Transformation of *Pseudomonas aeruginosa* Strain PAO with Bacteriophage and Plasmid DNA

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**Abstract**

A procedure has been developed which allows transformation of *P. aeruginosa* strain PAO with plasmid and bacteriophage DNA at a frequency of $10^{-6}$ per recipient cell. The method is similar in outline to that developed for *Escherichia coli*. It involves growing the recipient cells to $3-5 \times 10^8$ per ml in nutrient broth, washing the cells with $0.1 \text{ M MgCl}_2$, resuspending in $0.175 \text{ M CaCl}_2$ for 20 min, exposing to DNA for 1 h and then heat pulsing at 42°C for 1 min. Some plasmid markers are expressed immediately, whereas others require time for phenotypic expression.

**Introduction**

Bacterial transformation is a mechanism of genetic exchange involving the uptake of naked, exogenous DNA by a recipient bacterial cell, and the subsequent persistence of this genetic information within that cell and its descendants. Cells that are able to take up DNA are said to be competent. In a number of bacterial species including *Bacillus subtilis*, *Haemophilus influenzae* and *Streptococcus* spp. transformation occurs naturally, and cells may be rendered competent by manipulation of the growth conditions (Tomasz 1969).

Recently techniques have been developed for the induction of competence in other bacterial species such as *Escherichia coli* (Cohen et al. 1972), *Staphylococcus aureus* (Lindberg and Novick 1973), and *Pseudomonas putida* (Chakrabarty et al. 1975). Uptake of DNA does not necessarily ensure recombination of chromosomal DNA. In *E. coli*, for example, a *recB21 recC22 sbcB15* (or *sbcA6*)<sup>*</sup> recipient genotype is required to prevent degradation of the incoming DNA by the *recB* and *recC* endonuclease (Oishi and Cosloy 1972; Cosloy and Oishi 1973). However, the establishment of plasmid DNA is not affected by the *recB* and *recC* endonuclease.

The most topical use of plasmid transformation is in the cloning of DNA following *in vitro* construction of plasmid chimeras (Cohen and Chang 1974). It has also been used in the isolation of plasmid mutants following *in vitro* mutagenesis (Hashimoto and Sekiguchi 1976). A more general use is as a biological assay of plasmid DNA. This would prove to be a valuable adjunct to physical studies of plasmids in *Pseudomonas aeruginosa* strain PAO, particularly as this strain has been shown to harbour a number of cryptic plasmids (Pemberton and Clark 1973). It would also be invaluable.

<sup>*</sup> Genotype symbols are those used on the standard map of *E. coli* (Bachmann et al. 1976). The phenotype associated with *recB* and *recC* mutations is recombination deficiency and this is suppressed by *sbcA* and *sbcB* mutations.
in the characterization of plasmids suspected of carrying regions of the bacterial chromosome, particularly in organisms other than *Escherichia coli* which are not so well characterized genetically. For such studies it is necessary that the transformation system be reliable, so that some credence may be placed on negative results. Olsen and Hansen (1976) have transformed *P. aeruginosa* with plasmid DNA using a method developed for *Salmonella typhimurium* (Leberberg and Cohen 1974), but they do not report whether the method could be used routinely. M. Kageyama (personal communication) has also achieved plasmid transformation of *P. aeruginosa*. However, as different strains of *P. aeruginosa* show considerable genetic variation (Holloway 1975), it is quite likely that the optimum conditions will vary for different strains. As strain PAO (Holloway 1969) and its derivatives are widely used by *Pseudomonas* workers, we report here a series of experiments which define the optimum conditions for both bacteriophage and plasmid transformation of *P. aeruginosa* strain PAO.

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
<th>Molecular weight</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA01</td>
<td>Prototroph, <em>chl</em>-2</td>
<td>—</td>
<td>Holloway (1969)</td>
</tr>
<tr>
<td>PA08</td>
<td><em>met</em>-28, <em>ilo</em>-202, <em>str</em>-1</td>
<td>—</td>
<td>Isaac and Holloway (1968)</td>
</tr>
<tr>
<td>PAO25</td>
<td><em>leu</em>-10, <em>argF</em>10</td>
<td>—</td>
<td>Haas and Holloway (1976)</td>
</tr>
<tr>
<td>PAO38</td>
<td><em>leu</em>-38</td>
<td>—</td>
<td>Stanisich and Holloway (1969)</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R68</td>
<td>Cb, Km/Nm, Tc, Aer, Tra</td>
<td>$36 \times 10^6$</td>
<td>Holloway and Richmond (1973), Jacob <em>et al.</em> (1977)</td>
</tr>
<tr>
<td>pMG2</td>
<td>Sm, Su, Gm, Hg, Tra</td>
<td>$23 \times 10^6$</td>
<td>Shahrabadi <em>et al.</em> (1975), Jacoby and Shapiro (1977)</td>
</tr>
</tbody>
</table>

### Materials and Methods

The bacterial strains and plasmids used in this study are listed in Table 1.

#### Media

Nutrient broth (NB), nutrient agar (NA), minimal medium (MM), and TNM buffer (0·01 M tris, 0·15 M NaCl, 0·01 M MgSO$_4$, pH 7·4) have been described elsewhere (Stanisich and Holloway 1972). The tris--EDTA buffer used is 0·03 M tris, 0·005 M EDTA, 0·05 M NaCl (pH 8·0).

#### Chemicals

All chemicals used were reagent grade or better.

#### Bacteriophage

B3c is a clear plaque mutant of the temperate bacteriophage B3 (Holloway *et al.* 1960). It has a molecular weight of $25 \times 10^6$ (Holloway and Krishnapillai 1975).

General phage methods are described in Adams (1959). Large scale B3c lysates were prepared as described by Davison *et al.* (1964). After low speed centrifugation to remove bacterial debris...
the phage lysates were concentrated by centrifugation at 35 000 g for 2 h, and resuspension of the resulting phage pellet was carried out in 1% of the original volume of TNM buffer by standing at 4°C for 48 h.

Characterization of Transformants

Putative transformants were partially purified by patching on to selective plates, which after incubation were replicated to appropriately supplemented MM or NA plates. Inheritance of aeruginocin resistance was determined by replicating onto an NA plate spread with 0·3 ml of the aeruginocin AR41, prepared as described by Holloway et al. (1974). Transmissibility (Tra*) of antibiotic resistance was tested by replication onto an antibiotic supplemented MM plate freshly spread with 0·1 ml of an overnight NB culture of P. aeruginosa strain PA01. Chromosome mobilization ability (Cma*) was tested by replication onto an unsupplemented MM plate spread with PA038.

Extraction and Storage of Phage B3c DNA

Phage DNA was extracted from 7·5 ml of a phage suspension, titre 7 x 10^{11} plaque forming units per millilitre, by mixing with an equal volume of freshly distilled phenol saturated with 0·1 M disodium tetraborate. After centrifugation the aqueous layer was exhaustively dialysed against 0·14 M NaCl-0·001 M EDTA (pH 8·0). The DNA was precipitated with ethanol, spooled onto a glass rod, and finally dissolved in 2 ml of the tris-EDTA buffer. Sealed tubes of DNA in solution were stored at 4°C for up to 4 months without detectable decrease in transformation efficiency.

Extraction of Plasmid DNA

Plasmid DNA was extracted using a procedure similar to that described by Freifelder (1976). A 500-ml NB culture of stationary phase cells was harvested, washed three times in saline and resuspended in 3 ml of 25% (w/v) sucrose-2 x 10^{-5} M n-dodecylamine in tris-EDTA buffer. Meynell (1971) showed that n-dodecylamine sensitizes gram-negative cells to lysozyme. To this was added 0·2 ml of 0·25 M EDTA (pH 8·0) and 0·2 ml of lysozyme (10 mg/ml) in tris-EDTA buffer and after incubation at 37°C for 20 min 0·4 ml of 2% (w/v) sarcosyl (Ciba-Geigy, as Sarkosyl NL30) in tris-EDTA buffer was gently mixed in with a glass rod until viscosity effectively prevented further mixing. The resulting gel was centrifuged at 130 000 g for 90 min, yielding a supernatant that contained covalently closed and circular DNA and protein (Freifelder 1976). Protein was removed by passage through small columns of Sepharose 2B (Pharmacia Fine Chemicals) using 0·5 M ammonium acetate (pH 8·0) as the eluting fluid. Plasmid DNA came off just after the void volume, and was usually collected as a single fraction equal to 50% of the void volume. Plasmid extracts were stored frozen at -20°C.

DNA Assay

DNA extracts were routinely assayed by spectrophotometric analysis at 254 nm in a Zeiss PMQII spectrophotometer, using calf thymus DNA (Sigma) solutions of known concentration as standards. Protein contamination was routinely checked for by determining the ratio of absorbance at 254 nm and 280 nm. Absence of protein contamination was confirmed using the Folin–Ciocalteau reagent (Lowry et al. 1951) with bovine serum albumin (Sigma) as a standard.

Preparation of Competent Cells

Initially the method used was that described by Chakrabarty et al. (1975) for P. putida except that harvested cells were washed with 0·1 M MgCl_2 instead of 0·1 M NaCl. Cell growth was monitored using a Klett–Summerson photometric colorimeter. All glassware involved in preparing competent cells for transformation was washed in chromic acid and stored in a cold room with an ambient temperature of c. 4°C. All procedures were carried out in the cold room, tubes being held at 0°C in an ice bath.

Transformation Procedure

DNA solutions were dispensed into Wassermann tubes, and made up to the appropriate CaCl_2 concentration by addition of 1/10 volume of CaCl_2. Two volumes of competent cells were added and the contents gently mixed. Control tubes containing DNA only and cells only were always included. Cells were heat pulsed by partially immersing in water at 60°C for 10–15 s until the tube temperature reached 42°C (a thermometer in a temperature control tube was always included)
followed by transfer to a 42°C water bath for the appropriate time. After the heat pulse step the tubes were returned to the ice bath. Phage DNA-mediated transformation was detected by assaying for infectious centres on NA using an overnight NB culture of PAO1 as indicator. Plasmid DNA-mediated transformation was detected by assaying for transformants on NA supplemented with the appropriate antibiotic. Recipient cell survival was determined from the cells-only control tube. Unless otherwise stated transformation frequencies are calculated as infectious centres or transformants per surviving recipient cell.

**Enzymic controls**

Deoxyribonuclease I (Sigma Chemical Co.), ribonuclease A (type 1-A, Sigma Chemical Co.) and protease (type VI, Sigma Chemical Co.) were used as described by Cohen et al. (1972).

**Results**

**Determination of Optimum Conditions**

The primary aim of this study was to develop a reliable system for plasmid transformation in *P. aeruginosa*. However, initial experiments were carried out using B3c bacteriophage because of the ease with which large quantities of pure phage DNA can be obtained. B3c was chosen because of its molecular weight of $25 \times 10^6$. This is small enough to avoid serious problems of DNA shearing while pipetting, but large enough to reduce the risk of developing a system which could not accommodate the larger plasmids. PAO1 was used as the recipient for these experiments. Each parameter was varied in turn, and as each optimum value was determined, subsequent experimental conditions were altered to accommodate this information. Fig. 1 shows that as the DNA concentration is raised the transformation frequency increases linearly, indicating first-order kinetics. Saturation occurs at 1.15 µg/ml, and the input cell titre was 1.85 × 10^9 per ml. Assuming that non-viable cells adsorb DNA just as efficiently as surviving cells, this corresponds to 15.3 phage genomes per cell. In subsequent experiments a DNA concentration of 3.2 µg/ml was used to ensure saturation and allow for variation in input cell numbers.

The next parameter to be varied was CaCl_2 concentration. Fig. 2 shows that as the concentration is raised transformation frequency per surviving recipient cell increases, but that cell survival decreases markedly at high concentrations. This finding is quite repeatable and was the same when PAO8 was used as the recipient. As 175 mM CaCl_2 gave the greatest number of infectious centres it was chosen as
the optimum concentration. Subsequent attempts to increase cell survival at high CaCl\textsubscript{2} concentrations by resuspending cells at 100 mM CaCl\textsubscript{2} (which gave the highest survival) followed by dialysis against 200 mM CaCl\textsubscript{2} for varying lengths of time were not effective (C. H. Hamann and A. F. Morgan, unpublished data). The mechanism by which CaCl\textsubscript{2} induces competence is not understood (Taketa 1974) but it is clearly not simply a matter of the concentration at the time of exposure to DNA.

The effects of altering the duration of CaCl\textsubscript{2} treatment, and the time of exposure to DNA are presented in Table 2.

### Table 2: Effects of alterations to duration of (a) heat pulse, (b) CaCl\textsubscript{2} treatment, and (c) exposure to phage DNA

<table>
<thead>
<tr>
<th>(a) Heat pulse (s)</th>
<th>Transformants per 10\textsuperscript{6} surviving cells</th>
<th>(b) CaCl\textsubscript{2} treatment (min)</th>
<th>Transformants per 10\textsuperscript{6} surviving cells</th>
<th>(c) DNA exposure (min)</th>
<th>Transformants per 10\textsuperscript{6} surviving cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0·54</td>
<td>0</td>
<td>n.t.d.\textsuperscript{A}</td>
<td>10</td>
<td>0·7</td>
</tr>
<tr>
<td>30</td>
<td>0·88</td>
<td>5</td>
<td>0·58</td>
<td>20</td>
<td>1·3</td>
</tr>
<tr>
<td>60\textsuperscript{A}</td>
<td>1·2</td>
<td>10</td>
<td>1·2</td>
<td>40</td>
<td>1·8</td>
</tr>
<tr>
<td>90</td>
<td>1·0</td>
<td>20</td>
<td>1·5</td>
<td>60</td>
<td>2·1</td>
</tr>
<tr>
<td>120</td>
<td>1·2</td>
<td>30</td>
<td>1·5</td>
<td>90</td>
<td>1·9</td>
</tr>
</tbody>
</table>

\textsuperscript{A} No transformation detected.

**Recipient Cell Growth Stage**

In order to determine whether the growth regime of the recipient cells had a significant effect samples were taken at various times from an exponentially growing broth culture, the cells were treated to induce competence, and the transformation frequency was determined. Both cell survival and transformation frequency fell slightly as the cells approached stationary phase, the best results being obtained when the culture had a viable count of between 3 and 5 \(\times 10\textsuperscript{8}\) per ml.
Duration of Competence

*E. coli* cells treated with CaCl₂ can be maintained at 0°C for 10 days without significant loss of competence or cell viability (Taketo 1972). This has obvious logistical advantages when transformation is to be used as a routine assay. Accordingly, competent PA01 cells were prepared and held at 0°C. B3c DNA was added to samples at various times. Transformation frequency per surviving cell rose slightly over the first 6 h, but after 24 h it had fallen to less than 10% of the initial frequency, even though cell viability remained unchanged. Consequently competent PA0 cells need not be prepared immediately prior to use, but must be prepared fresh each day.

Summary of optimum transformation conditions

Initial cell density 3–5 × 10⁸ per ml; 0·1 mM MgCl₂ wash; 175 mM CaCl₂ treatment for 20 min; 60 min exposure to DNA, followed by a 1-min heat pulse.

![Fig. 3. Transformation of PAO25 with R68 DNA. After the void volume had passed through the column, 0·3 ml of eluate was collected directly into a tube containing 0·6 ml of competent cells, prepared as described on p. 684. After heat pulsing, the cells were diluted 10-fold into prewarmed broth and shaken at 37°C. At intervals the viable count was determined on NA (●), and on NA supplemented with 500 μg/ml carbenicillin (○), 250 μg/ml tetracycline (△), or 400 μg/ml neomycin (□). No transformants were seen at zero time when selection was made for tetracycline or neomycin resistance.](image)

Enzymic Controls

As was to be expected, transformation was unaffected by treating the B3c DNA with RNAase or protease or DNAase after the heat pulse, but DNAase treatment of the DNA prior to addition of competent PA01 cells abolished transformation.

Transformation with Plasmid DNA

For these experiments PAO25 was used as the recipient and plasmid DNA was extracted from PAO8 derivatives harbouring the appropriate plasmid. Chromosomally marked strains allow distinction between transformed cells and donor cells which have survived the plasmid extraction procedure. A preliminary experiment showed that transformation of PAO25 with phage B3c is the same as for PA01.

The first plasmid extracted was R68. The Sepharose column eluate was used undiluted. As the eluate contained 18·5 μg/ml DNA, it was assumed that saturation of the competent cells was achieved. Fig. 3 shows that of the antibiotic resistance
Transformation of *Pseudomonas aeruginosa*

markers carried by R68, only carbenicillin resistance can be selected for without allowing time for phenotypic expression. The β-lactamase gene carried by R68 is known to be expressed constitutively (Fullbrook et al. 1970). Fifty clones from each type of media and each time were tested for unselected markers. All had the nutritional requirements of the recipient and all were Cb⁺ Nm⁺ Tc⁺ Aer⁺ Tra⁺. Thus all transformants had apparently received complete plasmids. When the transformation procedure was repeated using DNA that had been exposed to mild shearing by means of pipetting several times, the transformation frequency dropped to 30%, but once again all transformants exhibited all the plasmid markers. Chakrabarty et al. (1975) reported that shearing of the closely related plasmid RP1 (Grinsted et al. 1972) resulted in *P. putida* transformants 40% of which had lost plasmid markers and which presumably carried deletions. We have never observed such deletions in transformants of *P. aeruginosa* strain PAO, irrespective of the plasmid used. This may reflect differences in nuclease activity between the two species.

**Fig. 4.** Effect of plasmid DNA concentration on transformation frequency. Plasmid pMG2 was isolated from a PAO8 derivative harbouring the plasmid. The Sepharose column eluate was assayed for DNA as described in Methods. 0-2-ml aliquots of the eluate, and of a series of two-fold dilutions, were added to tubes containing 0-4 ml of competent PAO25 cells. After adsorption and heat pulsing, quadruplicate 0-1-ml aliquots were plated from each tube onto NA supplemented with 500 μg/ml streptomycin. The intersect of the two lines occurs at a DNA concentration of 1.9 μg/ml. Input cell titre was $3.13 \times 10^9$ per ml, and cell survival was 50%.

**Effect of DNA Concentration on Transformation Frequency**

Because R68 is large enough to be sheared even by pipetting, the smaller plasmid pMG2 was used. Fig. 4 shows a very similar pattern to that obtained with B3c transfection. The saturating DNA concentration of 1.95 μg/ml corresponds to 16.4 plasmid molecules per cell. Both this value and the transformation frequency obtained are very close to the values obtained for transformation with B3c DNA.

**Discussion**

The protocol presented here can be used to transform *P. aeruginosa* strain PAO with phage or plasmid DNA at a frequency of $10^{-6}$ per surviving recipient cell. As is the case for *E. coli* the process has first-order kinetics. Cell survival is lower because of the higher concentrations of CaCl₂ required to render recipient cells competent. The frequencies obtained are quite reproducible but are 100-fold less than those reported for *E. coli* (Cohen et al. 1972; Taketo 1972), and 10–100-fold lower than those claimed for *P. putida* (Chakrabarty et al. 1975). However, in the latter instance it is not clear from the results whether the authors are observing a high frequency
of transformation or a low frequency followed by subsequent conjugal transmission of the plasmid. The reproducibility of the procedure reported here has allowed its use in the isolation of transfer-deficient mutants of the plasmid R91-5 following in vitro mutagenesis of the plasmid DNA (J. M. Carrigan, Z. M. Helman and V. Krishnapillai, personal communication).

The plasmid extraction method used here is simple, but is not applicable to all plasmids. We have made many attempts to isolate plasmid DNA from strains carrying the R68 derivative R68-45 (Haas and Holloway 1976), but only once was transformation achieved (data not shown). On the one successful occasion, however, all transformants that were tested carried apparently normal plasmids that exhibited all the R68-45 markers. Just why R68 should be easy and R68-45 difficult to isolate by this method is not clear. R68-45 is reported to be slightly larger than R68—37·4 × 10^6 compared to 36 × 10^6 (Jacob et al. 1977)—but the nature of the additional DNA is unknown. The plasmid extraction method used here would probably be unsuitable for plasmids that exist intracellularly as relaxation complexes, as sarcosyl is one of a number of agents known to destroy such complexes (Clewell and Helinski 1970; Helinski and Clewell 1971).

Attempts to apply this system to transformation of chromosomal markers were unsuccessful (data not shown). This is presumably due to exonuclease degradation of incoming linear DNA. Miller and Clark (1976) have shown that P. aeruginosa contains an exonuclease that is very similar to ExoV of E. coli, which is thought to be the product of the recB and recC genes (Tomizawa and Ogawa 1972). This exonuclease is possibly the cause of our failure to detect transformants that had lost unselected plasmid markers, due to degradation of any sheared plasmid DNA. Chromosomal transformation of P. aeruginosa probably awaits the isolation of the equivalent of the recB21 recC22 sbcB15 (or sbcA6) mutants of E. coli.

Acknowledgments

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References


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*Note Added in Proof*

Since submission of this manuscript the authors have had their attention drawn to the following publication: Sano, Y., and Kageyama, M. Transformation of *Pseudomonas aeruginosa* by plasmid DNA. *J. Gen. Appl. Microbiol.*, 1977, 23, 183–6. That paper reports a similar transformation frequency to that presented here, but describes different optimum conditions.