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Calcium-Requiring Step in the Uptake of Deoxyribonucleic Acid Molecules Through the Surface of Competent Pneumococci

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The conversion of surface-adsorbed deoxyribonucleic acid (DNA) molecules to a state in which they are inaccessible to exogenous deoxyribonuclease requires specifically calcium ions; magnesium ions cannot replace calcium ions. Virtually maximal levels of nuclease-resistant DNA binding and genetic transformation can be obtained in media free from magnesium and containing only calcium ions. It is suggested that the calcium-requiring process is the transport of DNA molecules across the plasma membrane. Magnesium ions stimulate both the loss of surface-adsorbed DNA to the medium and the extracellular degradation of DNA.

Methods have been developed recently that allow the recognition of several types of interactions between extracellular deoxyribonucleic acid (DNA) molecules and components of the cell surface during early stages of genetic transformation (6, 13). These interactions include the appearance of DNA receptors at the cell surface (in a process that requires the activator protein) (12); a glucose-dependent, "deep" binding of DNA molecules to a component(s) of the plasma membrane (12); nuclease-catalyzed release of surface-adsorbed DNA molecules to the medium (13); degradation of the adsorbed DNA by surface-located nucleases (3, 5, 9, 10); and an energy- and divalent cation-requiring uptake of the adsorbed DNA (5, 13).

In this communication we present evidence indicating that the process of DNA uptake (i.e., conversion of the cell-associated DNA to a nuclease-resistant form) requires specifically calcium ions.

MATERIALS AND METHODS

The bacterial strains used were all derivatives of the wild-type R36A strain of *Diplococcus pneumoniae*. A nuclease-defective mutant (*end-1 exo-2*) lacking about 90% of the wild-type nuclease activities but capable of normal levels of genetic transformation was kindly provided by S. Lacks (Brookhaven National Laboratory) (7). Most of the methods used have been described in detail in earlier publications. These methods include growth of bacteria in the competent and incompetent physiological states (16); the preparation of chemically defined and semisynthetic growth media (16); measurement of growth, viable cell counts, and scoring of transformants (17); activation of cells to competence and preparation of activator (18); and isolation of transforming DNA labeled with radioactive thymidine (13).

Detailed descriptions are available in recent publications for the two-stage DNA binding assay (11, 13), the procedures used to determine the activity of surface nucleases and total and deoxyribonuclease (DNase)-resistant DNA binding to the cells (11, 12). Sucrose density gradients (5 to 20% sucrose in 1.0 M NaCl solution) were run at 38,000 rpm for 3 h in polyallomer tubes, using an SW50.1 rotor of a model L3 50 ultracentrifuge (Beckman Spinco). Collection of samples was through a pinhole pierced through the bottom of the centrifuge tubes. Eight-drop fractions were collected onto glass-fiber filter disks (25-mm diameter, GFA) which subsequently were dried at 100 C. The commonly used toluene-based scintillator cocktail and a Mark II scintillation spectrometer (Nuclear-Chicago–Searle) were used to quantitate radioactivity.

RESULTS

Fox and Hotchkiss have described the requirement for calcium ions in pneumococcal transformation (4). The results illustrated in Table 1 reproduce the essential observation: genetic transformation of competent pneumococci can be inhibited by divalent cation complexing agents such as ethylenediaminetetraacetate (EDTA), and this inhibition can be reversed by calcium but not by magnesium ions. In the series of experiments described below, we tried to identify this calcium-dependent step more closely.

Growth. The synthetic and semisynthetic growth media used in our laboratory contain

TABLE 1. *Reversal of the EDTA inhibition of transformation by calcium ions*[a]

Medium for transformation	No. of transformed cells per ml
Growth medium	5.7×10^5
+ EDTA	0
+ EDTA + $CaCl_2$ (10 mM)	2.0×10^5
+ EDTA + $MgCl_2$ (20 mM)	0.009×10^5

[a] A wild-type culture (10 ml) stored at −80 C in the competent state was melted at 30 C, centrifuged at 10,000 × *g* for 5 min, and suspended in 50 ml of growth medium (pH 8, C medium containing 800 μg of bovine serum albumin per ml; cell concentration, 5×10^6 viable units per ml). Portions (1 ml) of the suspension received various additions (EDTA, 10 mM; $CaCl_2$, 10 mM; $MgCl_2$, 20 mM) and transforming DNA carrying the streptomycin resistance marker (0.1 μg/ml, representing a "saturating" DNA concentration) as indicated. After 10 min of incubation at 30 C, transformation was terminated by the addition of pancreatic DNase (crystalline, Worthington Biochemicals Corp.) at a concentration of 10 μg/ml. A 0.10-ml volume of each suspension was next diluted into 3 ml of full growth medium and incubated at 37 C for 90 min at which time the transformed cells were scored.

salts of three divalent cations: $MgCl_2$ (2.5 mM), $CaCl_2$ (0.02 mM), and $MnSO_4$ (0.0001 mM). Normal culture growth required the magnesium ions; calcium ions could not replace this requirement (Fig. 1). No requirement for manganous ions could be demonstrated. Virtually normal growth was obtained even when $CaCl_2$ was omitted from the medium; however, such media still contain calcium ions (5 μM) introduced in the form of calcium pantothenate. If calcium is required at all for pneumococcal growth, the maximum concentration needed is 5 μM.

Activation to competence. Incompetent pneumococci were treated with purified activator in growth medium lacking either calcium or magnesium or both. After activation, bacteria were diluted into complete growth media containing both divalent cations and supplemented with transforming DNA. Activation to the competent state required magnesium but not calcium ions (Table 2). On the other hand, the omission of calcium ions during contact of the cells with the transforming DNA (line 5 in the table) eliminated 90% of the potential transformants.

DNA binding and uptake. Competent pneumococci were suspended in growth media with different divalent cation compositions. Table 3 demonstrates several observations: (i) the omission of divalent cations stimulated the total amount of DNA bound by the cells and also caused a severe inhibition of DNA uptake. These observations were described in an earlier report (13). (ii) The addition of magnesium ions caused a dramatic loss of total cell-associated DNA (as compared with the amount bound in the absence of divalent cations) without any substantial increase in DNA uptake. (iii) The addition of calcium ions caused a maximum loss of about 50% of the total cell-bound DNA (as compared with the amount adsorbed to the cells in the absence of divalent cations); at the same time, DNA uptake was stimulated dramatically.

Figure 2 shows the kinetics of DNA adsorption by competent cells in the presence of EDTA, with and without calcium or magnesium ions. The maximum level of DNA bound was substantially lowered by magnesium ions (tube 3), in contrast to the relatively minor effect of calcium (tube 2). A likely explanation of this observation may be found in the experiment illustrated in Fig. 3.

Activity of surface nucleases. Competent cells were incubated with radioactive DNA in EDTA-containing media supplemented with either magnesium or calcium ions. After 30 min of incubation, the bacteria were removed by centrifugation, and the supernatants were

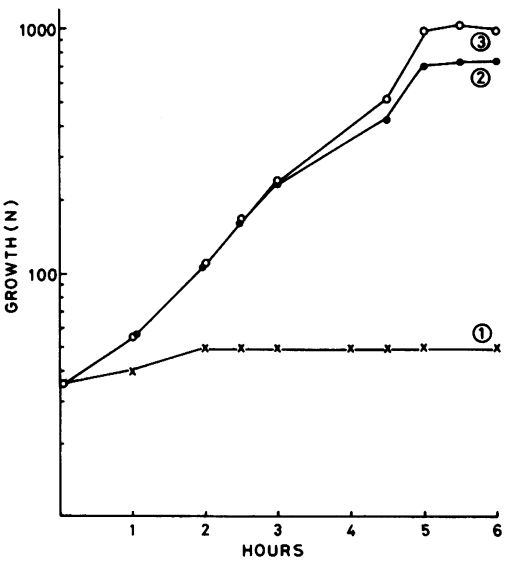

FIG. 1. *Effect of magnesium and calcium deprivation on growth. Chemically defined medium was used (17). No divalent cations were added to tube 1; tube 2 received only $MgCl_2$ (2.5 mM), tube 3 received both $MgCl_2$ (2.5 mM) and $CaCl_2$ (0.3 mM). A low level of calcium (5 μM) from calcium pantothenate was present in all media. Growth at 37 C was measured by nephelometry using a Coleman nephelometer.*

analyzed by sucrose density gradient centrifugation to determine the extent of DNA degradation by the surface-located nucleases of competent cells. Magnesium ions effectively reversed the EDTA inhibition of surface nuclease activity as indicated by the substantial reduction in the molecular size of the DNA (Fig. 3). The addition of calcium ions was less effective.

TABLE 2. *Effect of calcium deprivation during activation to competence*[a]

Divalent cations present during activation to competence	No. of transformed cells per ml
No activator + $MgCl_2$ + $CaCl_2$	<300
Activator + $MgCl_2$ + $CaCl_2$	1.4×10^5
+ $MgCl_2$ − $CaCl_2$	1.5×10^5
− $MgCl_2$ + $CaCl_2$	<300
+ $MgCl_2$ − $CaCl_2$; no $CaCl_2$ during transformation	0.14×10^5

[a] A wild-type culture (10 ml) stored at −80 C (in the incompetent physiological state) was melted, centrifuged (10,000 × *g*, 4 C, 5 min), and suspended in 10 ml of chemically defined growth medium (Cd [17]) lacking all three divalent cations ($MgCl_2$, $MnSO_2$, and $CaCl_2$) normally present. Portions (1 ml each) were pipetted into test tubes containing combinations of $MgCl_2$ (2.5 mM) and $CaCl_2$ (0.3 mM) plus purified activator (0.1 ml, containing 0.1 μg of protein). After incubation at 30 C for 20 min, all of the cell suspensions (except for the one illustrated on line 5) were diluted 10-fold into full growth medium containing transforming DNA, and transformants were scored as described in footnote *a* of Table 1. The culture illustrated on line 5 was exposed to DNA in calcium-deprived medium and was diluted into full growth medium only after treatment with DNase.

Effect of divalent cations in the "two-stage assay" of genetic transformation. Competent bacteria were allowed to adsorb radioactive transforming DNA in the absence of divalent cations. Adsorption was terminated by the addition of a large (100-fold) excess of nonradioactive heterologous DNA. The cell-DNA complexes were distributed into test tubes containing either magnesium or calcium ions or both. After various periods of incubation, samples were removed from the tubes to determine the amounts of cell-bound DNA and DNA taken up (into a form resistant to treatment with pancreatic DNase). Magnesium ions alone or in combination with calcium catalyzed an extensive and rapid loss of cell-bound DNA (Fig. 4A); this loss was slower and less extensive in the tube supplemented with calcium only. DNA uptake was poor in the presence of magnesium ions only, whereas calcium ions alone stimulated uptake to nearly maximal levels (i.e., to levels approaching that observable in complete growth medium).

Figure 5 shows the results of a two-stage assay similar in design to the one illustrated in Fig. 4, except that DNA uptake was monitored by diluting portions of the cell-DNA complexes into complete growth medium containing DNase. Transformed cells were scored after an additional 90 min of incubation in

TABLE 3. *Effect of divalent cations on DNA binding and uptake*[a]

Conditions during DNA binding and uptake	Total DNA bound (counts/min per ml)	DNA uptake (counts/min per ml)
Growth medium + $MgCl_2$ (2.5 mM) + $CaCl_2$ (0.3 mM)		
	5,000 (10 ng)	3,500 (7 ng)
− $MgCl_2$ − $CaCl_2$	23,970	617
+ $MgCl_2$(1 mM)	13,806	871
(10 mM)	5,302	461
+ $CaCl_2$(0.1 mM)	18,228	2,141
(0.5 mM)	15,429	3,413
(1.0 mM)	12,592	3,536

[a] A frozen competent culture (10 ml each) of wild-type cells (1.4×10^8 colony-forming units per ml) was melted and transferred into fresh chemically defined (Cd_{en} [17]) medium containing no divalent cations and no vitamin solution (to eliminate the calcium ions present in the calcium pathotenate). The medium was made up with tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.05 M, pH 8) instead of the usual phosphate buffer. Portions (2 ml each) of the cell suspension were pipetted into glass centrifuge tubes containing various combinations of $MgCl_2$ and $CaCl_2$ as well as radioactive DNA (0.1 μg of DNA labeled biosynthetically with tritiated thymidine; specific radioactivity of DNA, 10^6 dpm per μg). After incubation at 30 C for 20 min, half of each cell suspension (1 ml) was centrifuged (10,000 rpm, 10 min), washed with medium three times, and finally precipitated with cold 10% trichloroacetic acid. The precipitate was collected on glass-fiber filter disks (GFA, Whatman), dried, and used for the determination of total cell-bound DNA. The other half of each cell suspension was first treated with pancreatic DNase (10 μg/ml, 37 C 10 min) and was subsequently processed for the quantitation of DNA bound in a nuclease-resistant form (DNA uptake). The amount of cell-bound DNA is given as counts per minute per milliliter; some of the data (controls) are also expressed as nanograms per milliliter.

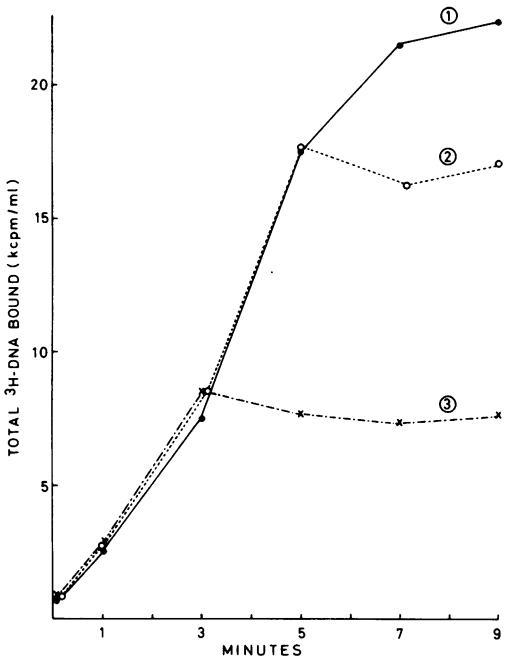

FIG. 2. *Effect of divalent cations on the kinetics of DNA adsorption. Wild-type competent cells (5 × 10^8 viable units per ml) were suspended in chemically defined medium (Cd_{en} [17]) from which all divalent cations had been omitted. Portions (3 ml each) were pipetted into three test tubes; every tube received EDTA (10 mM) and radioactive DNA (labeled with [^{3}H]thymidine; 0.1 μg and 6 × 10^6 dpm per ml). Tube 1 received no further additions; tube 2 received $CaCl_2$ (10 mM); $MgCl_2$ (10 mM) was added to tube 3. The suspensions were incubated at 30 C, and samples (0.2 ml) were removed at the time intervals indicated for the determination of total cell-bound DNA by the method described in footnote a of Table 3.*

the full growth medium. Figure 5 demonstrates the relatively high efficiency of calcium ions in promoting DNA uptake. In contrast, magnesium ions alone were relatively ineffective.

DISCUSSION

The observations described above confirm the earlier report of Fox and Hotchkiss (4) concerning the need for calcium ions in pneumococcal transformation. Our results indicate that the calcium-dependent step is the conversion of cell-bound DNA to a form resistant to added nuclease, i.e., presumably the transport of DNA through the cell membrane. Cellular growth, activation to the competent state, and adsorption of DNA to the surface receptors show no comparable requirement for calcium ions. Magnesium ions cannot replace calcium in stimulating the uptake of DNA (into a nuclease-resistant form) or the emergence of genetic transformants. On the other hand, the presence of magnesium ions seems to stimulate greatly the loss of cell-bound DNA into the medium and the degradation of DNA in the medium. Both of these processes are catalyzed by surface-located pneumococcal nucleases, which are known to require magnesium ions (6). It is important to note that the loss of DNA into the medium and DNA degradation are limited in the presence of calcium ions alone, while DNA uptake and transformation approach the maximum levels attainable. The presence of both magnesium and calcium ions improves the levels of genetic transformation by 30% to twofold over that obtained in the presence of calcium only.

The biochemical nature of the calcium-dependent step in DNA uptake is not known at the present time. It does not seem to involve the major pneumococcal nucleases since both the exo- and the endonuclease require magnesium and not calcium ions for activity. Calcium ions did not have any stimulating effect on crude or dialyzed pneumococcal exonuclease or endonuclease (measured as ribonucleic acid-inhibitable nuclease) irrespective of whether or not soluble enzyme or enzyme bound to the plasma membrane was assayed; at high calcium concentrations (1 mM), a moderate (30%) inhibition of exonuclease activity was observed

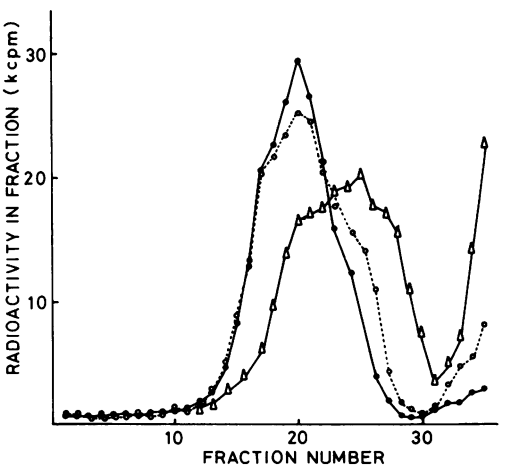

FIG. 3. *Effect of divalent cations on the activity of surface-located nucleases. Competent bacteria (1.4 × 10^8 colony-forming units per ml) were incubated with [^{3}H]DNA (0.1 μg, 3 × 10^5 dpm) at 30 C in the presence of 10 mM EDTA (●), EDTA plus 10 mM $CaCl_2$ (○), or EDTA plus 10 mM $MgCl_2$ (△). After 10 min, the bacteria were removed by centrifugation. The cell-free supernatant fluids received 10 mM EDTA and were analyzed on 5 to 20% linear sucrose gradients as described in the text.*

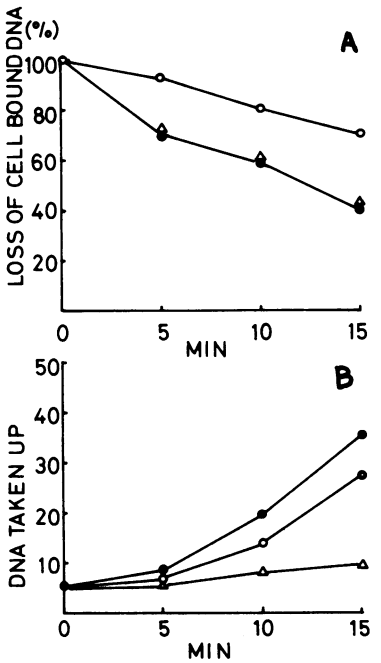

FIG. 4. *Effect of divalent cations on DNA release and DNA uptake by competent cells. Competent cells of the nuclease-defective mutant (end-1 exo-2) were suspended in chemically defined medium (Cd_{rn} [17]) from which divalent cations had been omitted. The bacteria received DNA (0.1 μg and 6 × 10^6 dpm per ml) and were incubated at 30 C for 10 min. Next, the cell-DNA complexes were centrifuged and suspended in fresh Cd_{rn} medium free from divalent cations and containing excess heterologous DNA (thymus DNA, 100 μg/ml) to prevent further attachment of radioactive DNA molecules. Portions (3 ml each) were pipetted into three test tubes, one of which (●) received both $MgCl_2$ (2.5 mM) and $CaCl_2$ (0.3 mM). A second tube (○) received only $CaCl_2$ (0.3 mM); only $MgCl_2$ (2.5 mM) was added to the third tube (△). The suspensions were incubated at 30 C, and samples were removed at the time intervals indicated for the determination of total cell-bound DNA (A) and DNA uptake (B) by methods described in footnote a of Table 3.*

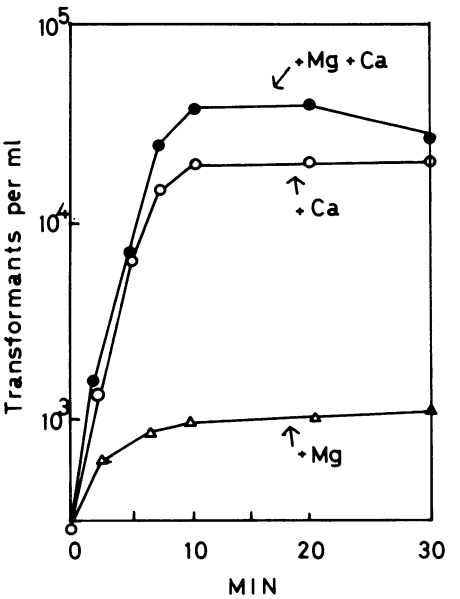

FIG. 5. *Effect of calcium and magnesium ions on the emergence of transformants in the two-stage transformation assay. The design of this experiment was the same as that described in the legend of Fig. 4, except that the non-radioactive, transforming DNA preparation (carrying the streptomycin resistance marker) was used. The cell-DNA complexes were suspended in three different media, containing either calcium or magnesium ions or both. The suspensions were incubated at 30 C, and at various time intervals 0.1-ml samples were removed into 1 ml of full growth medium containing 10 μg of DNase per ml. Transformed cells were scored as described in footnote a of Table 1.*

(unpublished data). We also tested a nuclease-defective pneumococcal mutant ("noz" type mutant, according to Lack's criteria [7]), which showed only about 0.1 to 1% residual nuclease activity and reduced transformability (1 to 10% of the wild type). This mutant still exhibited a calcium requirement for genetic transformation.

DNA bound and absorbed by pneumococci in the absence of magnesium ions (or in the presence of EDTA plus calcium) was reisolated and analyzed by sucrose density gradient centrifugation. Such molecules also showed the reduction in size observable in the presence of magnesium ions (unpublished data).

It is interesting to note that a requirement for relatively high concentrations of calcium ions have also been reported in several other bacterial transformation systems (2, 14) as well as in some systems of transfection (2, 15).

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