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Transformation and Transfection of *Pseudomonas aeruginosa*: Effects of Metal Ions

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The ability of different metal ions to promote transformation of *Pseudomonas aeruginosa* by deoxyribonucleic acid of the plasmid RP1 was examined. $CaCl_2$, $MgCl_2$, and $MnCl_2$ were found to promote such transformation, although at different frequencies and with the optimum response at different concentrations. Only $MgCl_2$ promoted transfection of *P. aeruginosa* by the linear deoxyribonucleic acid of phage F116. $CaCl_2$ was demonstrated to allow adsorption and entry into the cell of F116 deoxyribonucleic acid such that it became resistant to exogenous deoxyribonuclease, but phage production occurred only when $MgCl_2$ was provided. Inactivation of linear phage deoxyribonucleic acid taken up in the absence of $MgCl_2$ was observed. The transfection frequencies at various concentrations of $MgCl_2$ were compared, and the optimum response occurred at the concentration which promoted the highest frequency of transformation by RP1 deoxyribonucleic acid.

A wide variety of techniques has been used to allow the uptake of phage DNA by *Escherichia coli* (for review see Benzinger [1]). One of the techniques was that of Mandel and Higa (11), who showed that treatment with cold calcium chloride enabled *E. coli* to take up the purified DNA of phages lambda and P2 and to produce viable phage. Although the highest efficiencies of transfection were not obtained by this particular technique, it does have the advantage of being a simple system in which the bulk of the cells remain viable. Recent work has shown that purified plasmid DNA and chromosomal DNA will also transform *E. coli* after cold $CaCl_2$ treatment (4, 5). Successful transformation of *E. coli* by linear DNA was achieved by using a *recB recC* recipient with recombination proficiency restored by an additional suppressor mutation such as *sbcA* or *sbcB* (5).

Genetic transformation of *Pseudomonas aeruginosa* with linear chromosomal DNA has been reported (7), but this work has proved difficult to repeat (2, 19). In a recent report (19), the Ca^{2+} technique was adapted to *P. aeruginosa* so as to allow transformation by the DNA of the plasmid RP1. In the same report, it was also observed that $MgCl_2$ could be used in place of $CaCl_2$ to allow transformation of RP1 DNA. Mercer and Loutit (13) have shown Ca^{2+}-dependent transformation of *P. aeruginosa* strain 1 by the plasmid RP1 and also by DNA of the conjugative plasmid FP2. In a subsequent report (14), they observed that treatment with $MgCl_2$ allowed a restrictionless mutant of *P. aeruginosa* strain 1 to be transfected by the DNA of phage F116 and transformed by purified chromosomal DNA. In this paper we compare the response of $CaCl_2$-treated and $MgCl_2$-treated cells of *P. aeruginosa* to circular plasmid DNA and linear phage DNA. In addition, the processes of uptake and expression of linear phage DNA are investigated.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. OT683, a restrictionless derivative of *P. aeruginosa* strain 1 (14) was used as the recipient in plasmid DNA transformation studies. In transfection studies, however, a Res^+ derivative of strain 78, OT500 (12), was generally used. Transfection was performed by using DNA of the generalized transducing phage of *P. aeruginosa*, F116 (6). Plasmid RP1 was obtained from D. F. Bacon, Massey University, Palmerston North, New Zealand.

Media. Growth media used were brain heart infusion broth (Difco Laboratories), supplemented with 4 g of potassium nitrate per liter, and Luria broth (18). Where a solid medium was used, brain heart infusion broth (Difco) was solidified with 2% Davis agar.

Preparation of phage and plasmid DNA. Phage F116 was grown and harvested as described by Booker and Loutit (2), using OT100 (10) as a host. Phage DNA was purified by pronase (Calbiochem) digestion, phenol extraction, and dialysis against 0.05 M Tris-hydrochloride, pH 7.5 (20). Plasmid DNA was isolated by the method of Stanisich and Bennett (21).

Transformation and transfection. Transformation by plasmid DNA was performed as described by Chakrabarty et al. (3) with the following modifications: overnight growth of the recipient was in brain heart broth; the first wash was performed with a solution of

the same composition as that used in the subsequent treatment; and the heat pulse was carried out at 37°C. After we allowed 60 min of incubation for the expression of drug resistance, the transformation mixtures were plated on brain heart agar plates containing either 1 mg of carbenicillin per ml, 200 μg of tetracycline per ml, or 300 μg of kanamycin per ml. Transfection was performed in the same manner but without the 60 min for expression. Samples were plated on brain heart agar and examined for plaques after incubation at 37°C for 24 h.

Electrophoresis. The examination of the DNA content of crude lysates by agarose gel electrophoresis was performed by the method of Meyers et al. (15), except that electrophoresis was carried out in cylindrical gels (5 by 100 mm) at 2 mA per gel for 2.5 h. The same electrophoretic conditions were used for the examination of *Hin*dIII restriction endonuclease digests.

RESULTS

Metal ion-dependent transformation by RP1. Transformation of *P. aeruginosa* to carbenicillin resistance was achieved with RP1 DNA after treatment with $CaCl_2$, $MgCl_2$, or $MnCl_2$. No such transformation was observed when NaCl, CsCl, zinc acetate, $CoCl_2$, or $CdCl_2$ was used. The optimum concentration for $CaCl_2$ and $MnCl_2$ was approximately 0.05 M, whereas $MgCl_2$ produced an optimum response at 0.15 M (Fig. 1). Figure 1 also demonstrates the considerably higher frequency of transformation achieved after treatment with $MgCl_2$. The frequency of transformation in response to various concentrations of $MnCl_2$ followed a curve very similar to that shown for $CaCl_2$.

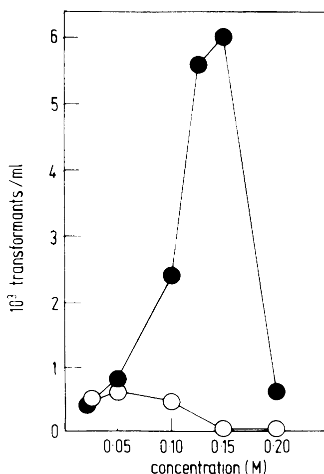

FIG. 1. *Effect of $MgCl_2$ and $CaCl_2$ concentration on RP1 transformation. Cells (OT683) were treated with $MgCl_2$ (●) or $CaCl_2$ (○) as described in the text, and exposed to RP1 DNA (2.8 μg/ml). Transformants were selected on brain heart agar containing carbenicillin.*

The carbenicillin-resistant clones recovered after transformation by RP1 DNA also acquired resistance to the other resistance markers of the plasmid, namely, tetracycline, kanamycin, and ampicillin. These resistances were transferred by conjugation at a frequency of 1 per 10^5 donor cells, when mated by the procedure of Loutit and Marinus (10). When a transformed clone was examined by agarose gel electrophoresis of a crude lysate, it was found to have acquired a DNA satellite band which was not present in the untreated carbenicillin-sensitive parent. This band had the same mobility as the satellite band present in a strain carrying RP1 and the same mobility as a sample of purified RP1 DNA.

Transfection by F116 DNA. In the light of studies on the uptake of linear DNA by *E. coli* (5), it was thought that transfection of *P. aeruginosa* by linear phage DNA after $CaCl_2$ treatment was not likely to be successful. However, the stimulation of plasmid DNA transformation of *P. aeruginosa* by Mg^{2+} encouraged us to examine the effect of this metal ion on linear DNA transfection. Miller et al. (17) reported that the DNA of phage F116 is a linear duplex of molecular weight $(38.0 \pm 2) \times 10^6$, and this phage was used for the investigation.

The results of exposure of $CaCl_2$- or $MgCl_2$-treated cells to purified DNA of the phage F116 are shown in Table 1. It can be seen that no plaques were detected with $CaCl_2$-treated cells, but quite high levels of transfection were observed with $MgCl_2$-treated cells. The absence of response to $CaCl_2$ was not apparent in the transformation of treated cells exposed to circular RP1 DNA (Table 1). It seemed that transfection by linear DNA was dependent upon a high concentration of $MgCl_2$ and that $CaCl_2$ could not replace $MgCl_2$ even to produce a lower frequency of transfection. When intact phage was added in place of a DNA preparation either at 0°C or during the heat pulse in an otherwise normal transfection experiment, the efficiency of plating

TABLE 1. *Stimulation of transformation and transfection by $CaCl_2$ and $MgCl_2$*

DNA	Selection for:	Colonies or plaques per cell[a]	
		Ca^{2+}	Mg^{2+}
RP1	Cb^{r}[b]	2.4×10^{-6}	8.4×10^{-6}
RP1	Plaques	$<1.0 \times 10^{-8}$	$<1.0 \times 10^{-8}$
F116	Plaques	$<1.0 \times 10^{-8}$	8.0×10^{-6}

[a] Values indicate transformant colonies or transfectant plaques per viable cell. Cells (OT683) were treated with $CaCl_2$ (0.05 M) or $MgCl_2$ (0.15 M) as described in the text and were exposed to RP1 or F116 DNA (2 μg/ml).

[b] Cb^r, Carbenicillin resistance.

observed was the same as that achieved with an overnight brain heart broth culture or cells treated at 0°C with 0.15 M $MgCl_2$. In addition, when an equal volume of Luria broth containing 0.05 M $CaCl_2$ was added after the heat pulse, and incubation of the phage-cell mixture at 37°C continued for 18 h, the phage titer rose from 5 × 10^3 to 5 × 10^8 plaque-forming units per ml. This indicated that intact phage was able to replicate in cells treated with 0.05 M $CaCl_2$ and that the failure of $CaCl_2$ to promote transfection was not a result of inhibition of normal F116 phage production.

The phage produced in the transfection experiments was harvested and purified, and the DNA was extracted. This DNA had the same *Hin*dIII fragmentation pattern as that of the original DNA (results not shown), confirming that the process observed was indeed transfection. We then decided to examine the difference between the Ca^{2+}-stimulated and the Mg^{2+}-stimulated responses to linear phage DNA.

Ca^{2+}-stimulated adsorption of linear DNA. It was assumed that the process of transformation or transfection involved an adsorption of DNA to cells during the incubation at 0°C, followed by entry into the cell and expression of the DNA during the heat pulse and subsequent growth. Metal ions such as Ca^{2+} or Mg^{2+} might be involved in either or both of these two stages. A possible explanation for the lack of transfection by F116 DNA after $CaCl_2$ treatment was that Ca^{2+} could not promote adsorption of linear as opposed to circular DNA to cells during the incubation at 0°C. To test this, cells and DNA of F116 were mixed in the presence of cold $CaCl_2$, then transferred to warm (37°C) $MgCl_2$ for the heat pulse and plated to demonstrate transfection. The results (Table 2) indicate that $CaCl_2$ promoted the adsorption of F116 DNA to cells during the cold incubation and thus allowed transfection, provided $MgCl_2$ was present during the subsequent heat pulse. The results also show that treatment with a salts solution in place of $CaCl_2$ did not promote transfection even if Mg^{2+} was present during the heat pulse, indicating the positive role of Ca^{2+} in the first stage of this process. Treatment of cells and F116 DNA with cold $MgCl_2$ followed by transfer to warm $CaCl_2$ produced no transfection, confirming that the failure of $CaCl_2$ to promote transfection is related to its inability to stimulate the second stage of the transfection process, i.e., the entry or expression or both of the DNA.

Ca^{2+}-stimulated entry of linear DNA into the cell. A change in the susceptibility of the transfection process to exogenously supplied DNase was taken as an indicator of entry of the linear DNA into the cell. $CaCl_2$-treated and $MgCl_2$-treated cells were compared for their ability to promote such entry into the cell. The results (Table 3) demonstrated that with either Ca^{2+} or Mg^{2+} treatment the DNA was largely in a DNase-sensitive state at the end of the cold incubation period. The 20 plaques recorded with $MgCl_2$-treated cells exposed to DNase at 0 min of heat pulse can be approximated to a negative response, since it represents only 1.3% of the level obtained with no DNase treatment and simply reflects the efficiency of the $MgCl_2$-stimulated response.

After 2 min of incubation at 37°C, the DNA was no longer susceptible to DNase. This occurred with both $MgCl_2$-treated and $CaCl_2$-treated cells, but for the latter, $MgCl_2$ had to be

TABLE 2. *Comparison of the actions of $CaCl_2$ and $MgCl_2$ in transfection*

Stage 1[a]	Stage 2[b]	Plaques/ml
Ca^{2+}	Ca^{2+}	0
Ca^{2+}	Mg^{2+}	35
Ca^{2+}	Ca^{2+}[c]	0
Mg^{2+}	Mg^{2+}	165
Mg^{2+}	Ca^{2+}	0
Mg^{2+}	Mg^{2+}[c]	925
Salts solution[d]	Salts solution[c]	0
Salts solution	Mg^{2+}	0

[a] Cells (OT500) were treated as indicated and held with F116 DNA (2 μg/ml) at 0°C for 60 min.

[b] The mixtures from stage 1 were centrifuged at 5,000 × *g* for 10 min at 4°C, suspended in warm (37°C) $CaCl_2$ (0.05 M) or $MgCl_2$ (0.15 M), and held in a 37°C water bath for 2 min before plating.

[c] In this experiment, the mixtures from stage 1 were not centrifuged but transferred directly to a 37°C water bath, held there for 2 min, and plated.

[d] The salts solution consisted of 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.01% $MgSO_4$, and 0.1% $(NH_4)_2SO_4$.

TABLE 3. *Conversion of phage DNA to a DNase-resistant state*

Stage 1[a]	Stage 2[b]			Plaques/ml
	0 min	2 min	4 min	
Ca^{2+}		DNase	Mg^{2+}	140
Ca^{2+}	DNase		Mg^{2+}	0
Ca^{2+}			Mg^{2+}	130
Ca^{2+}				0
Mg^{2+}		DNase		1,230
Mg^{2+}	DNase			20
Mg^{2+}				1,500
Mg^{2+}		DNase	Ca^{2+}	1,100

[a] As described in Table 2, footnote *a*.

[b] Mixtures from stage 1 were transferred to a 37°C water bath. At the indicated times, DNase (1/10 volume of DNase I at 50 μg/ml in 50 mM Tris-hydrochloride-5 mM, $MgCl_2$, pH 7.4), Mg^{2+} (1/6 volume of 1 M $MgCl_2$), or Ca^{2+} (1/20 volume of 1 M $CaCl_2$) was added. All mixtures were plated after 6 min at 37°C.

supplied at some point during the heat pulse stage for plaques to occur. In these experiments, $MgCl_2$ was added directly to $CaCl_2$-treated cells, rather than transferring the cells to $MgCl_2$ by centrifugation and suspension. The occurrence of plaques by this procedure indicated that $CaCl_2$ did not play an inhibitory role, but simply failed to produce transfection in the way that $MgCl_2$ did.

Inactivation of linear DNA in $CaCl_2$-treated cells. The apparent ability of $CaCl_2$ to stimulate entry of linear DNA into the cell but not to allow full transfection suggested the possibility that the linear DNA was being inactivated in Ca^{2+}-treated cells but was not inactivated if $MgCl_2$ was present.

Cells treated with $CaCl_2$ and exposed to F116 DNA at 0°C in the normal manner were placed in a 37°C water bath for various lengths of time before being plated. $MgCl_2$ was added to a final concentration of 0.15 M 2 min before the end of each heat pulse. The same experiment was performed with $MgCl_2$-treated cells but without the addition of $MgCl_2$ during the heat pulse.

The results (Fig. 2) show that continued incubation at 37°C in the presence of $CaCl_2$ and absence of $MgCl_2$ drastically reduced the level of transfection observed. This falloff in the level of transfection was considerably less when the prolonged heat pulse was performed in the constant presence of 0.15 M $MgCl_2$.

The results presented are the average of three separate experiments, and although there was considerable experiment-to-experiment variation, the levels observed with incubation in the constant presence of $MgCl_2$ were always higher than the corresponding levels achieved with the later addition of $MgCl_2$. The low value after 12 min in the presence of $MgCl_2$ alone is not regarded as significant. The falling levels of transfection observed with increasing incubation in the absence of $MgCl_2$ were consistent with the F116 DNA being inactivated by some agent, possibly a DNase. Presumably, 0.05 M $CaCl_2$ (unlike 0.15 M $MgCl_2$) was unable to protect linear DNA from inactivation by this unknown agent.

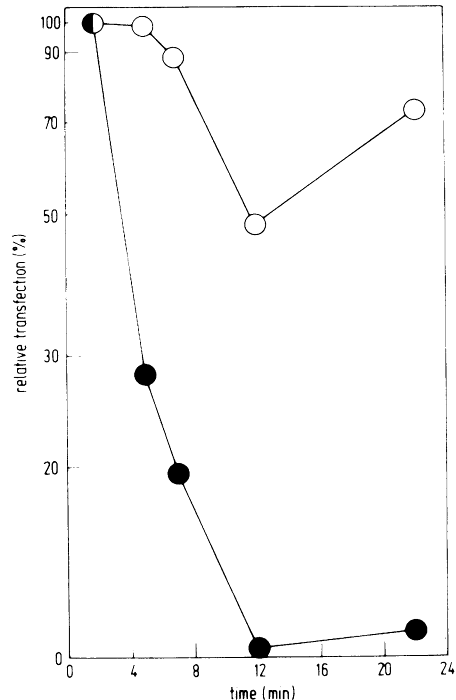

FIG. 2. *Inactivation of transfecting DNA during a 37°C heat pulse. Values represent the number of plaques at each time as a percentage of the number observed after a 2-min heat pulse under the same conditions. Cells (OT500) were treated with $CaCl_2$ (●) or $MgCl_2$ (○) as described in the text and exposed to F116 DNA (8 μg/ml) before being held in a 37°C water bath for the indicated time. Cells treated with $CaCl_2$ had $MgCl_2$ added 2 min before the end of the heat pulse so as to give a final $MgCl_2$ concentration of 0.15 M.*

Optimum $MgCl_2$ concentration for F116 DNA transfection. Although transformation by circular plasmid DNA had been shown to have an $MgCl_2$ optimum of 0.15 M, it was conceivable that transfection by linear phage DNA might respond optimally to some other concentration of $MgCl_2$. It was also possible that each of the two major stages of transfection—adsorption at 0°C and uptake at 37°C—might have a different $MgCl_2$ optimum.

To investigate these points F116 DNA was exposed to cells at 0°C in the presence of 0.05 M $CaCl_2$, 0.05 M $MgCl_2$, or 0.15 M $MgCl_2$. These mixtures were then heat pulsed in the presence of various concentrations of $MgCl_2$.

The results (Table 4) reveal that the highest level of transfection was achieved when 0.15 M $MgCl_2$ was used in both stages, suggesting that 0.15 M was overall the optimum concentration of $MgCl_2$ for transfection as well as for transformation by plasmid DNA. This was confirmed by a more detailed examination of the frequencies of transfection over the range of 0.025 to 0.4 M $MgCl_2$ (results not shown). Cells treated at 0°C with $CaCl_2$ showed their highest level of transfection when $MgCl_2$ was provided at a concentration of 0.15 M during the heat pulse, demonstrating that this was also the optimum $MgCl_2$ concentration for the expression of linear DNA at 37°C. Similarly, cells treated at 0°C with 0.05 M $MgCl_2$ and with 0.15 M $MgCl_2$ at 37°C showed

TABLE 4. *Effect of $MgCl_2$ concentration on transfection*

Stage 1[a]	Plaques/0.1 ml[b]		
	Stage 2: 0.05 M Mg^{2+}	Stage 2: 0.15 M Mg^{2+}	Stage 2: 0.30 M Mg^{2+}
0.50 M Ca^{2+}	0	50	27
0.05 M Mg^{2+}	30	416	203
0.15 M Mg^{2+}		700	258

[a] See Table 2, footnote *a*.

[b] Mixtures from stage 1 were transferred to a 37°C water bath and additions were made (where necessary) to give the indicated Mg^{2+} concentration. After 2 min at 37°C, samples were plated.

a lower level of transfection than did cells treated with 0.15 M $MgCl_2$ at both stages, indicating an optimum response to 0.15 M $MgCl_2$, during the adsorption at 0°C.

DISCUSSION

After $CaCl_2$ treatment and exposure to RP1 DNA, cells of *P. aeruginosa* acquired a plasmid so as to become indistinguishable from the parent strain from which the RP1 DNA was derived. This $CaCl_2$-dependent transformation by plasmid DNA is similar to that reported for *E. coli*, although the frequencies achieved with *P. aeruginosa* are somewhat lower. The transformation of *P. aeruginosa* after treatment with Mg^{2+} and Mn^{2+} is in contrast to the response of *E. coli* to these metal ions. There has been no report of a beneficial effect of $MnCl_2$ on the transformation of *E. coli*, and $MgCl_2$ (5 mM) increases only slightly the frequency of transformation by ϕA replicative-form DNA when used in conjunction with $CaCl_2$ (22). $MgCl_2$ increases the frequency of plasmid DNA transformation of *Salmonella*, but only when used as a treatment before $CaCl_2$ treatment (9). The well-studied transformation systems of *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* have requirements for both Ca^{2+} and Mg^{2+} but at low concentrations (8), and the lack of a requirement for low-temperature incubation makes it difficult to compare the different systems.

The ATP-dependent DNase of *E. coli* is thought to degrade incoming linear DNA, thus prohibiting transformation or transfection by linear DNA unless the enzyme is inactive. A similar ATP-dependent DNase has been detected in *P. aeruginosa* (16). In our early studies it was assumed that, as in *E. coli*, this enzyme would prevent transfection by linear phage DNA. Transfection by linear DNA of phage F116 was achieved, however, after $MgCl_2$ treatment but not after $CaCl_2$ treatment.

The work reported here reveals that both $CaCl_2$ and $MgCl_2$ can promote adsorption and transport into the cell of linear phage DNA. In $CaCl_2$-treated cells, this DNA is then rapidly inactivated, such that transfection does not occur. This inactivation might represent degradation by the ATP-dependent DNase. The manner in which 0.15 M $MgCl_2$ protects linear DNA from inactivation is unclear. One possibility is that the high $MgCl_2$ concentration inhibits the ATP-dependent DNase activity. Miller and Clark (16) have reported that the optimum Mg^{2+} concentration for this enzyme is quite low (10 mM with 60% efficiency at 20 mM). It is also not clear why the DNA introduced by normal infection is not susceptible to inactivation in the same way as DNA introduced by transfection. One suggestion for this could be the early production of a repressor for ATP-dependent DNase, although Miller and Clark (16) were unable to demonstrate inactivation of the ATP-dependent DNase activity of *P. aeruginosa* after infection by phage F116. Nevertheless, our results indicate that some protective mechanism must exist.

We have previously reported transformation of *P. aeruginosa* by chromosomal DNA (14). This was achieved only when $MgCl_2$ treatment was applied, as is the case with transfection. The recipient used in those studies was OT683, a restrictionless mutant. Although the observation of transformation of this and other related restrictionless strains is readily repeatable, we have as yet not succeeded in transforming Res^+ strains with linear chromosomal DNA. Transfection by linear phage DNA or transformation by plasmid DNA occurs in both Res^+ and Res^- recipients.

The mechanism by which Ca^{2+} treatment at low temperature promotes transformation of *E. coli* is unknown. The similarity of the response of *P. aeruginosa* to Ca^{2+} treatment suggests a common mechanism of DNA uptake. The response of *P. aeruginosa* to $MgCl_2$ and $MnCl_2$, however, is perhaps indicative of differences in the transformation mechanisms.

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