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Optimal Conditions for Transformation of *Azotobacter vinelandii*

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Optimal transformation of *Azotobacter vinelandii* OP required a 20-min incubation of the competent cells with deoxyribonucleic acid at 30°C in buffer (pH 6.0 to 8.0) containing 8 mM magnesium sulfate. Nitrogen-fixing transformants of nitrogen fixation-deficient recipients could be plated immediately on selective medium, but transformants acquiring rifampin and streptomycin resistance required preincubation in nonselective medium. The three phenotypes achieved an approximately equal and stable frequency after 17 h (six generations) of growth in nonselective medium.

Recently a liquid medium for the production of competent *Azotobacter vinelandii* was described (12). This induction procedure utilizes an iron-limited Burk medium containing ammonium acetate and glucose (OFe+N medium) and provides a major improvement in the *Azotobacter* transformation procedure. Formerly, a combined competence induction and transformation medium (plate assay) was the only procedure for genetic exchange in this organism (11). The plate assay was used to map nitrogenase-deficient (Nif^-) lesions (1) and to perform intergeneric transformation of *A. vinelandii* with *Rhizobium* spp. DNA (2, 10). In these studies the transformant cells and the transformation frequencies were examined, but the mechanisms of DNA binding, uptake, and recombination could not be studied by the plate assay, which required a solid support (11).

A. vinelandii OP strain UW1, a previously described (1), nitrogenase-deficient (Nif^-), rifampin- and streptomycin-sensitive strain (obtained from W. Brill, University of Wisconsin, Madison), was used as a recipient in the present study. Donor DNA was prepared from *A. vinelandii* ATCC 12837 strains 113 (Nif^+ Rif^+) and 114 (Nif^+ Str^+) by lysis in 15 mM saline citrate buffer (pH 7.0) containing 0.05% sodium dodecyl sulfate as described previously (11). Prototrophic (Nif^+) cells were maintained or selected on Burk nitrogen-free medium (pH 7.2) containing 1% glucose (11). Burk buffer refers to Burk medium without glucose. The Nif^- strains were grown on Burk medium containing 1.1 g of ammonium acetate per liter (BBGN medium).

The Nif transformation frequency was 3.95×10^{-3} when recipient cells were induced to competence in the OFe+N liquid competence medium and were assayed by the plate method. The same competent cells also could be transformed in a liquid assay containing Burk buffer. The frequency of transformation was dependent on the magnesium concentration in the assay (Fig. 1). The Nif transformation frequencies were comparable in the plate and the liquid assays when the magnesium concentration of the Burk buffer was increased from the normal 0.81 mM to 8.5 mM.

Increasing the magnesium concentration to 40 mM did not further increase the transformation frequency. The results confirm the previous observation that magnesium was essential for the transformation of *A. vinelandii* (11). There was no transformation in the liquid assay without the addition of magnesium or DNA, or when DNase was added at zero time. Unlike other genera that require magnesium for transformation (4, 7, 8, 13, 15), calcium would not substitute for magnesium in the transformation assay. Whether magnesium is required for the binding of DNA to the competent cell or for uptake of the transforming DNA remains to be elucidated.

The crude lysate DNA was purified by precipitating 1 volume of crude DNA with 3 volumes of 95% ethanol at 4°C. The precipitated DNA was wound onto a sterile glass rod, drained, and redissolved in sterile 15 mM saline (pH 7.0), and the DNA concentration was estimated by the Burton procedure (3). When equal amounts of purified or crude DNA were used in the liquid assay, the resultant Nif transformation frequencies were 6.16×10^{-3} and 5.76×10^{-3}, respectively. It was proposed previously that crude lysate DNA was the best donor for the transformation of *A. vinelandii* and that purification of the DNA was very detrimental to the transformation frequency (11). The earlier erroneous conclusion was probably due to the inability of

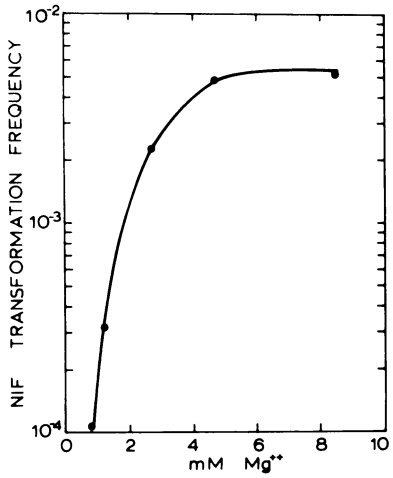

FIG. 1. *Requirement for magnesium in the transformation assay. Competent strain UW1 cells were transformed with strain 113 crude DNA in a liquid assay containing 0.81 mM to 8.5 mM $MgSO_4$. The liquid transformation assay consisted of mixing 50 μl of competent cells (2 × 10^7 cells per ml of assay) with 50 μl of DNA (2.7 μg/ml of assay) in 0.3 ml of Burk buffer containing a final concentration of magnesium sulfate as indicated (pH 7.2). The assay mixture was incubated for 20 min at 30°C, and then 100 μl of DNase I (4 μg/ml of assay) was added. This mixture was then plated immediately for viable cells on BBGN medium or for transformants on nitrogen-free Burk medium. Transformation frequency was calculated as the number of Nif^+ transformants per the number of viable cells plated on selective medium.*

the plate assay to generate a highly competent population (12) rather than to a fault in the DNA purification. This result also indicates that it is unlikely that the crude lysate DNA contained any chemical or cell fraction that was required for transformation.

Competent strain UW1 cells were able to bind DNA into the DNase-insensitive state over a wide range of pH values (Fig. 2). The optimum assay pH was between 6.0 and 8.0, and transformation was more sensitive to a lower pH than a higher pH. The recipient cell viability remained unchanged in all of the assays. This range was much wider than that previously reported for the plate assay, which was a composite optimum for competence induction, transformation, and recombination (11). The iron, molybdate, calcium, and phosphate present in the Burk buffer were not required in the transformation assay, since the other buffers in the optimal pH range lacked these ions.

Transformation of strain UW1 also was possible over a wide range of temperature from 20 to 35°C, with an optimum at 30°C (Fig. 3). Transformation was more sensitive to increased temperature, showing 96% inhibition at 7°C above the optimum compared to 96% inhibition at 13°C below the optimum. There was no transformation at 4°C, and the frequencies at 42 and 46°C were 3.82×10^{-6} and 1.17×10^{-6}, respectively. The viability of the recipient cells in the assays was not affected from 4 to 42°C. At 46°C, however, there was a 98% decrease in viability. Because of the disproportionate loss of transformation versus viability at 37 and 42°C, it was suspected that a temperature-sensitive event was involved in transformation. This was proven to be the case by preincubating competent strain UW1 cells at 17, 25, 30, 37, and 42°C for 30 min, and then incubating for 20 min at 30°C with DNA present. The frequencies of Nif transformants from the assays preincubated at 17, 25, and 30°C were identical to the 30°C value reported in Fig. 3. Upon preincubation at 37 and 42°C, however, the Nif transformation frequencies were 6.53×10^{-5} and 1.57×10^{-6}, respectively. Identification of this temperature-sensitive "competence factor" is in progress (unpublished data).

The Nif marker proved to be a useful transformation indicator because cells could be plated

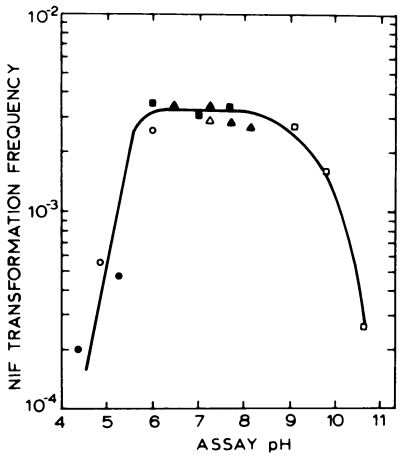

FIG. 2. *The effect of assay pH on transformation frequency. Competent strain UW1 cells were preincubated for 10 min in 10 mM buffers containing 20 mM $MgSO_4$ (final assay concn). The buffers used were acetate (●), citrate-phosphate (○), potassium phosphate (■), Good [piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), pH 6.4; 3-(N-morpholino)propane sulfonic acid (MOPS), pH 7.3; N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.7; N-2-hydroxyethyl-piperazine-N'-3-propane sulfonic acid (HEPS), pH 8.1; 5] (▲), Burk buffer (△), and carbonate-bicarbonate (□). The cells were then transformed with strain 113 crude DNA in the same buffer for 20 min at 30°C. Transformation frequency was estimated as described in Fig. 1.*

directly from the assay onto selective medium. There apparently was enough carry-over of NH_4^+ in the recipient cell pools to permit phenotypic expression of Nif on the nitrogen-free selective medium. As shown in Fig. 4, however, Rif and Str resistance was first expressed after 3 h of incubation in nonselective medium, which coincided with the onset of cell division. As the culture grew (mean generation = 1.8 h) in the nonselective medium, the apparent frequencies of the antibiotic resistances increased with time while the frequency of Nif decreased with time. The frequency of all three markers stabilized after approximately six generations or after 11 h of exponential growth. Because there was a 6-h lag before the onset of exponential growth, the total incubation required before marker frequency stabilization was approximately 17 h. This marker frequency stabilization period could have been caused by a lag in (i) DNA uptake, (ii) DNA integration, (iii) nuclear segregation, (iv) cell growth and division, (v) product synthesis, or (vi) turnover of sensitive products, if they were relatively stable, or a combination of any of these. The lag of *Azotobacter* transformant frequency stabilization was much closer to that of *Bacillus subtilis* (5 h, eight generations) (5) than that of *Haemophilus influenzae* (no lag) (9) or *Streptococcus pneumoniae* (5 min) (14).

Clearly there are not sufficient data in this initial report to determine unequivocably which of the factors in the preceding list are responsible for this delay in *Azotobacter*. Lags in DNA uptake and integration were unlikely to account for the delay because nitrogenase activity, as estimated by acetylene reduction (10), was detected within 80 min of DNA addition. The 3-h lag before cell division in the nonselective medium probably affected the timing of the first appearance of the Rif and Str transformants. The initial frequencies of these two markers also may be determined by the turnover rate of their respective products and by the number of cell divisions required to yield a phenotypically resistant cell. It has been estimated that *A. vinelandii* may contain 10 to 50 genome equivalents per cell (H. L. Sadoff, B. Shimei, and S. Ellis, Abstr. Annu. Meet. Am. Soc. Microbiol., Abstr. H99, p. 151, 1977). This would require a long period of nuclear segregation before the production of genotypically homogeneous progeny with a stable phenotype frequency during cell division.

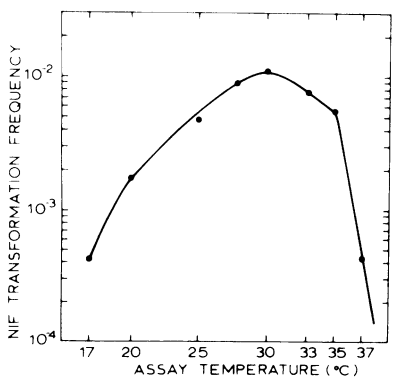

FIG. 3. *The effect of assay temperature on transformation frequency. Competent strain UW1 cells were preincubated in Burk buffer containing 8 mM $MgSO_4$ (final assay concn) for 10 min at the appropriate temperature, then transformed with strain 113 crude DNA for 20 min at 30°C. Transformation frequency was estimated as described in Fig. 1.*

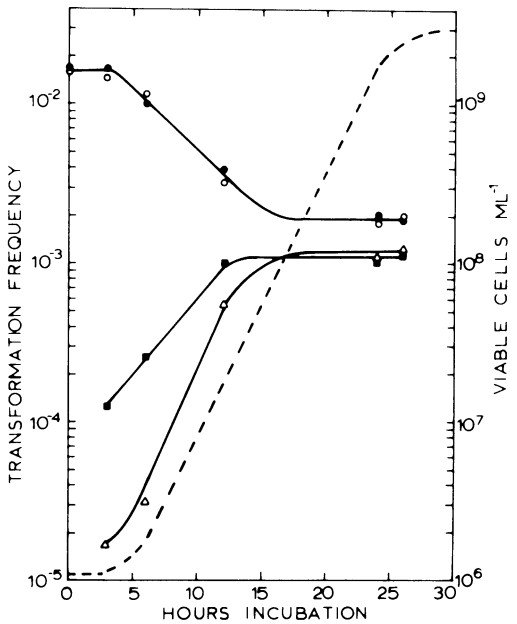

FIG. 4. *The time required for phenotype frequency stabilization. Competent strain UW1 cells were transformed with strain 113 or strain 114 crude DNA for 20 min at 30°C. After the addition of DNase, 0.2 ml of the assay tube contents was added to a 10-ml Erlenmeyer flask containing 4.8 ml of BBGN medium, and this was incubated at 30°C in a water bath rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Selective media for Rif and Str transformants consisted of the BBGN medium containing 20 μg of rifampin per ml or 20 μg of streptomycin per ml, respectively. At time intervals, the transformation frequencies of the Nif marker (●, strain 113 DNA; ○, strain 114 DNA), Str marker (△), and Rif marker (■) were estimated as described in Fig. 1. The viable count of the BBGN cultures was also determined (dotted line).*

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LITERATURE CITED

1. **Bishop, P. E., and W. J. Brill.** 1977. Genetic analysis of *Azotobacter vinelandii* mutant strains unable to fix nitrogen. J. Bacteriol. **130**:954-956.
2. **Bishop, P. E., F. B. Dazzo, E. R. Appelbaum, R. J. Maier, and W. J. Brill.** 1977. Intergeneric transfer of genes involved in *Rhizobium* legume symbiosis. Science **198**:938-940.
3. **Burton, K.** 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**: 315-323.
4. **Cosloy, S. D., and M. Oishi.** 1973. The nature of the transformation process in *Escherichia coli* K12. Mol. Gen. Genet. **124**:1-10.
5. **Fox, M. S., and R. D. Hotchkiss.** 1960. Fate of transforming deoxyribonucleate following fixation by transformable bacteria. Nature (London) **187**:1002-1006.
6. **Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izaea, and R. M. M. Singh.** 1966. Hydrogen ion buffers for biological research. Biochemistry **5**:467-477.
7. **Khan, N. C., and S. P. Sen.** 1974. Further observations on genetic transformation in *Pseudomonas*. J. Gen. Microbiol. **83**:251-259.
8. **Kloos, W. E.** 1969. Factors affecting transformation of *Micrococcus lysodeikticus*. J. Bacteriol. **98**:1397-1399.
9. **Nester, E. W., and B. A. D. Stocker.** 1963. Biosynthetic latency in early stages of deoxyribonucleic acid transformation of *Bacillus subtilis*. J. Bacteriol. **86**:785-795.
10. **Page, W. J.** 1978. Transformation of *Azotobacter vinelandii* strains unable to fix nitrogen with *Rhizobium* spp. DNA. Can. J. Microbiol. **24**:209-214.
11. **Page, W. J., and H. L. Sadoff.** 1976. Physiological factors affecting transformation of *Azotobacter vinelandii*. J. Bacteriol. **125**:1080-1087.
12. **Page, W. J., and M. von Tigerstrom.** 1978. Induction of transformation competence in *Azotobacter vinelandii* iron-limited cultures. Can. J. Microbiol. **24**:1590-1594.
13. **Rudin, L., J. Sjöström, M. Lindberg, and L. Philipson.** 1974. Factors affecting competence for transformation in *Staphylococcus aureus*. J. Bacteriol. **118**:155-164.
14. **Voll, M. J., and S. H. Goodgal.** 1965. Loss of activity of transforming deoxyribonucleic acid after uptake by *Haemophilus influenzae*. J. Bacteriol. **90**:873-883.
15. **Young, F. E., and J. Spizizen.** 1963. Incorporation of deoxyribonucleic acid in the *Bacillus subtilis* transformation system. J. Bacteriol. **86**:392-400.