Heat Sensitivity of *Azotobacter vinelandii* Genetic Transformation

JAMES L. DORAN AND WILLIAM J. PAGE*

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada, T6G 2E9

Heating competent *Azotobacter vinelandii* at 37 or 42°C resulted in a total loss of competence with no loss of viability. The transformation process was relatively insensitive to heating at either temperature once DNase-resistant DNA binding was nearly complete. Although competent and 42°C-treated cells bound equivalent amounts of [^{32}P]DNA in a DNase-resistant state, no donor DNA marker (*nif*) or radioactivity was detected in the envelope-free cell lysate of heated cells, suggesting that DNA transport across the cell envelope was a heat-sensitive event. Competence was reacquired in a 42°C-treated culture after 2 h of incubation at 30°C by a process which required RNA and protein syntheses. The release of a surface glycoprotein, required for competence, from cells treated at 42°C occurred in an insufficient amount to account for the total loss of competence. Recovery of competence in 42°C-treated cells and further transformation of competent cells were prevented by the exposure of cells to saturating amounts of transforming DNA. Further DNase-resistant DNA binding, however, still occurred, suggesting that there were two types of receptors for DNase-resistant DNA binding to competent *A. vinelandii*. DNase-resistant DNA binding was dependent on magnesium ions, and at least one receptor type did not discriminate against heterologous DNA.

Competence for bacterial transformation designates a physiological state in which cells are able to bind exogenous DNA and transport it across the cell envelope. Competence defined as the ability of cells to bind DNA in a DNase-resistant state (21) is not applicable to *Azotobacter vinelandii*, as both transformable and nontransformable cells share equally in this ability (this study). One approach to the study of the physiological aspects of *Azotobacter* competence is to determine the basis for treatments which are detrimental to competence (29). It has been shown that heating competent *A. vinelandii* at 37 or 42°C reduces competence (32). An envelope glycoprotein with an apparent molecular weight of 60,000 (60K), which is required for competence (29), is removed from the cells by washing with distilled water at 38°C (34). We report here that 60K glycoprotein removal is primarily effected by washing cells with distilled water and that the amount released upon heating cells in buffer is insufficient to account for competence loss at 37 to 42°C.

The treatment of competent *Haemophilus influenzae* at 42°C results in a reduction in the ability of cells to bind DNA in a DNase-resistant state (1). Heating competent *Streptococcus pneumoniae* at 35 to 40°C has the same effect, and this ability is regenerated at 30°C by a

process requiring protein synthesis (18). Similarly, heating competent *Streptococcus sanguis* (Challis) at 48°C for 20 min decreases the ability of cells to irreversibly bind DNA (33) and also reduces their ability to discriminate against heterospecific DNA and low-efficiency homospecific markers (9). Competent *Bacillus subtilis* is unable to bind DNA in a DNase-resistant state after brief heating at 50°C (25), and donor marker survival is also sensitive to heating (25, 26). The results of this investigation indicate that competent *A. vinelandii* heated at 42°C for 20 min is unable to transport donor DNA across the cell envelope but is fully capable of binding DNA in a DNase-resistant state. The survival and expression of newly transported donor DNA marker are insensitive to heating at 42°C.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The transformation recipient *A. vinelandii* OP strain UW1 (Nif⁻ capsule⁻) and the parental strain UW (Nif⁺ capsule⁻) were provided by W. Brill (University of Wisconsin, Madison). *A. vinelandii* ATCC 12837 strains 113 (Nif⁺ rifampin resistant) and 114 (Nif⁺ streptomycin resistant) and the diazotrophs *Azotobacter chroococcum* ATCC 7486, *Azotobacter paspali* ATCC 23368, *Azotobacter beijerinckii* ATCC 19360, *Azomonas macrocytogenes* ATCC 12335, *Azomonas insignis*, *Beijerinckia indica* ATCC 12523, *Rhizobium meliloti* 17A, *Rhizobi-*

um trifolii CC10, *Klebsiella pneumoniae* ATCC 13883, and *Clostridium pasteurianum* NCIB 9486 were used as sources of donor *nif* DNA. *C. pasteurianum* was grown under anaerobic conditions at 37°C on modified Burk medium containing 2% sucrose and 2 µg biotin per liter. *K. pneumoniae* was grown at 37°C on nutrient agar (Difco Laboratories, Detroit, Mich.) containing 1% glucose. *Rhizobium* species were grown on yeast extract agar, containing 1% glucose, at 30°C. *A. paspali* and *A. beijerinckii* were grown at 30°C on N-free Burk medium containing 1% sucrose as the C source. Other Nif+ strains were grown at 30°C on N-free Burk medium (30). Strain UW1 was grown on Burk medium containing 1.1 g of ammonium acetate per liter. Liquid cultures were shaken at 170 rpm in a water bath Gyrotory shaker (model G-76; New Brunswick Scientific Co., New Brunswick, N.J.).

Crude lysate DNA preparation. Crude lysate DNA was prepared by suspending cells from slant cultures in 15 mM saline–15 mM sodium citrate buffer, pH 7.0 (SSC), containing 0.05% sodium dodecyl sulfate, and heating at 60°C for 60 min (30). Heating was extended for up to 6 h for organisms other than *A. vinelandii*. In some cases, DNA was partially purified by phenol extraction, RNase treatment, and removal of oligoribonucleotides and capsular material as described by Marmur (24). DNA concentration was estimated using a modified (14) Burton assay (4).

Transformation assay. Competence for genetic transformation was induced in strain UW1 by 18 to 22 h of growth in Fe-limited Burk medium as described previously (31). Except where noted, competent cells were transformed in Burk buffer, pH 7.2, containing 8 mM MgSO4 (transformation assay buffer, [32]), with 10 µg of crude lysate DNA per 10^8 cells. This amount of DNA was approximately 10 times that required to saturate the cells. Similarly, [^{32}P]DNA was used at two to three times the saturating concentration. The reaction was stopped by the addition of DNase I (final concentration, 4 µg/ml) and incubation at 30°C for 5 min.

Cells to be transformed a second time with additional DNA were centrifuged and suspended in 1 volume of transformation assay buffer and then treated with trypsin, to degrade residual DNase, followed by trypsin inhibitor (final concentrations, 4 µg/ml), each for 10 min at 30°C. The cells were collected again, resuspended in transformation assay buffer, and exposed to the second DNA species. Competence and competence recovery in 42°C-treated cells were not sensitive to trypsin at this concentration.

Nif+ transformants were detected by plating cells directly on N-free Burk medium. Rifampin-resistant and streptomycin-resistant transformants required 20 h of incubation in Burk medium for phenotypic frequency stabilization (32) before plating on selective Burk medium containing 20 µg of rifampin or streptomycin per ml. Transformation frequency was calculated as the number of transformants detected per total number of viable cells plated on selective medium. All determinations were made at least twice.

Preparation of sterile culture supernatant. It was often necessary to remove competent cells from their original growth medium after a specific treatment and resuspend them for subsequent incubation. Resuspending competent cells in fresh Fe-limited Burk medium resulted in lower levels of competence and

poorer competence recovery than resuspending competent cells in filter-sterilized, original growth supernatant (unpublished data). In all instances, either competent culture supernatant or 42°C-treated competent culture supernatant served equally well as a resuspension medium.

Concentration of culture supernatants and distilled water washes of competent cells. Culture supernatant fluids were concentrated for the analysis of glycoprotein released by competent cells and Ca-limited competent cells as described previously (29). Distilled water washes of competent cells performed at either 30 or 42°C also were concentrated as previously described (29).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (20) under the conditions described previously (29). Proteins were stained as described by Fairbanks et al. (13) with Coomassie blue R250 in isopropanol–acetic acid.

Time of expression of nitrogenase activity in newly transformed cells. Cells from a competent culture of strain UW1 were washed twice by centrifugation, using 0.5 volume of Fe-limited, N-free Burk medium to remove excess ammonia, and were resuspended to the original volume in filter-sterilized culture supernatant obtained from strain UW grown for 20 h in Fe-limited, N-free Burk medium. A 5-ml amount of this cell suspension was dispensed into each of 12 10-ml Erlenmeyer flasks, and the cultures were incubated at 30°C for 4 h to starve the competent cells for nitrogen. The cultures were then transferred to centrifuge tubes containing 1.5 ml of strain 113 crude lysate DNA (60 µg of DNA per ml) and 18 ml of transformation assay buffer. After 20 min of incubation at 30°C, the reaction was stopped with 1.0 ml of 100-µg/ml DNase I. The cells were harvested by centrifugation, suspended in 5 ml of Fe-limited, N-free Burk medium, and incubated in 10-ml Erlenmeyer flasks at 30°C for the expression of nitrogenase activity. At 20-min intervals, beginning immediately, flasks were sealed with serum stoppers and nitrogenase activity was measured by the acetylene-ethylene reduction assay (16) as previously described (28). Portions of each culture were plated to determine the viable number and frequency of Nif+ transformants. Nitrogenase specific activity was calculated as nanomoles of ethylene formed per hour per 10^8 Nif+ transformants.

Recovery of donor DNA from transformed cells. Transformation assays were set up in five pairs of 15-ml conical centrifuge tubes, each containing 3.0 ml of transformation assay buffer and 50 µg of strain 113 crude lysate DNA. One tube of each pair received 0.5 ml of competent strain UW1 culture, and the other received 0.5 ml of the same culture treated at 42°C. After 1, 10, and 30 min of incubation at 30°C, DNA binding was stopped by the addition of 1 ml of 20-µg/ml DNase I, and each pair of tubes was placed on ice. DNase was digested with trypsin as described above, except that incubations were for 2 min at 22°C, a temperature at which transformation occurs very poorly (32). The cells were collected by centrifugation at 4°C, and crude lysates were prepared. Cell envelopes were removed by centrifugation at 20,000 rpm for 60 min, and the supernatant was decanted and stored at 4°C. DNA binding in the remaining two pairs of tubes was stopped after 30 min. DNase was inacti-

vated as described above, using 5-min incubation periods at 30°C. The cells were collected by centrifugation, suspended in 0.5 ml of sterile culture supernatant, and incubated with shaking at 30°C in 10-ml Erlenmeyer flasks. Envelope-free cell lysates were prepared from these samples 60 and 90 min after the initiation of transformation.

The envelope-free cell lysates were assayed for *nif* transforming activity by mixing each 0.5 ml of lysate with 1 ml of freshly prepared strain UW1 competent cell suspension in 6 ml of transformation assay buffer. The reaction was stopped after 20 min of incubation at 30°C by the addition of 1.0 ml of 20-μg/ml DNase I. As the number of Nif$^+$ transformants was expected to be small, the recipient cells were concentrated 7.5-fold before plating on selective medium.

Preparation of purified [^{32}P]DNA. Crude lysate DNA was prepared from strain UW1 grown for 2 days in modified Burk medium containing 1 mM phosphate buffer, pH 7.2, and 2.5 mCi of ^{32}P (55% of which became incorporated). The crude lysate was treated with proteinase K and further purified as previously described (24). [^{32}P]DNA was purified free of residual contaminating ^{32}P-labeled oligoribonucleotides by 4.5 h of electrophoresis at 50 V in 0.5% agarose–20 mM Tris–2 mM EDTA–50 mM sodium acetate (pH 7.8) (TEA) gels. [^{32}P]DNA detected by autoradiography with Kodak X-Omat X-ray film was extracted by gel dissolution in KI and DNA binding to hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories, Mississauga, Ontario, Canada) (37). The hydroxylapatite-[^{32}P]DNA was washed free of agarose and KI as described previously (37), except that it was not formed into a column as better recoveries of [^{32}P]DNA were obtained by batch treatment. Treatment with 1 M sodium phosphate–1 mM EDTA released 20 to 30% of [^{32}P]DNA, which was dialyzed twice in 48 h against 4 liters of SSC at 4°C. Samples concentrated by dialysis against polyethylene glycol (PEG 20,000) were analyzed by agarose gel electrophoresis. All radioactivity detected by autoradiography was present in high-molecular-weight DNA. Treatment of the sample with DNase I rendered 98% of the radioactivity soluble in cold 10% trichloroacetic acid. The specific activity of the [^{32}P]DNA preparation varied with different batches and the age of the preparation. Nonradioactive purified DNA also was prepared by this method.

[^{32}P]DNA binding and uptake. Cells from a competent strain UW1 culture and from a 42°C-treated, formerly competent culture were concentrated 10-fold into 0.5 ml of sterile competent culture supernatant and mixed with 3 ml of transformation assay buffer containing 0.5 ml of [^{32}P]DNA. After 10 min of incubation, the cells were pelleted and washed once by centrifugation with 4 ml of transformation assay buffer, suspended in the same, and treated with DNase I for 5 min. The amount of unabsorbed [^{32}P]DNA and the amount of radioactivity released by DNase treatment of cells were determined by adding 4 ml of aqueous sample to 6 ml of Monophase 40 liquid scintillation fluor (Packard Instrument Co., Inc., Downers Grove, Ill.) and measuring radioactivity with a Searle model 6880 liquid scintillation counter. Residual DNase in the transformed cell suspension was digested with trypsin, which in turn was inactivated with trypsin inhibitor as described above. The cell pellet was suspended in 4 ml of sterile culture superna-

tant. A crude lysate (total volume, 4 ml) prepared from 0.5 ml of this suspension was used to determine the amount of [^{32}P]DNA bound to cells in a DNase-resistant state. The remaining 3.5 ml of cell suspension was shaken for 2 h in a 10-ml Erlenmeyer flask to allow completion of the transformation process. ^{32}P released into the culture supernatant was measured. The location of bound [^{32}P]DNA was analyzed further after spheroplasts were formed by suspending cells in 3.5 ml of 50 mM Tris–50 mM EDTA–10% (wt/vol) sucrose, pH 8.0, and incubating for 60 min at 30°C with 100 μg of lysozyme per ml. Pelleted spheroplasts were lysed by twice suspending them in 5 ml of distilled water. The lysates were pooled and centrifuged at 5,000 rpm for 10 min to collect unbroken cells which, typically, were absent. The lysate was then centrifuged at 20,000 rpm for 70 min to obtain the spheroplast membranes, and the radioactivity in this pellet and in the supernatant was measured.

The spheroplast lysate was analyzed to determine whether all radioactivity was present as high-molecular-weight DNA. DNA and RNA, precipitated by adding 10 ml of lysate to 30 ml of 95% ethanol and placing at −20°C for 24 h, were collected by centrifugation at 4,000 rpm for 60 min, and the amount of soluble ^{32}P remaining in the supernatant was measured. The ethanol-precipitable material was subjected to electrophoresis on a 0.4% agarose–TEA gel for 10.5 at 20 V. Lambda *c*I857 DNA was used as a molecular weight indicator. The gel was stained with ethidium bromide, and DNA and RNA bands were detected by fluorescence (35). ^{32}P-labeled material was detected by autoradiography for 9 days at −70°C as described earlier but with du Pont Cronex Lightning-Plus intensifying screens.

Chemicals. All fine chemicals were reagent grade, and most were obtained from Fisher Scientific Co., Edmonton, Alberta, Canada, except for enzymes and trypsin inhibitor, which were from Sigma Chemical Co., St. Louis, Mo. Agarose was supplied by Mandel Scientific Co., Calgary, Alberta, Canada. Carrier-free [^{32}P]phosphoric acid in aqueous solution was supplied by Amersham Corp., Arlington Heights, Ill. Acrylamide and N,N'-methylenebisacrylamide were from Eastman Kodak Co., Rochester, N.Y. All other electrophoresis chemicals were obtained from Bio-Rad Laboratories.

RESULTS

Loss of competence at elevated temperatures and competence recovery. Holding a competent culture of *A. vinelandii* at 37 or 42°C resulted in an exponential loss of competence (Fig. 1). Although competence loss at 37°C occurred at a slower rate after a lag of 20 min, both treatments resulted in a complete phenotypic loss of competence. The treatment of cells at either temperature had no effect on viability, and holding competent cells at 30°C had no effect on competence.

Transformable cells were regenerated in 42°C-treated culture by incubation at 30°C (Fig. 2). The maximum level of competence attained was
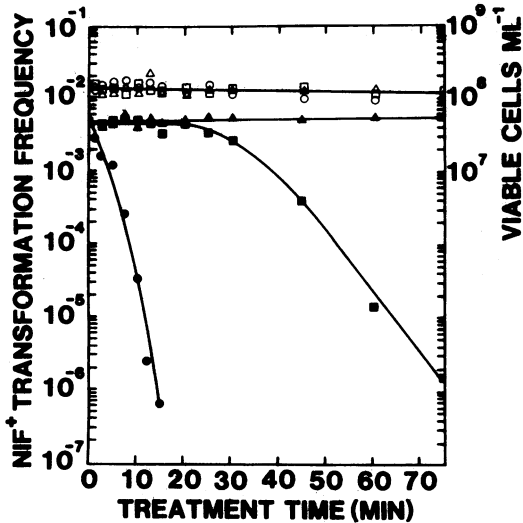
FIG. 1. Loss of competence at elevated temperatures. A competent strain UW1 culture was held at 30°C (△), 37°C (□), or 42°C (○). At intervals, samples of the cells were removed, equilibrated to 30°C, and assayed for transformation frequency (closed symbols) and viable number (open symbols).

FIG. 2. Recovery of competence in 42°C-treated cultures. A culture of competent strain UW1 (Nif+ transformation frequency, 1.7×10^{-3}) was held at 42°C for 25 min to eliminate competence. Samples of the 42°C-treated culture were incubated with chloramphenicol (final concentration, 25 μg/ml) (□), rifampin (final concentration, 1 μg/ml) (△), or no further additions (○). After 30 min of incubation at 30°C, the cells were harvested by centrifugation and suspended in the original volume of original competent culture supernatant. Incubation was continued, and at intervals samples were removed for transformation assay.

not always as high as that before heating. This probably reflected the normal decay of competence which occurs after an extended period of incubation in Fe-limited medium (31). There was no change in the number of viable cells during the period of competence recovery. The recovery of competence appeared to require RNA and protein syntheses because recovery was delayed when cells were treated with rifampin or chloramphenicol (Fig. 2). Rifampin and chloramphenicol at these concentrations had no effect on the maintenance of competence in an unheated culture.

It has been reported that a 60K envelope glycoprotein required for competence (29) was completely removed from A. vinelandii by washing with distilled water at 38°C but not at 33°C (34). It was possible, therefore, that synthesis of the 60K glycoprotein was the step necessary for competence recovery in 42°C-treated cells. Although the culture supernatant of the 42°C-treated competent culture contained somewhat more 60K glycoprotein (Fig. 3, lane 4) than did the competent culture supernatant (Fig. 3, lane 3), it was much less than was removed by washing competent cells with distilled water at 30°C (lane 2). Washing cells with distilled water resulted in a fivefold reduction in competence (transformation frequency, 2.4×10^{-4}), whereas 42°C-treated cells were noncompetent. Cells pregrown in Ca-limited competence medium developed a very low level of competence (trans-
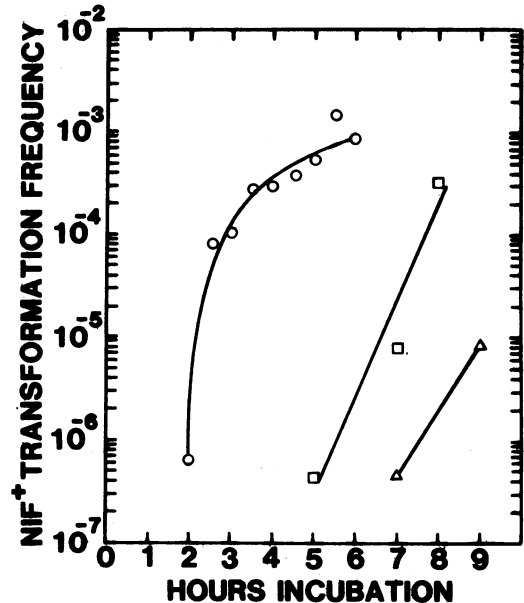
formation frequency, 1.8×10^{-6}) and released a similar large amount of glycoprotein into the culture supernatant (Fig. 3, lane 1). Although 60K glycoprotein loss has been correlated with competence loss (29), clearly, glycoprotein release from 42°C-treated competent cells was insufficient to account for the complete loss of competence observed. These data also indicated that the release of glycoprotein from cells was mediated primarily by washing with distilled water and not by treatment at 42°C.

Effect of elevated temperatures on transformed cells. Competent cells exposed to a 10-fold excess of donor DNA became nearly saturated with DNA bound in a DNase-resistant form after 5 to 10 min (Fig. 4). DNase-resistant DNA binding was prevented by adding DNase 1 s but not 2 s after adding DNA. Shifting cells to 37°C during this period of rapid DNase-resistant DNA binding had little or no effect on the transformation frequency, whereas heating at 37°C after this time produced a slight, but consistently observed, enhancement. Shifting the transformation assay to 42°C after DNase-resistant
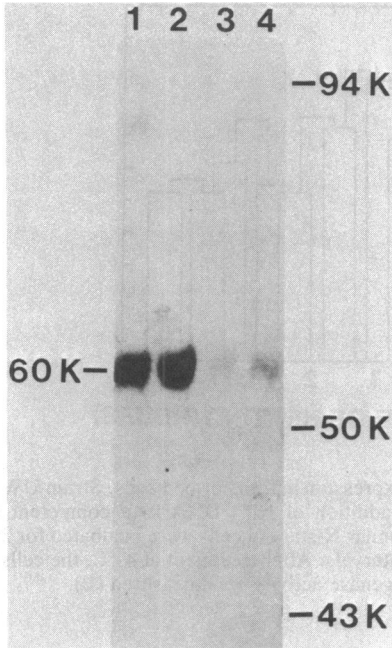
FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of culture supernatants and 30°C distilled water washes of competent cells. All samples were concentrated to a standard volume per 10^8 cells per ml in the original culture, and 50 μl of each was added to the gel. Lanes 1, 3, and 4 contained concentrated culture supernatants from strain UW1 (1) grown in Ca- and Fe-limited Burk medium, (3) grown in Fe-limited Burk medium, and (4) pregrown in Fe-limited Burk medium and treated at 42°C for 25 min. Lane 2 contained concentrated 30°C distilled water washes of competent cells. The molecular weight standards phosphorylase *a* (94,000), bovine immunoglobulin (50,000), and ovalbumin (43,000) were used to determine the molecular weights shown.
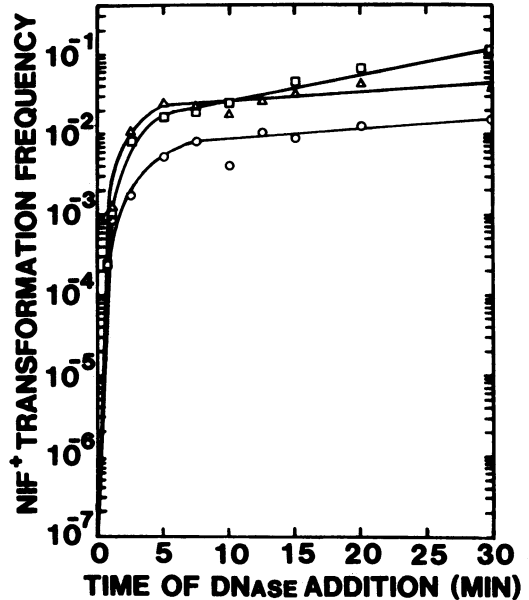

FIG. 4. Treatment of competent cells at 30, 37, or 42°C after DNase-resistant DNA binding. At zero time, crude lysate DNA was mixed with strain UW1 to initiate transformation at 30°C. At intervals thereafter, DNase I was added, and the assay mixtures were immediately transferred to preheated tubes at 30°C ($\triangle$), 37°C ($\square$), or 42°C ($\bigcirc$) and held at this temperature for 30 min before plating on selective medium.

DNA binding produced only a small, but consistent, decrease in transformation frequency.

The failure to detect transformants in 42°C-treated, formerly competent cells could not be attributed to a temperature-sensitive event in the subsequent steps of the transformation process. Newly acquired nitrogenase genes in transformed, nitrogen-starved strain UW1 were expressed 60 to 80 min after the addition of transforming DNA (Fig. 5A). Neither the survival of the donor DNA *nif* marker in the recipient cell nor the expression of the *nif* genes (Fig. 5B) was particularly sensitive to heating at 42°C.

Recovery of *nif* marker from recipient cells. Donor (*nif*) activity was recovered from transformed envelope-free cell lysates 1 min after mixing donor DNA with the strain UW1 recipient (transformation frequency, 5.8×10^{-6}). The Nif$^+$ transforming activity of the competent cell

lysates increased with time during the 30-min exposure of cells to donor DNA and during 60 min of further incubation after the addition of DNase (maximum frequency, 2.4×10^{-4}). The second recipient was transformed at a frequency of 3.5×10^{-2} when exposed to a similar concentration of original donor DNA. No Nif$^+$ donor activity was recovered from similarly prepared 42°C-treated cell lysates. These results suggested that DNA uptake was the heat-sensitive event.

Binding and uptake of [^{32}P]DNA. Competent cells, pretreated at 42°C, bound equivalent amounts of [^{32}P]DNA as unheated competent cells ($P = 0.05$). Two-thirds of the [^{32}P]DNA bound to either cell type was insensitive to DNase. The fate of this DNA after incubation of the cells for a period sufficient to allow uptake and integration of DNA was determined (Table 1). During the 2-h incubation period after the termination of DNase-resistant DNA binding, both cell types released similar amounts of radioactivity, 95% of which was soluble in cold 8% trichloroacetic acid. Approximately 17% of the radioactivity bound to competent cells was transported into the cell cytoplasm, but there was essentially no [^{32}P]DNA uptake in 42°C-
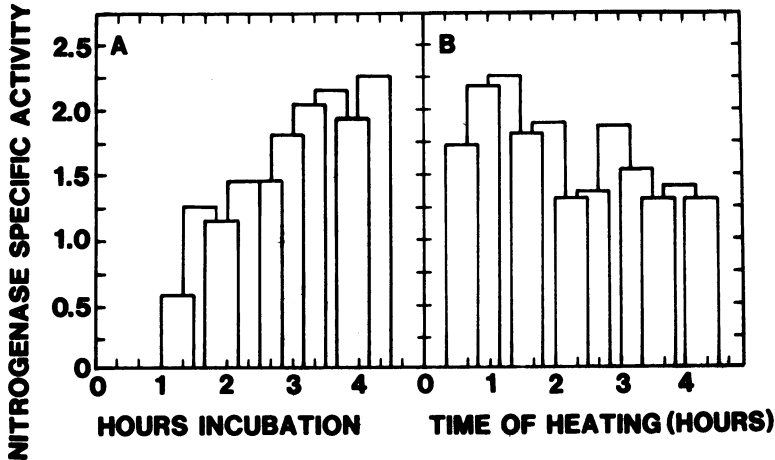
FIG. 5. Effect of 42°C heating on *nif* marker survival and *nif* expression in transformed cells. Strain UW1 was assayed for nitrogenase activity at 20-min intervals after the addition of Nif⁺ DNA to a competent (Nif⁺ transformation frequency, 1.6 × 10⁻²), N-starved culture (A). Similar N-starved cells were incubated for 20 min with donor DNA and then shifted to 42°C for 30 min at 20-min intervals. After treatment at 42°C, the cells were transferred to 30°C to give a total incubation of 5 h before nitrogenase activity was measured (B).

treated cells. The very small amount of radioactivity which appeared in the cytosol fraction of 42°C-treated cells was quite possibly washed from the spheroplast membranes when they were removed by centrifugation. A greater proportion of the [^{32}P]DNA remained bound to the 42°C-treated cell envelope, consistent with a failure to transport DNA into these cells. All of the ^{32}P-labeled material in the cytosol of the transformed cells was ethanol precipitable. The chromosomal and [^{32}P]DNA in osmotically lysed spheroplasts migrated as a tight band (molecular weight > 1.7 × 10⁷) during electrophoresis in agarose. These results were consistent with the hypothesis that 42°C-treated cells were unable to transport DNA across the cell envelope.

DNA binding to competent and noncompetent cells. It could not be determined whether cells which were competent before 42°C treatment

were the same cells which reacquired competence during subsequent incubation. Competent *A. vinelandii* cells which were exposed to saturating strain 113 (Rifʳ) DNA before 42°C treatment were not transformed to streptomycin resistance with DNA from strain 114 at any time during the 8-h postheating incubation at 30°C. Exposure of 42°C-treated cells to saturating Rifʳ DNA prevented subsequent transformation by Strʳ DNA, and Rifʳ transformants were not detected during the 8-h incubation. Competent cells exposed to an excess of strain 113 DNA also could not be transformed subsequently with strain 114 DNA, even 8 h after the first exposure to strain 113 DNA.

In each of the three cases outlined above, preexposure of cells to an excess of purified DNA did not prevent these cells from binding a second [^{32}P]DNA in a DNase-resistant state. Approximately equivalent amounts of [^{32}P]DNA

TABLE 1. Binding and uptake of [^{32}P]DNA[a]

Recipient cells[b]	DNase-resistant ^{32}P bound		^{32}P released during 2-h incubation		^{32}P released upon spheroplast formation		^{32}P bound to spheroplast membranes		^{32}P in cell cytosol	
	cpm[c]	%	cpm	%	cpm	%	cpm	%	cpm	%
Competent	3,280 (±215)[d]	100	197 (±35)	6.0	2,309 (±473)	70.4	212 (±29)	6.4	562 (±120)	17.2
42°C treated	2,945 (±510)	100	136 (±18)	4.6	2,002 (±128)	68.0	780 (±23)	26.5	26.7 (±3.0)	0.95

[a] The procedure is described in the text.
[b] Competent cells were transformed to Nif⁺ at a frequency of 4.9 × 10⁻². No competent cells were detected in the 42°C-treated culture.
[c] Measurements of radioactivity are counts per minute per 10⁸ cells.
[d] Standard deviation.

were bound after the first and second exposure of competent cells to DNA. Precompetent strain UW1 grown in Fe-limited Burk medium for 4, 8, and 12 h (31) bound as much [^{32}P]DNA in a DNase-resistant state (1.2 × 10^5 cpm per 10^8 cells) as did the competent population which was present after 16 and 24 h of incubation. [^{32}P]DNA also was bound in a DNase-resistant state by noncompetent cells (31) prepared by growth in Fe-sufficient Burk medium (9.1 × 10^4 cpm per 10^8 cells) or in Fe-limited Burk medium containing glutamate as the sole N source (5.9 × 10^5 cpm per 10^8 cells). The ability of *A. vinelandii* to bind DNA in a DNase-resistant state was, therefore, not a competence-specific characteristic. DNase-resistant DNA binding was dependent on magnesium ions. Competent cells which had been washed free of excess magnesium ions and exposed to DNA in the absence of magnesium ions bound less than 1.0 × 10^2 cpm of [^{32}P]DNA per 10^8 cells, and no transformants were generated.

Heterologous DNA competition. Competent strain UW1 was transformed to Nif$^+$ at a high frequency with saturating DNA from *A. vinelandii* strains 113 (frequency, 1.0 × 10^{-2}), 114 (frequency, 2.5 × 10^{-2}), or UW (frequency, 8.4 × 10^{-3}). Transformation with saturating concentrations of DNA prepared from *A. chroococcum* and *A. paspali* resulted in frequencies of 4.0 × 10^{-3} and 2.2 × 10^{-3}, respectively. Strain UW1 was transformed at a very low frequency by saturating DNA from *A. beijerinckii* (frequency, 3.2 × 10^{-5}), *B. indica* (frequency, 1.5 × 10^{-6}), *R. meliloti* (frequency, 1.4 × 10^{-6}), and *R. trifolii* (frequency, 3.3 × 10^{-6}). Strain UW1 was not transformed by DNA isolated from the distantly related bacteria *A. insignis* and *A. macrocytogenes* or the unrelated bacteria *K. pneumoniae* and *C. pasteurianum*.

Several of the homologous and heterologous DNA species were partially purified and tested for their ability to compete with strain 113 DNA. Competing DNA was mixed with an equivalent amount (1 µg per 10^8 cells) of strain 113 DNA, and the frequency of Rif$^+$ marker transformation was assayed. Nontransforming *A. macrocytogenes* DNA and very poorly transforming *B. indica* DNA decreased the Rif$^+$ transformation frequency by 50 to 52%. The ability of these two DNA species to block transformation was not altered significantly when recipient cells were exposed to the heterologous DNA 20 min before they were exposed to strain 113 DNA. DNA from *A. beijerinckii*, *A. paspali*, and *A. vinelandii* strain 114 similarly decreased transformation by strain 113 DNA by 55, 59, and 67%, respectively. Although the ability of these related DNA species to compete with strain 113 DNA did not correlate precisely with their ability to transform

strain UW1, the relative order was the same. These DNA species blocked transformation more efficiently when cells were exposed to blocking DNA before the addition of strain 113 DNA, and the relative order of blocking ability remained unchanged. Treatment of cells with DNase after exposure to heterologous or homologous DNA and before the addition of strain 113 DNA did not alter the blocking ability of the competing DNA species. Blocking by these DNA species also was dependent on magnesium ions.

Bacteriophage φW-14 DNA bound in a DNase-resistant state to competent *A. vinelandii* blocked transformation by an equivalent amount (1 µg) of strain 113 homologous DNA more effectively than any other heterologous DNA, including heterospecific DNA species. Under these conditions, φW-14 DNA produced a 64% decrease in the Rif$^+$ transformation frequency. The ability of φW-14 DNA to block transformation by homologous DNA was concentration dependent, such that transformation of strain UW1 was completely blocked by exposing 10^8 cells to greater than 50 µg of φW-14 DNA.

DISCUSSION

Genetic tranformation of *A. vinelandii* was notably similar to that of *H. influenzae*. DNase-resistant DNA binding to both organisms demonstrated a brief lag (from 1 to 3 s), was saturated after 5 to 10 min of exposure to excess DNA (11, 39), and occurred outside the cytoplasmic membrane (10, 17). In both transformation systems, DNA uptake was not accompanied by the release of an equivalent amount of acid-soluble deoxyribonucleotides from the cell surface (40). Therefore, a nuclease such as that active in gram-positive transformation systems (19, 27), which generates single-stranded DNA during uptake, does not have an equivalent role in *A. vinelandii* transformation. Single-stranded DNA probably is not an acceptable donor in this system because heat-denatured *A. vinelandii* DNA (60 µg of DNA per ml of SSC at 100°C for 25 min) is 1,200-fold less active in transformation than native DNA (data not shown). Since biologically active donor DNA was recovered from transformed *A. vinelandii* as early as 1 min after the DNA addition, it is likely that the donor DNA is transported in a double-stranded form, typical of gram-negative transformation systems (1, 2, 38).

In contrast to other heat-sensitive transformation systems (9, 25, 26, 33), the major effect of heating competent *A. vinelandii* was limited to a single period before the completion of DNase-resistant DNA binding. Despite this, heat-treated, formerly competent cells were not impaired in their ability to bind DNA in a DNase-resistant

state. The small, but consistently observed, enhancement of transformability at 37°C that occurred concurrently with the completion of DNase-resistant DNA binding indicated that DNase-resistant DNA binding and the heat-sensitive step were closely linked temporally. DNase-resistant DNA binding clearly preceded the heat-sensitive step in the transformation process. The significant observation was that little or no radioactivity and no biologically active donor DNA marker were recovered from the cytoplasm of 42°C-treated cells which had bound saturating amounts of [^{32}P]DNA in a DNase-resistant state. The data support the hypothesis that the heat-sensitive event was involved in DNA transport across the cell envelope. The existence of a DNA uptake protein (7, 41) which is particularly heat labile may explain the need for protein synthesis during competence recovery. The nature of the heat-sensitive event in *A. vinelandii* transformation may be unique. In other heat-sensitive transformation systems, the loss of transformability is almost totally attributable to a loss of the ability to bind DNA on the cell surface in a DNase-resistant state (1, 9, 25, 33).

Similar to *Neisseria gonorrhoeae* (12), *A. vinelandii* cells exposed to homologous DNA were unable to be transformed by a second DNA species. A surprising observation was that these cells were capable of further DNase-resistant DNA binding. These results suggest that there may be two types of DNA binding to competent *A. vinelandii*, both of which convey resistance to DNase. We propose that DNA receptors of type 1 are responsible for the possibly less specific form of DNase-resistant DNA binding, which may be preliminary to DNA binding for transformation but which occurs in the absence of transformation and that type 2 receptors are those which bind transforming DNA during the initial stages of the transformation process. These two DNA receptor types are therefore somewhat analogous to those described for *N. gonorrhoeae* (12). Both types of DNA binding receptors are heat stable as 42°C-treated cells participated in both types of DNase-resistant DNA binding. Furthermore, 42°C-treated cells failed to transport DNA, indicating that both types of receptors must be located in the cell envelope. The existence of type 1 receptors may explain the phenomenon of DNase-resistant DNA binding to noncompetent cells, a characteristic apparently unique to *A. vinelandii*. Obviously, whether noncompetent cells possess a type 2 receptor cannot be determined until such a receptor has been physically identified.

Competent *A. vinelandii* cells discriminate against transformation by heterologous DNA, a characteristic unique to gram-negative organisms which are naturally grown to competence (5, 12, 15, 36). A possible exception to this rule was the anomalous behavior of φW-14 DNA, a DNA species which also is exceptionally competitive in the *B. subtilis* transformation system (24). When competent *A. vinelandii* cells are generated by the artificial system used to produce competent *Escherichia coli* cells (6), however, they show the same lack of discrimination against heterologous DNA as does *E. coli* (3, 8).

Competent *A. vinelandii* demonstrated a certain lack of discrimination against binding heterologous DNA in a DNase-resistant state, which may be explained by the existence of type 1 receptors. The ability of heterologous nontransforming DNA to inhibit transformation by homologous DNA was proportional to the concentration of the two competing DNA species but was not enhanced by the prior exposure of cells to blocking DNA, unlike the case with competing transforming DNA. These results suggest either that DNA binding to type 1 receptors was preliminary to binding to type 2 receptors or that DNA binding to type 1 receptors hindered transforming DNA binding to type 2 receptors.

It is possible that type 2 receptors were responsible for discrimination against heterologous DNA other than φW-14 DNA, preventing or limiting its uptake. Thus, type 2 receptors would resemble the surface receptors of competent *H. influenzae* (10, 11, 17) and *N. gonorrhoeae* (12), which recognize specific uptake sequences on transforming DNA. These sequence-specific receptors are reputed to bind and transport one molecule of DNA each, but *A. vinelandii* doubly transformed by unlinked markers is often observed (unpublished data), suggesting that there are several type 2 receptors per competent cell. The lag before the loss of transformation competence during 37°C treatment suggests that there are also several copies of a heat-labile DNA uptake site per competent cell.

The lack of transformants in a 42°C-treated culture, containing prebound homologous DNA, after incubation for a period sufficient to otherwise allow competence recovery suggests that DNA uptake may have to occur concomitantly with DNA binding to type 2 receptors. The rapid recovery of donor DNA marker (*nif*) from competent cells demonstrated that DNA transport was occurring very soon after DNase-resistant DNA binding. DNA binding to type 2 receptors may alter the receptor, preventing it from becoming properly associated with the DNA uptake mechanism at a later time. Type 2 receptors, therefore, may be synthesized only once in the competence cycle, or if they are synthesized during competence recovery, they may be inactivated by DNA prebound to the cell.

LITERATURE CITED

1. **Barnhart, R. J., and R. M. Herriott.** 1963. Penetration of deoxyribonucleic acid into *Haemophilus influenzae*. Biochim. Biophys. Acta **76**:25–39.
2. **Biswas, G. D., and P. F. Sparling.** 1981. Entry of double-stranded deoxyribonucleic acid during transformation of *Neisseria gonorrhoeae*. J. Bacteriol. **145**:638–640.
3. **Brown, M. G. M., J. R. Saunders, and G. O. Humphreys.** 1981. Lack of specificity in DNA binding and uptake during transformation of *Escherichia coli*. FEMS Microbiol. Lett. **11**:97–100.
4. **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.
5. **Carlson, C. A., L. S. Pierson, J. J. Rosen, and J. L. Ingraham.** 1983. *Pseudomonas stutzeri* and related species undergo natural transformation. J. Bacteriol. **153**:93–99.
6. **Cohen, S. N., A. C. Y. Chang, and L. Hue.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110–2114.
7. **Concino, M. F., and S. H. Goodgal.** 1981. *Haemophilus influenzae* polypeptides involved in deoxyribonucleic acid uptake detected by cellular surface protein iodination. J. Bacteriol. **148**:220–231.
8. **David, M., M. Tronchet, and J. Dénarié.** 1981. Transformation of *Azotobacter vinelandii* with plasmids RP4 (IncP-1 group) and RSF1010 (IncQ group). J. Bacteriol. **146**:1154–1157.
9. **Deddish, P. A., and A. W. Ravin.** 1974. Relation of macromolecular synthesis in streptococci to efficiency of transformation by markers of homospecific and heterospecific origin. J. Bacteriol. **117**:1158–1170.
10. **Deich, R. A., and L. C. Hoyer.** 1982. Generation and release of DNA-binding vesicles by *Haemophilus influenzae* during induction and loss of competence. J. Bacteriol. **152**:855–864.
11. **Deich, R. A., and H. O. Smith.** 1980. Mechanism of homospecific DNA uptake in *Haemophilus influenzae* transformation. Mol. Gen. Genet. **177**:369–374.
12. **Dougherty, T. J., A. Asmus, and A. Tomasz.** 1979. Specificity of DNA uptake in genetic transformation of gonococci. Biochem. Biophys. Res. Commun. **86**:97–104.
13. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2616.
14. **Giles, K. W., and A. Meyers.** 1965. An improved diphenylamine method for estimation of deoxyribonucleic acid. Nature (London) **206**:93.
15. **Graves, J. F., G. D. Biswas, and P. F. Sparling.** 1982. Sequence-specific DNA uptake in transformation of *Neisseria gonorrhoeae*. J. Bacteriol. **152**:1071–1077.
16. **Hardy, R. W. F., D. Holstein, E. K. Jackson, and R. C. Burns.** 1968. The acetylene-ethylene assay for N_2-fixation: laboratory and field evaluation. Plant Physiol. **43**:1085–1207.
17. **Kahn, M. E., G. Maul, and S. H. Goodgal.** 1982. Possible mechanism for donor DNA binding and transport in

Haemophilus. Proc. Natl. Acad. Sci. U.S.A. **79**:6370–6374.
18. **Lacks, S., and B. Greenberg.** 1973. Competence for deoxyribonucleic acid uptake and deoxyribonuclease action external to cells in the genetic transformation of *Diplococcus pneumoniae*. J. Bacteriol. **114**:152–163.
19. **Lacks, S., B. Greenberg, and M. Neuberger.** 1974. Role of a deoxyribonuclease in the genetic transformation of *Diplococcus pneumoniae*. Proc. Natl. Acad. Sci. U.S.A. **71**:2305–2309.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lerman, L. S., and L. J. Tolmach.** 1957. Genetic transformation. I. Cellular incorporation of DNA accompanying transformation in *Pneumococcus*. Biochim. Biophys. Acta **26**:68–82.
22. **López, P., M. Espinosa, M. Piechowska, and D. Shugar.** 1980. Influence of bacteriophage PBS1 and φW-14 deoxyribonucleic acids on homologous deoxyribonucleic acid uptake and transformation in competent *Bacillus subtilis*. J. Bacteriol. **143**:50–58.
23. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
24. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
25. **McCarthy, C., and E. W. Nester.** 1969. Heat-sensitive step in deoxyribonucleic acid-mediated transformation of *Bacillus subtilis*. J. Bacteriol. **97**:162–165.
26. **McDonald, W. C.** 1971. Initial temperature sensitivity of streptomycin-dependent and tryptophan transformants of *Bacillus subtilis*. Can. J. Microbiol. **17**:823–825.
27. **Mulder, J. A., and G. Venema.** 1982. Transformation-deficient mutants of *Bacillus subtilis* impaired in competence-specific nuclease activities. J. Bacteriol. **152**:166–174.
28. **Page, W. J.** 1982. Optimal conditions for induction of competence in nitrogen-fixing *Azotobacter vinelandii*. Can. J. Microbiol. **28**:389–397.
29. **Page, W. J., and J. L. Doran.** 1981. Recovery of competence in calcium-limited *Azotobacter vinelandii*. J. Bacteriol. **146**:33–40.
30. **Page, W. J., and H. L. Sadoff.** 1976. Physiological factors affecting transformation of *Azotobacter vinelandii*. J. Bacteriol. **125**:1080–1087.
31. **Page, W. J., and M. von Tigerstrom.** 1978. Induction of transformation competence in *Azotobacter vinelandii* iron-limited cultures. Can. J. Microbiol. **24**:1590–1594.
32. **Page, W. J., and M. von Tigerstrom.** 1979. Optimal conditions for transformation of *Azotobacter vinelandii*. J. Bacteriol. **139**:1058–1061.
33. **Ravin, A. W., and M. Ma.** 1972. Specific effects of heating of transformable streptococci on their ability to discriminate between homospecific, heterospecific, and hybrid deoxyribonucleic acid. J. Bacteriol. **109**:616–625.
34. **Schenk, S. P., and C. F. Earhart.** 1981. Characterization of the predominant *Azotobacter vinelandii* envelope protein. J. Bacteriol. **146**:398–403.
35. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry **12**:3055–3063.
36. **Sisco, K. L., and H. O. Smith.** 1979. Sequence specific DNA uptake in *Haemophilus* transformation. Proc. Natl. Acad. Sci. U.S.A. **76**:972–976.
37. **Smith, H. O.** 1980. Recovery of DNA from gels. Methods Enzymol. **65**:371–380.
38. **Stuy, J. H.** 1965. Fate of transforming DNA in the *Haemophilus influenzae* transformation system. J. Mol. Biol. **13**:554–570.

39. **Stuy, J. H., and D. Stern.** 1964. The kinetics of DNA uptake by *Haemophilus influenzae*. J. Gen. Microbiol. **35:**391–400.
40. **Stuy, J. H., and B. Van der Have.** 1971. Degradation of adsorbed transforming DNA by *Haemophilus influenzae*.

J. Gen. Microbiol. **65:**147–152.
41. **Sutrina, S. L., and J. J. Scocca.** 1979. *Haemophilus influenzae* periplasmic protein which binds deoxyribonucleic acid: properties and possible participation in genetic transformation. J. Bacteriol. **139:**1021–1027.