected on the basis of resistance to kanamycin, rifampicin or prototrophy for IVL (using

<table>
<thead>
<tr>
<th>Description</th>
<th>Selection</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal DNA from A. calcoaceticus AAC1</td>
<td>Rifampicin resistance</td>
<td>1 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chromosomal DNA from A. calcoaceticus AAC1</td>
<td>Prototrophy for IVL</td>
<td>1.5 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chromosomal DNA from A. calcoaceticus BD413-ivl10::pAVA213-8</td>
<td>Kanamycin resistance</td>
<td>2 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasmid pAVA213-8</td>
<td>Kanamycin resistance</td>
<td>9 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasmid pAVA213-38</td>
<td>Kanamycin and ampicillin resistance</td>
<td>1.2 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasmid pAVA213-41</td>
<td>Kanamycin resistance</td>
<td>1 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Single-stranded plasmid pAVA213-8</td>
<td>Kanamycin resistance</td>
<td>7 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasmid pGV1</td>
<td>Kanamycin resistance</td>
<td>8 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasmid pWH1274</td>
<td>Ampicillin resistance</td>
<td>2 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
strain BD413-ivl10, auxotrophic for isoleucine, valine and leucine), selection for rifampicin resistance yielded transformation frequencies that were a factor of 100 lower than the transformation frequencies obtained with the other selection markers. Thus, the rifampicin-resistant marker proved to be a poor selection marker when used in transformation experiments with Acinetobacter. This was also seen when the transformation efficiency of an Acinetobacter culture, using the rifampicin-resistant marker, was followed during growth in batch culture (Fig. 1). When the transformation frequency, used as an indicator for the level of competence for natural transformation, was monitored formation frequency, used as an indicator for the level of growth in batch culture (Fig. 1). When the trans-

The following diagram shows the plasmid map of pAVA213-8 and pAVA213-41.

![Plasmid Map](image)

**Fig. 2.** Plasmid map of pAVA213-8 and pAVA213-41. E, EcoRI; Ev, EcoRV; H, HindIII; P, PstI; S, Sall. The restriction maps do not show all the HindIII sites that are present in these plasmids.

When Acinetobacter was transformed with plasmid pAVA213-8 (Fig. 2). This is a pUC18-based plasmid, containing a 6.4 kb chromosomal DNA fragment from A. calcoaceticus in which an nptII gene (encoding kanamycin resistance) has been inserted (Palmen et al., 1992). pUC18-derived plasmids do not, or only very poorly, replicate in Acinetobacter. When Acinetobacter is transformed with pAVA213-8, kanamycin-resistant transformants originate only after insertion of the kanamycin marker into the chromosome. The insertion of the kanamycin marker is facilitated by the flanking chromosomal fragments and can take place in two ways: (i) via replacement recombination, resulting from homologous recombination events on both flanking sequences, or (ii) via a Campbell-like mode of insertion, resulting from a single recombination event. In the case of a Campbell-like integration, the entire plasmid will be integrated, whereas replacement recombination results in insertion of the kanamycin marker only. Transformants arising from a Campbell-like integration will be kanamycin and ampicillin resistant. Ampicillin resistance is encoded by the vector part of pAVA213-8 and will only be retained when the entire plasmid is integrated into the chromosome. Comparing the number of kanamycin-resistant transformants with kanamycin- and ampicillin-resistant transformants within one transformation experiment (Table 2) showed that replacement integration of the kanamycin marker of pAVA213-8 occurs 1000 times more frequently than Campbell-like integration.

To investigate which mode of integration is preferred, a new construct was devised, named pAVA213-41 (Fig. 2), in which the kanamycin-resistance gene is incorporated between the chromosomal insert of pAVA213-8 (without the kanamycin marker) and the vector part of the plasmid. Integration of the kanamycin marker of this construct into the recipient chromosome can only occur via a Campbell-like mechanism. Interestingly, transformation frequencies obtained with pAVA213-41 (Table 2) were about 50–100 times lower than with pAVA213-8. This indicates that replacement recombination is more efficient than Campbell-like integration.

To test whether single-stranded chromosomal Acinetobacter DNA could give rise to transformants, the chromosomal insert of pAVA213-8, containing the kanamycin marker, was cut out by restriction with EcoRI and PstI, and subsequently denatured by heating for 10 min at 95 °C. Restriction of pAVA213-8 with these enzymes had no effect on the transformation efficiency (result not shown). Transformation with single-stranded DNA though (Table 2), resulted in a very much lower transformation frequency. The residual number of transformants is probably due to the presence of contaminating double-stranded copies, still present in
Table 3. Transformation frequencies of *Acinetobacter calcoaceticus* BD413, grown in mineral medium containing different carbon sources and grown on LB-medium

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid (60 mM)</td>
<td>1.1 x 10^{-2}</td>
</tr>
<tr>
<td>Glucose (55 mM)</td>
<td>4.7 x 10^{-2}</td>
</tr>
<tr>
<td>Ethanol (1%)</td>
<td>5.5 x 10^{-2}</td>
</tr>
<tr>
<td>Hexadecane (1%)</td>
<td>0.9 x 10^{-2}</td>
</tr>
<tr>
<td>Luria-Bertani medium</td>
<td>5.5 x 10^{-2}</td>
</tr>
</tbody>
</table>

Cultures were transformed with pAVA213-8 via the standard procedure and selected for kanamycin resistance.

The denatured DNA sample, although it cannot be excluded that some single-stranded DNA is taken up.

Growing *A. calcoaceticus* BD413 on different carbon sources had no effect on competence induction. From Table 3 it can be concluded that competence is induced to the same extent in minimal media supplemented with 40 mM-potassium phosphate buffer of various pH values. The pH was determined at the time of sampling for the transformation assay. Samples were transformed at the culture pH (O), and, after transfer of the cells into standard fresh medium, were transformed at pH 6.7, (\(\nabla\)).

Effect of pH on competence induction

*A. calcoaceticus* is capable of growing in a pH range from 5 to 8. It is worthwhile to see if competence is also induced during growth in this pH range. From Fig. 3 it can be seen that if cells are transformed when growing at a pH below 6.5, the transformation frequency is reduced moderately at pH 6.0 and severely at pH 5.4. This does not mean that competence induction is inhibited at acidic pH, since cells that are grown at acidic pH and subsequently transferred to a pH of 6.7 exhibit transformation frequencies comparable to cells grown at a neutral pH (Fig. 3). To exclude competence induction after transfer of the cells into the fresh medium at pH 6.7, the complementary experiment was also performed. Cells were grown to competence at pH 7.0 and transformed at pH 7.0, 6.0 and 5.3. Here also, a reduction of the transformation frequency was observed when the cells were transformed at an acidic pH (results not shown). Thus, below a pH of 6.5, it is not competence induction that is inhibited, but the process of DNA uptake and/or integration.

Effect of DNA concentration on transformation

To study the relationship between the amount of DNA added and the efficiency of transformation, *A. calcoaceticus* was transformed with different concentrations of plasmid pGV1 (3.95 kb) and pAVA213-8 (10.5 kb) DNA, and with chromosomal DNA from *A. calcoaceticus* BD413-lv110::pAVA213-8. The results are shown in Fig. 4. The transformation frequency is plotted on a logarithmic scale because of the large dynamic range of five orders of magnitude in which the transformation process can be assayed. For clarity, an insert has been added in which the DNA concentrations are re-plotted logarithmically. It is clear that transformation follows saturation kinetics. Plasmid pGV1 DNA saturates above 20 µg (ml cells)^{-1}, whereas chromosomal DNA and plasmid pAVA213-8 saturate at approximately 1–2 µg (ml cells)^{-1}. This latter observation was to be expected since transformants resulting from pAVA213-8 and chromosomal DNA are
generated via the same mechanism. Both types of DNA are taken up via the same uptake system [see Effect of competing DNA on transformation efficiency (below) and Lorenz et al. (1992)], and both have to integrate their selective marker into the chromosome. The difference in the maximal level of transformation between chromosomal DNA and pAVA213-8 DNA can be explained by taking into account the ratio between DNA fragments selected for (i.e., containing the kanamycin marker) and competing fragments without the kanamycin marker. Plasmid pAVA213-8 contains, in addition to the kanamycin marker, 9 kb of DNA, whereas chromosomal DNA contains approximately 4000 kb of DNA per kanamycin marker (the exact size of the chromosome has not been determined yet). Thus, pAVA213-8 contains more copies of the selective marker per μg DNA than chromosomal DNA, resulting in an increased maximal transformation frequency.

### Transformation as a function of the length of the incubation period with plasmid DNA

To obtain more information about DNA uptake as a function of incubation time, *Acinetobacter* was transformed with pAVA213-8 and transformation was interrupted by addition of DNAase I, at various times after addition of DNA (Fig. 5). When DNAase I was added before addition of DNA, no transformants were found upon selection for kanamycin resistance. The first transformants are already present after incubation for 1 min (Fig. 5b; note that the figure is a logarithmic plot). The number of transformants increased linearly up to incubation for 2 h (Fig. 5c) before transformation saturated. After 3 h, about 25% of the cells in the culture had been transformed to kanamycin resistance (Fig. 5a).

### Effect of divalent cations on transformation

In *Bacillus subtilis* (Mulder & Venema, 1982a,b), *Streptococcus pneumoniae* (Lacks et al., 1975) and *Azotobacter vinelandii* (Page & von Tigerstrom, 1979), transformation is dependent on the presence of Mg²⁺ and/or Ca²⁺ ions. After treatment of *A. calcoaceticus* with EDTA, to remove divalent cations, transformation was severely inhibited (Table 4). Addition of 5 mM-Mg²⁺ partially restored transformation up to 3% of the wild-type level. Addition of 5 mM-Ca²⁺ or 0.5 mM-Mn²⁺ restored transformation to 1%. Simultaneous addition of 5 mM-Mg²⁺ and 5 mM-Ca²⁺ restored transformation up to 7%. If EDTA-treated cells were resuspended in minimal medium without a carbon source, transformation was restored up to 7.7%. The minimal medium contains 0.81 mM-Mg²⁺ and 0.068 mM-Ca²⁺. This indicates that at 5 mM, Mg²⁺ and Ca²⁺ were present at saturating concentrations. When EDTA-treated cells were suspended in LB-medium, transformation was

![Fig. 5. Transformation frequency of *A. calcoaceticus* BD413 as a function of the incubation time with pAVA213-8 DNA. (a), (b) and (c) are referred to in the text.](image)

### Table 4. Effect of divalent cations on transformation

<table>
<thead>
<tr>
<th>EDTA treatment</th>
<th>Resuspended in:</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no</td>
<td>Mineral medium</td>
<td>100</td>
</tr>
<tr>
<td>yes</td>
<td>MOPS buffer</td>
<td>0.1</td>
</tr>
<tr>
<td>yes</td>
<td>MOPS buffer + 5 mM-MgCl₂</td>
<td>3.3</td>
</tr>
<tr>
<td>yes</td>
<td>MOPS buffer + 5 mM-CaCl₂</td>
<td>1.2</td>
</tr>
<tr>
<td>yes</td>
<td>MOPS buffer + 5 mM-MgCl₂ + 5 mM-CaCl₂</td>
<td>7.0</td>
</tr>
<tr>
<td>yes</td>
<td>MOPS buffer + 0.5 mM-MnCl₂</td>
<td>1.1</td>
</tr>
<tr>
<td>yes</td>
<td>MOPS buffer + 0.5 mM-ZnCl₂</td>
<td>0.004</td>
</tr>
<tr>
<td>yes</td>
<td>MOPS buffer + 0.5 mM-CuCl₂</td>
<td>No viable count</td>
</tr>
<tr>
<td>yes</td>
<td>Mineral medium without lactic acid</td>
<td>7.7</td>
</tr>
<tr>
<td>yes</td>
<td>Mineral medium with lactic acid</td>
<td>8.5</td>
</tr>
<tr>
<td>yes</td>
<td>LB medium</td>
<td>31</td>
</tr>
</tbody>
</table>
restored up to 31% of the original level. The effect of
divalent cations on transformation was studied in MOPS
buffer without addition of an energy source. To test
whether supplying an energy source could explain the
improved transformation in LB-medium, cells were
treated with EDTA and resuspended in minimal medium
containing 60 mM-Lactic acid. The resulting transforma-
tion frequency of 8.5% was comparable to the value
obtained after resuspending the cells in minimal medium
without a carbon source. This indicates that after
transformation with EDTA and resuspending the cells in
MOPS buffer, transformation is not limited by a lack of
energy. The factor responsible for the extra restoration
of transformation in LB-medium has not yet been
identified. Another interesting observation was that
addition of 0.5 mM-Zn²⁺ specifically inhibited trans-
formation. Zn²⁺ might be of use as a specific inhibitor
of transformation. Addition of 0.5 mM-Cu²⁺ to EDTA-
treated cells proved to be toxic for the cells as it severely
reduced the viable count.

Effect of energization of competent cells on the
transformation efficiency

It is generally assumed that DNA uptake during natural
transformation is an energy-requiring process (Grinius,
1987). Growing Acinetobacter in the presence of different
concentrations of dinitrophenol (DNP), a compound
that dissipates the proton motive force, showed that
growth is moderately inhibited at a concentration of
1 mM and completely inhibited at 2.5 mM-DNP (Table 5).
When DNP was added to a competent culture (grown to
competence via the standard procedure in LB-medium)
10 min before addition of DNA, transformation was
inhibited at DNP concentrations higher than 1 mM
(Table 5). This shows that energy supply, in the presence
of 1 mM-DNP, becomes limiting for both growth and
transformation, with growth being more sensitive. The
energy requirement of transformation could also be
demonstrated with the protonophores 5-chloro-3-tert-
butyl-4-hydroxybenzilidene malononitrile (SF6847; data not shown).

<table>
<thead>
<tr>
<th>DNP concn (mM)</th>
<th>Growth rate (h⁻¹)</th>
<th>Final OD₅₄₀</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>0.83</td>
<td>2.9</td>
<td>1.6 x 10⁻²</td>
</tr>
<tr>
<td>0.5</td>
<td>0.79</td>
<td>2.5</td>
<td>1.9 x 10⁻²</td>
</tr>
<tr>
<td>1.0</td>
<td>0.52</td>
<td>1.8</td>
<td>1.0 x 10⁻²</td>
</tr>
<tr>
<td>2.5</td>
<td>No growth</td>
<td></td>
<td>5.5 x 10⁻²</td>
</tr>
<tr>
<td>5.0</td>
<td>No growth</td>
<td></td>
<td>1.9 x 10⁻³</td>
</tr>
</tbody>
</table>

Effect of competing DNA on transformation frequency

Acinetobacter is capable of transformation using both
chromosomal and plasmid DNA. To tackle the question
whether or not both types of DNA are transported via
the same uptake system, Acinetobacter was transformed
with plasmid pGV1 in the presence of different
concentrations of chromosomal Acinetobacter DNA.
The transformation frequencies resulting from uptake
and maintenance of pGV1 were monitored (Table 6). Transformation with 2 µg pGV1, without addition of
chromosomal DNA, was used as a reference. Increasing
amounts of chromosomal Acinetobacter DNA resulted
in decreasing transformation frequencies with pGV1.
This means that chromosomal DNA and plasmid DNA
compete for the same uptake system. Due to the
difference in fragment length of chromosomal (a large
range of sizes) and pGV1 (3.95 kb) DNA, addition of
2 µg chromosomal DNA to 2 µg plasmid DNA does not result in a 50% inhibition of transformation. When
chromosomal DNA from Pseudomonas stutzeri was used
as competing DNA, the same level of inhibition of
transformation with pGV1 was observed. This indicates
that Acinetobacter does not discriminate between hom-
ologous and heterologous DNA at the stage of binding
and uptake. Similar results were obtained by Lorenz et al., 1992.

Effect of DNA topology

Whether or not DNA in Acinetobacter is translocated in
a single- or double-stranded form has yet to be
determined, and this issue is still rather controversial
for other Gram-negative bacteria (Doran et al., 1987;
Goodgal, 1982). One way to obtain more information
about this process is to investigate the effects of plasmid
topology on transformation. When undigested pGV1
DNA was used as transforming DNA, the transforma-
tion frequency was 8.4 x 10⁻⁴ (Table 7). Transformation
with monomers of pGV1 gave the same efficiency of
transformation as a mixture of mono- and multimers
(data not shown). This means that transformation with

Table 6. Effect of competing chromosomal DNA on
efficiency of transformation with plasmid pGV1 DNA

<table>
<thead>
<tr>
<th>Competing DNA</th>
<th>Transformation frequency</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.1 x 10⁻⁴</td>
<td>100</td>
</tr>
<tr>
<td>0.5 µg A. calcoaceticus</td>
<td>2.3 x 10⁻⁵</td>
<td>324</td>
</tr>
<tr>
<td>2.0 µg A. calcoaceticus</td>
<td>7.7 x 10⁻⁵</td>
<td>108</td>
</tr>
<tr>
<td>5.0 µg A. calcoaceticus</td>
<td>4.7 x 10⁻⁶</td>
<td>6.7</td>
</tr>
<tr>
<td>100 µg A. calcoaceticus</td>
<td>1.6 x 10⁻⁶</td>
<td>2.2</td>
</tr>
<tr>
<td>100 µg P. stutzeri</td>
<td>1.6 x 10⁻⁶</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Table 7. Effect of DNP on transformation frequency
and growth of Acinetobacter calcoaceticus BD413

<table>
<thead>
<tr>
<th>DNP concn (mM)</th>
<th>Growth rate (h⁻¹)</th>
<th>Final OD₅₄₀</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.83</td>
<td>2.9</td>
<td>1.6 x 10⁻²</td>
</tr>
<tr>
<td>0.5</td>
<td>0.79</td>
<td>2.5</td>
<td>1.9 x 10⁻²</td>
</tr>
<tr>
<td>1.0</td>
<td>0.52</td>
<td>1.8</td>
<td>1.0 x 10⁻²</td>
</tr>
<tr>
<td>2.5</td>
<td>No growth</td>
<td></td>
<td>5.5 x 10⁻²</td>
</tr>
<tr>
<td>5.0</td>
<td>No growth</td>
<td></td>
<td>1.9 x 10⁻³</td>
</tr>
</tbody>
</table>
Table 7. Effect of plasmid topology on transformation efficiency with plasmid pGV1

<table>
<thead>
<tr>
<th>Plasmid form</th>
<th>Topology</th>
<th>Transformation frequency</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undigested</td>
<td></td>
<td>5.4 x 10^{-4}</td>
<td>100</td>
</tr>
<tr>
<td>Smal digested (2 μg)</td>
<td>Blunt</td>
<td>1.2 x 10^{-6}</td>
<td>0.23</td>
</tr>
<tr>
<td>PstI digested (2 μg)</td>
<td>3' Overhang</td>
<td>5.8 x 10^{-7}</td>
<td>0.11</td>
</tr>
<tr>
<td>EcoRI digested (2 μg)</td>
<td>5' Overhang</td>
<td>4.0 x 10^{-7}</td>
<td>0.074</td>
</tr>
<tr>
<td>ClaI digested (2 μg)</td>
<td>5' Overhang</td>
<td>8.5 x 10^{-8}</td>
<td>0.016</td>
</tr>
<tr>
<td>EcoRI digested (10 μg) + ClaI digested (1 μg)</td>
<td></td>
<td>3.2 x 10^{-5}</td>
<td>3.7</td>
</tr>
</tbody>
</table>

plasmid DNA in *Acinetobacter* is not dependent on the presence of multimers, as in *Bacillus subtilis* (Canosi et al., 1978; de Vos et al., 1981). When pGV1 was restricted with EcoRI or ClaI (creating 5' overhanging ends), PstI (creating 3' overhanging ends) or Smal (creating blunt ends), transformation was severely inhibited (pGV1 has a unique restriction site for each of these enzymes). When, on the other hand, a mixture of plasmids was used, restricted with different enzymes (i.e. EcoRI and ClaI), transformation could be partially restored. The EcoRI and ClaI site are oppositely located in the intact plasmid. Recircularization of the EcoRI-digested plasmid after transformation is most likely facilitated by a ClaI-digested copy of the plasmid and vice versa. The second copy allows closure of the restriction site generated by the first enzyme, resulting in a partially double-stranded plasmid, which can be repaired by the enzymic machinery of the host cell. From these results, it is not certain yet whether DNA enters in single- or in double-stranded form. If plasmid DNA were to enter single-strandedly, recircularization would be promoted by homologous base-pairing of the single-stranded DNA, bridging the two restriction sites. On the other hand, multiple plasmid DNA fragments entering linearly in a double-stranded fashion could recircularize via homologous recombination. The latter possibility implies that plasmids are not able to transform a recombination-deficient strain. However, a previously constructed RecA-deficient *A. calcoaceticus* strain AAC400 (derived from BD413) displayed almost wild-type transformation frequencies with plasmid DNA. The transformation frequency of *A. calcoaceticus* BD413 obtained with plasmid pWH1274 was 2.0 x 10^{-3}, whereas for AAC400 a value of 5.7 x 10^{-4} was obtained. This proves that plasmid recircularization is a RecA-independent mechanism and suggests, together with the results given above, that DNA enters in a single-stranded fashion.

To prove that transforming DNA is taken up single-strandedly, the following experiment was devised (adapted from Stachel et al., 1986). The RecA-deficient *Acinetobacter* strain (AAC400-iv110, Palmen et al., 1992) was incubated with pAVA213-8 DNA. Subsequently, DNA was extracted from these cells, electrophoresed and blotted under non-denaturing conditions onto a nitrocellulose filter. Under these conditions only single-stranded DNA or double-stranded DNA to which protein is bound, is transferred to the filter. The filter was

Fig. 6. Southern hybridization of *A. calcoaceticus* AAC400-iv110 DNA transformed with pAVA213-8 DNA. Lane 1, non-transformed AAC400-iv110; lane 2, AAC211 transformed with pAVA213-8; lane 3, AAC400-iv110 transformed with pAVA213-8; lane 4, 50 ng single-stranded pAVA213-8; lane 5, 200 ng single-stranded pAVA213-8; lane 6, 1000 ng single-stranded pAVA213-8. ss, Single-stranded pAVA213-8; ds, double-stranded pAVA213-8.
subsequently hybridized, using pAVA213-8 DNA as a probe. In lanes 4, 5 and 6 of Fig. 6, single-stranded pAVA213-8 DNA (respectively 50, 200 and 1000 ng) was layered as a positive control. In lane 6, a hybridization band of double-stranded pAVA213-8 can be observed also, probably because of the large amount of DNA that was blotted. This signal is not visible in lanes 4 and 5. In lane 3, containing DNA extracted from AAC400-iv110 after incubation with pAVA213-8, a clear hybridizing signal is present. This signal is absent in lane 1, which contained DNA from an AAC400-iv110 culture that had not been incubated with pAVA213-8 DNA.

One could argue that the hybridizing material originated directly from the added DNA. This is unlikely, however, because the plasmid DNA used was purified via equilibrium centrifugation in a caesium chloride gradient and no single-stranded DNA could be detected in that sample. To further check whether the signal observed in lane 3 is transformation related, strain AAC211, a transformation-deficient mutant (Palmen et al., 1992) of strain BD413-iv110, was incubated with pAVA213-8. DNA extracted from AAC211 after incubation, was layered in lane 2. From these cells, no hybridizing DNA could be extracted. These results lead to the conclusion that the signal observed really represents single-stranded DNA present inside the cells. Thus, the incoming DNA is converted into a single-stranded form during uptake for natural transformation in A. calcoaceticus.

Discussion

The main characteristic of genetic transformation is the change in genotype of the recipient organism after incorporation, or in the case of plasmid DNA, replication, of the DNA taken up. To be able to detect transformation via a change in genotype, a clearly selectable marker is a prerequisite. Our first experiments were devised to optimize the selection marker and the kind of DNA (chromosomal or plasmid DNA) bearing the marker. From these experiments, we concluded that rifampicin resistance is a very poor marker for use in A. calcoaceticus. The maximal transformation frequencies with this marker are low and activity seems to be dependent on the growth phase of the culture (Fig. 1). A large increase in transformation efficiency was obtained when plasmid pAVA213-8, containing a kanamycin-resistance marker flanked by Acinetobacter sequences, was used as transforming DNA. Compared to transformations with other plasmids like pGV1, pWH1274 or pKT210 (not shown), pAVA213-8 yields transformation frequencies that are about 50 times higher. pAVA213-8 does not replicate in Acinetobacter and the kanamycin-resistance marker must recombine into the chromosome to be able to transform Acinetobacter into a kanamycin-resistant state. Integration of the kanamycin-resistance gene is facilitated by the flanking Acinetobacter sequences. From the transformation frequencies obtained with plasmid pAVA213-8, pGV1 and pWH1274, one has to conclude that the process of integration of the marker into the chromosome via homologous recombination is much more efficient than the process of plasmid recircularization. This effect can also be seen in the experiment on the affinity of transformation for chromosomal and plasmid DNA (Fig. 4). Uptake of a plasmid that has to recircularize in order to be propagated saturates at much higher DNA concentrations than uptake of pAVA213-8 or chromosomal DNA. Chromosomal DNA is often used in transformation assays of naturally transformable bacteria. The use of pAVA213-8-like transformation constructs may increase the observed maximal transformation frequencies in these organisms too.

The main purpose of this investigation was to determine basic characteristics of natural transformation in Acinetobacter and to compare this transformation system with the systems reported in other Gram-negative (Haemophilus and Neisseria) and Gram-positive (Bacillus and Streptococcus) bacteria. From the competition experiments, it is concluded that plasmid DNA and chromosomal DNA are taken up via the same system and that Acinetobacter does not discriminate between heterologous and homologous DNA. This is also found for the transformation systems of the Gram-positive genera Bacillus and Streptococcus, and differs from the transformation system of Haemophilus and Neisseria, which both discriminate between heterologous and homologous DNA via specific sequences present on their DNA (Goodgal, 1982; Goodman & Scocca, 1991). From our inhibitor studies we can conclude that natural transformation is an energy-requiring process in Acinetobacter, as it is in Bacillus and Streptococcus (Grinius, 1987). Our data indicate that growth is more sensitive to dissipation of the protonmotive force than transformation (Table 5).

DNA transport across the cytoplasmic membrane in natural transformation occurs in a single-stranded fashion in Bacillus (Piechowska & Fox, 1971; Davidoff-Abelson & Dubnau, 1973), and Streptococcus (Morrison & Guild, 1973) and possibly also in Haemophilus (Kahn et al., 1983). We have shown here that this is also the case in Acinetobacter. From the hybridization experiment, one can conclude that DNA is taken up in a single-stranded fashion. The hybridizing signal in lane 3, representing single-stranded pAVA213-8, is rather broad and is not contained in a single sharp band; neither are the single-stranded controls. This could be the result of one or more of a number of factors. (i) The plasmid was
taken up only partially, leading to an array of plasmid sizes present in the extracted DNA. (ii) Mechanical shear during the phenol/chloroform extraction steps could fracture the DNA, again leading to smaller fragments. (iii) Re-hybridization of the single-stranded fragments during the isolation procedure could lead to increased fragment sizes. (iv) The formation of secondary structure could occur, leading to unpredictable electrophoretic behaviour. (v) Single-stranded nicks present in the pAVA213-8 DNA could give rise to an array of fragment sizes after denaturation of the DNA. Since chromosomal and plasmid DNA are taken up via the same system, both will enter the cell single-strandedly. This is in agreement with the results of the plasmid topology experiments, which suggested the conversion of double-stranded into single-stranded DNA during uptake. This probably also explains why integration via replacement recombination is favoured over Campbell-like integration. After uptake, a closed circular double-stranded plasmid is converted into a linear single-stranded DNA molecule. In the case of pAVA213-41, integration of the entire plasmid can occur only after recircularization of the plasmid and a subsequent homologous recombination event. Recircularization can be promoted by the resident chromosome, provided the plasmid was linearized during uptake in the Acinetobacter fragment of pAVA213-41. Or, if the plasmid was linearized in the vector part or the kanamycin-resistance gene of the plasmid, it can recircularize via a second copy of the plasmid restricted at a separate site. This process of recircularization is not necessary in the case of integration via replacement recombination. The incoming single-stranded DNA molecule of pAVA213-8 can directly pair with the resident chromosome and integrate via two RecA-dependent recombination events, provided that the plasmid was not linearized within about 1 kb of the kanamycin-resistance marker. The process of recircularization has a lower efficiency than the process of homologous recombination, as shown in the saturation experiment with chromosomal and plasmid DNA (Fig. 4).

_Bacillus subtilis_ and _Streptococcus pneumoniae_ are capable of binding single- and double-stranded DNA (Smith _et al._, 1985; Lacks, 1977), but only double-stranded DNA is capable of transforming these organisms (Rudolph _et al._, 1986; Lacks, 1962). During uptake, one of the strands of the DNA helix is degraded by membrane-bound nucleases and the complementary strand is internalized (Lacks _et al._, 1975; Lacks & Neuberger, 1975). Uptake of DNA is inhibited after removal of divalent cations by EDTA treatment. Mg\(^{2+}\) proved to be necessary for the correct function of the membrane-bound nucleases (Lacks _et al._, 1975; Mulder & Venema, 1982a,b), whereas Ca\(^{2+}\) is additionally required for uptake of DNA in _S. pneumoniae_ (Seto & Tomasz, 1976). Transformation of _Haemophilus_, on the other hand, is not inhibited after EDTA treatment (Noteborn _et al._, 1981). Transformation in _Acinetobacter_ also shows a requirement for Mg\(^{2+}\) and/or Ca\(^{2+}\) and/or Mn\(^{2+}\). From this, combined with the fact that DNA enters _Acinetobacter_ in a single-stranded form, we expect the involvement of a membrane-located nuclease in DNA uptake during natural transformation in _Acinetobacter_ too.

From Fig. 5, an approximate DNA uptake rate can be calculated. pAVA213-8 is a plasmid of 10.5 kb. The first transformants arise within 1 min of incubation with DNA. The minimum DNA fragment size needed to transform _Acinetobacter_ into a kanamycin-resistant form is about 3.5 kb. The kanamycin marker is 1.4 kb and about 1 kb of flanking homologous sequences on each side of the marker are needed for integration into the chromosome (R. Palmen and others, unpublished results; L. A. Gregg-Jolly & L. N. Ornston, unpublished results). Thus, at least 3.5 kb had to be taken up in 1 min to give rise to a kanamycin-resistant transformant. This results in a minimal uptake rate of about 60 nucleotides s\(^{-1}\). This value does not include the time required for binding and therefore is an underestimate. This rate is comparable with uptake rates determined for _Bacillus_ and _Streptococcus_ which are in the order of 80–180 nucleotides s\(^{-1}\) (Grinius, 1987; Dubnau, 1991). Uptake rates in _Haemophilus_ are much higher (500–30000 nucleotides s\(^{-1}\); Grinius, 1987). The high nucleotide uptake rate and EDTA-insensitivity of transformation in _Haemophilus_ is probably due to the specific uptake mechanism via so called transformasomes (Kahn _et al._, 1982). A transformasome is a vesicle-like structure at the surface of the cell envelope. These vesicles can bind double-stranded DNA and are subsequently transported from the outer to the inner membrane, where the DNA is released into the cytoplasm. DNA uptake via transformasomes has only been detected in _Haemophilus_ so far.

With respect to the above-mentioned properties, the transformation system of _Acinetobacter_ does not resemble the transformation system studied in species of the Gram-negative genera _Haemophilus_ or _Neisseria_. On the contrary, it is more similar to the systems in _Bacillus_ and _Streptococcus_. Differences between the Gram-positive system and the _Acinetobacter_ system are mainly restricted to the regulation of competence development. No competence factor has been detected in _Acinetobacter_ (Juni, 1978), in contrast to _Streptococcus_. Competence in _A. calcoaceticus_ BD413 is maximal in the exponential growth-phase (Fig. 1). It is not as strictly regulated as competence induction in a number of other organisms,
which induce competence only under specific growth conditions. Competence induction in *A. calcoaceticus* BD413 seems to be independent of the growth medium and pH. Further studies will be addressed to the regulation of competence induction and to the nature of the mechanism of uptake of DNA and the energization of this process.

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References


