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Role of a Deoxyribonuclease in the Genetic Transformation of *Diplococcus pneumoniae*

(competence/DNA transport/transformation-defective mutations)

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ABSTRACT **Two steps in the uptake of DNA by *Diplococcus pneumoniae* were characterized by analyzing mutants defective in transformation. A strain deficient in the two major deoxyribonucleases of *D. pneumoniae* takes up DNA normally and converts it to single strands within the cell and oligonucleotide fragments outside the cell. Extracts of this strain contain a residual deoxyribonuclease that produces similar oligonucleotide fragments *in vitro*. This enzyme is missing in transformation-defective mutants blocked in the second or entry step. Cells of this mutant class bind large amounts of DNA to their surface in a form accessible to external agents. Another class of nontransformable mutants fails to bind DNA at all. Their deoxyribonuclease content is unchanged, and they are apparently blocked in the first or binding step of DNA uptake. The binding step requires a source of energy and prior activation of the cells by competence factor. Entry may be independent of these requirements and may come about by action of the deoxyribonuclease on one strand of DNA with energy for the transport of the intact strand deriving from hydrolysis of the degraded strand. The enzyme may thus act as a DNA translocase.**

An early step in the genetic transformation of bacteria is the entry of donor DNA into the recipient cell. Prior to entry, DNA appears to be bound to the cell surface in a form sensitive to external agents (1–4). During entry, double-stranded donor DNA is converted to single strands in both *Diplococcus pneumoniae* (5, 6) and *Bacillus subtilis* (7, 8). The concomitant appearance of donor DNA degradation products in pneumococcal cells suggested the role of a cellular DNase in the entry process (5). Degradation products also appear outside the cells, in proportion to the amount of DNA taken up (3, 9, 10).

In a double mutant of *D. pneumoniae*, which is deficient in the two major DNase activities of the wild type, uptake of DNA and its fate after entry were normal (11). We set out to fractionate and characterize the residual DNase components in this strain, in particular to compare the products of the residual enzymes with the products formed external to the cell during DNA uptake. We selected mutants of the strain that were more deficient in DNase and, also, mutants that were defective in transformation. Both kinds of mutant were examined with respect to DNase composition, genetic transformability, DNA uptake, and binding of DNA to the outside of the cell.

MATERIALS AND METHODS

Bacterial Strains and Media. Strain designations do not necessarily indicate genotype. R6end1exo2 and trt1hex4 both are *end-1,exo-2* (9, 11). Moend1exo2 and T6trt1hex4 are maltose-negative derivatives. Moend1exo2hex3 is a *hex* mutant (11). Transformation-defective mutants were obtained in a parental strain after treatment with 1-methyl-3-nitro-1-nitrosoguanidine, and the mutations were transferred by transformation to another strain. A DNase plate assay (11) was used to select *noz* mutants that failed to form a colorless zone in agar containing DNA and methyl green after 4 days at 37°. Selection of *ntr* mutants was accomplished by screening for transformation on agar plates (9). The growth medium (12) and the minimal medium for DNA uptake (9) have been described. When added, trypsin was present at 2 μg/ml. Cultures were grown for all purposes to OD_{650} about 0.2. The number of colony-forming units (CFU) and the amount of protein in the cells were determined from the relationship: OD_{650} of 0.2 = 10^8 CFU = 60 μg of protein per ml.

Transformation. The general procedure has been presented (9). To test for transformability, cultures grown at 37° were placed at 30° for 15–40 min, diluted 10-fold into growth medium, treated with DNA at 30° for 10 min, and incubated at 37° for 100 min before they were scored for streptomycin-resistant transformants. Transformability is here expressed as the number of such transformants per 100 CFU present at the time of addition of DNA. For the transfer of *noz* and *ntr* mutations, recipient cells were diluted 100-fold, treated with mutant DNA for 60 min at 30°, and incubated for 3 hr at 37° to allow segregation of the mutant type.

Binding and Uptake of DNA. [^{32}P]DNA prepared from *Escherichia coli* (6) was added at 0.3 μg/ml to cultures that had been incubated at 30°. Samples were chilled at intervals; the initial sample was chilled before addition of [^{32}P]DNA. The cells were washed twice by centrifuging and layering medium over the cells without suspending them. The cells were then suspended and a portion was taken for determination of total radioactivity associated with the cells. The remainder was treated for 10 min at 30° in medium with pancreatic DNase at 2 μg/ml before centrifugation, resuspension, and sampling of DNase-resistant counts. Uptake is defined as [^{32}P]DNA associated with the cells in a DNase-resistant form. Binding is defined as the difference between the total [^{32}P]DNA associated with the cells and the DNase-resistant uptake.

Fractionation of DNases. Cultures grown at 37° were transferred to 30° for the final 40 min of growth, after which all steps were at 0–5°. Washed cells were suspended in 0.5 M NaCl in buffer (composed of 10 mM Tris·HCl, pH 7.5–

Abbreviations: CM-cellulose, carboxymethyl-cellulose; CFU colony-forming units.

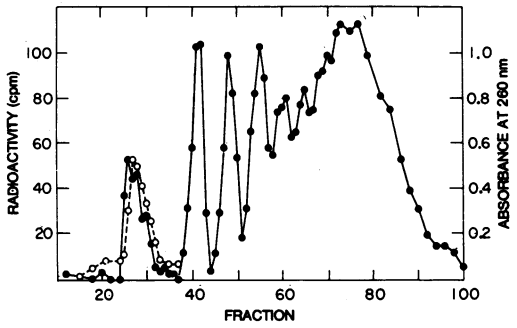

FIG. 1. Oligonucleotide fragments produced external to cells during uptake of DNA. Cells of strain trt1hex4, incubated 40 min at 24° in minimal medium with [^{32}P]DNA (76 cpm/ng) at 0.3 μg/ml, took up 9.6% of the DNA and rendered 8.1% acid-soluble in the medium. The acid-soluble material was chromatographed on DEAE–cellulose (1 × 5-cm column). Fractions contain 4 ml. (●) Radioactivity; (○) absorbance of mononucleotide (5′-dAMP) marker.

3 mM 2-mercaptoethanol–0.1 mM EDTA) and extracted in a French pressure cell. Brij-35 was added to 0.1% and the extract was centrifuged. The sediment was extracted overnight with buffer containing 0.5 NaCl and centrifuged. The supernatant fluids were combined and fractionated on a column of agarose (Biogel A-0.5 m) by eluting with 0.5 M NaCl in buffer, which in this and subsequent steps also contained 1 mM $MnCl_2$. Pooled fractions were dialyzed and fractionated on carboxymethyl (CM)-cellulose and DEAE–cellulose by eluting with increasing concentrations of NaCl in buffer. For routine separations of the DNases, extracts were diluted 10-fold in buffer and fractionted on CM-cellulose with stepwise increments of NaCl concentration.

DNase was assayed by the release of acid-soluble radioactivity in mixtures containing [^{32}P]DNA at 5 μg/ml and bovine-serum albumin at 0.4 mg/ml in buffer containing 1 mM $MnCl_2$ and 20–80 mM NaCl. One unit of DNase corresponds to the release of 1 μg of acid-soluble [^{32}P]DNA per hr at 30°. DNA polymerase and protein were assayed as before (11).

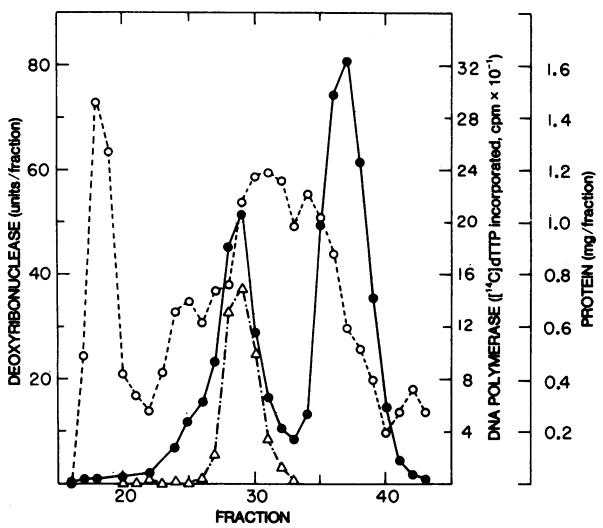

FIG. 2. Fractionation of DNases by gel filtration on agarose. An extract of Moendlexo2 from 400 ml of culture was applied to a column of Biogel A-0.5 m (2 × 70-cm) and eluted with 0.5 M NaCl in buffer. Fractions contain 4 ml. (●) DNase activity; (△) DNA polymerase; (○) protein. Values for cpm have been multiplied by 10^{-1}, as indicated.

Analysis of DNase Products. Samples containing perchloric acid-soluble degradation products of [^{32}P]DNA, found in the medium after DNA uptake by cells or produced *in vitro* by purified enzyme fractions, were neutralized with KOH, separated from the precipitate of $KClO_4$, and diluted with water prior to chromatography on DEAE–cellulose with increasing NaCl concentration in the presence of 7 M urea (13). Radioactivity in the eluted fractions was determined as Cerenkov radiation in a scintillation counter.

RESULTS

DNA Degradation Products External to Cells. When pneumococcal cells take up DNA, an approximately equal amount of donor DNA is found degraded to acid-soluble material outside the cells. Both DNA uptake and this external DNase action require prior induction of competence and the presence of sugar and magnesium or manganous ions (9). The acid-soluble products found in the medium were analyzed by chromatography under conditions that separate oligonucleotides on the basis of length. Fig. 1 shows that the products consist of oligonucleotides ranging from one to about ten residues in length in roughly equal proportions by weight.

Residual DNase in the end-1,exo-2 *strain.* Extracts of strain R6end1exo2 prepared by deoxycholate lysis of cells grown at 37° contain only about 3% of the DNase activity found in strain R6, and these *end,exo* extracts are devoid of the high endonuclease and exonuclease activities of the wild strain (11). However, growth of cells at 30° and preparation of extracts with a French pressure cell both result in greater DNase activity in extracts of *end-1,exo-2* strains. Extracts prepared in this manner were fractionated by gel filtration on agarose and by ion exchange on substituted celluloses. Recoveries of applied DNase activity were about 100%, except as specified.

Two peaks of DNase activity were obtained in the agarose fractionation (Fig. 2). The activity of the first peak is associated with a DNA polymerase, as reported (11). This polymerase-exonuclease gives rise predominantly to mononucleotides and dinucleotides. The material in the second peak consists of two enzymes which are separated on CM-cellulose (Fig. 3). In this step, total activity in the fractions amounted

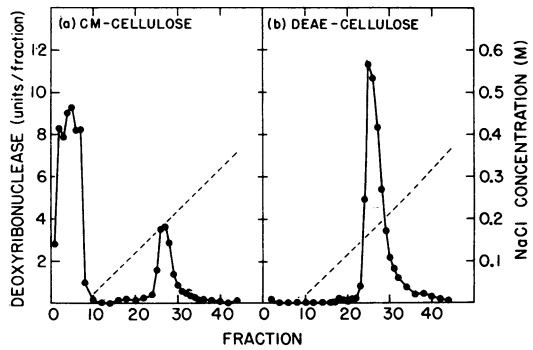

FIG. 3. Fractionation of DNases by ion exchange on cellulose derivatives (columns, 1 × 5-cm; 4-ml fractions collected). (*a*) Fractions 34–40 from the gel filtration shown in Fig. 2 were pooled and dialyzed. A sample was placed on CM-cellulose and eluted with increasing NaCl concentration. (*b*) Fractions 1–8 from *a* were pooled, and a portion was chromatographed on DEAE–cellulose. (●) DNase; (– – –) NaCl concentration.

TABLE 1. *Properties of transformation-defective strains*

Strain	Relevant genotype	DNase plate assay	Transformability ($str^R/10^2$ CFU)	Binding of DNA (ng/10^8 CFU)	Uptake of DNA (ng/10^8 CFU)
Moendlexo2	normal	+	2.4	6	23
T6trt1hex4	normal	+	2.1	3	20
R6end1exo2ntr11	*noz-11*	−	0.02	24	2
T6trt1hex4noz11	*noz-11*	−	0.06	16	4
R6end1exo2ntr19	*noz-19*	−	0.007	55	2
T6trt1hex4noz19	*noz-19*	−	0.005	23	2
Moendlexo2ntr35	*noz-35*	±	0.0007	35	1
Moendlexo2ntr48	*noz-48*	±	0.003	38	2
T6trt1hex4ntr48	*noz-48*	±	0.003	35	2
Moendlexo2noz1	*noz-1*	−	0.09	4	7
trt1hex4noz1	*noz-1*	−	0.26	10	7
Moendlexo2ntr37	*ntr-37*	+	<0.0001	0	0
Moendlexo2hex3ntr37	*ntr-37*	+	<0.0001	0	0
Moendlexo2ntr40	*ntr-40*	+	<0.0001	0	0
Moendlexo2ntr43	*ntr-43*	+	<0.0001	0	0
R6end1exo2ntr9	*noz-9,ntr-9*	−	<0.0001	0	0
Moendlexo2hex3ntr9	*ntr-9*	+	<0.0001	0	0
T6trt1hex4noz9	*noz-9*	−	0.006	15	2
T6trt1hex4noz9ntr9	*noz-9,ntr-9*	−	<0.0001	0	0

Original mutant strains are listed without indentation; derivatives obtained by transformation are indented. All strains carrying *noz* mutations were selected by the DNase plate assay except for those carrying *noz-35* and *-48*, which were selected by the plate transformation technique, as were the strains carrying only *ntr*. Transformability values are the mean from 3–10 tests. Binding (DNase-sensitive) and uptake (DNase-resistant) results are from a single experiment. All three parameters were measured after 10 min with DNA at 30° as described in *Methods*.

to only half the activity applied. A negatively charged enzyme, which does not adsorb to CM-cellulose, elutes as a single peak from DEAE–cellulose. This enzyme is an exonuclease that produces only mononucleotides; it is four times as active with Mn^{2+} as with Mg^{2+} ions. The third activity elutes as a single peak from CM-cellulose. It is equally active with Mn^{2+} and Mg^{2+}, and it produces oligonucleotide fragments (Fig. 4). Since it can cleave internal bonds in DNA, this enzyme will be called an endonuclease, although it may preferentially attack at ends of DNA since the proportion of small oligonucleotides in the acid-soluble products is independent of the extent of hydrolysis. The distribution of oligonucleotides produced by the enzyme resembles the distribution of oligonucleotides found outside the cell during DNA uptake (Fig. 1), which suggests that this enzyme may play a role in DNA uptake.

Mutants Defective in Transformation. Two classes of transformation-defective mutants were found. One type, called *noz*, was obtained mainly be selecting clones that failed to form colorless zones in the DNase plate assay. Another type, called *ntr*, was found among clones that failed to undergo transformation on agar plates. Some of these clones corresponded to *noz*-type mutants.

Mutants of the *noz* class show a reduced but definite level of transformability, which appears to be characteristic of the mutation (Table 1). The level of transformability is generally reduced to 0.1–1% of normal, but the *noz-1* mutation only causes a reduction to about 10%. Correlated with the reduced transformability is a reduced uptake of DNA by the cell. However, *noz* mutants are able to bind large amounts of DNA to the outside of the cell in a form susceptible to externally added DNase. Most of the bound DNA is double-stranded and can be removed by vigorous agitation (data not shown).

The kinetics of binding and uptake by normal and *noz* cells are compared in Fig. 5. Much of the apparently DNase-resistant uptake associated with mutant cells may represent degraded bound DNA still adhering to the surface since this material failed to give a sharp peak in CsCl density gradients. Growth of cells in the presence of trypsin prevents binding as

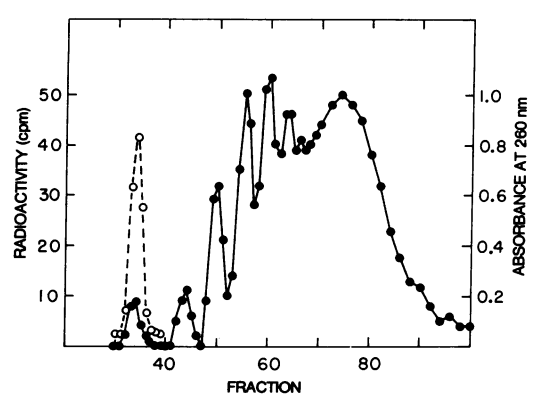

FIG. 4. Oligonucleotide fragments produced by the residual endonuclease. The enzyme purified on CM-cellulose converted 18% of the [^{32}P]DNA (6 cpm/ng) substrate to an acid-soluble product, which was chromatographed as in Fig. 1. (●) Radioactivity; (○) absorbance of 5′-dAMP marker.

TABLE 2. *DNase content of transformation-defective strains*

Strain	Transformation frequency ($str^R/10^2$ CFU)	DNase content (units/mg of protein)		
		Residual endonuclease	Total exonuclease	Polymerase-exonuclease
T6trt1hex4	0.3	1.8	8.5	1.6
T6trt1hex4	1.2	3.3	10.2	
T6trt1hex4	2.1	1.4	8.9	
Moend1exo2	3.2	1.9	23.2	7.1
Moend1exo2	1.7	4.1	33.8	
Moend1exo2 (grown with trypsin)	0.0005	2.8	29.6	8.5
Moend1exo2ntr37	<0.0001	3.2	20.9	5.5
Moend1exo2ntr40	<0.0001	4.6	33.4	
Moend1exo2ntr43	<0.0001	2.4	20.5	
Moend1exo2hex3ntr9	<0.0001	5.4	16.2	
Moend1exo2noz1	0.14	0.1	4.6	3.1
trt1hex4noz1	0.15	0.1	3.9	
T6trt1hex4noz9	0.002	0.1	6.6	1.1
T6trt1hex4noz11	0.017	0.0	6.9	
T6trt1hex4noz19	0.0004	0.0	3.8	
Moend1exo2ntr35	0.0007	0.2	5.2	
Moend1exo2ntr48	0.005	0.1	8.4	2.3
T6trt1hex4ntr48	0.0004	0.1	4.2	

Samples of cultures were tested for transformability before extraction. Extracts were fractionated on CM-cellulose. Total exonuclease was measured as DNase activity not retained by the column; the endonuclease was eluted by 0.25 M NaCl. Polymerase-exonuclease was separated by gel filtration.

well as uptake. Trypsin presumably acts by destroying the competence factor, thereby preventing activation of the cells for competence (14). Trypsin itself does not interfere with uptake by competent cells (9, 14), nor does it interfere with binding by *noz* strains containing the *trt* gene, which renders them permanently activated (9). Thus, activation by competence factor is required for binding by *noz* mutants. Cells suspended in a minimal medium devoid of a nitrogen source are able to bind DNA, and magnesium ions are not essential (Fig. 6). Binding does require sugar, presumably as a source of energy.

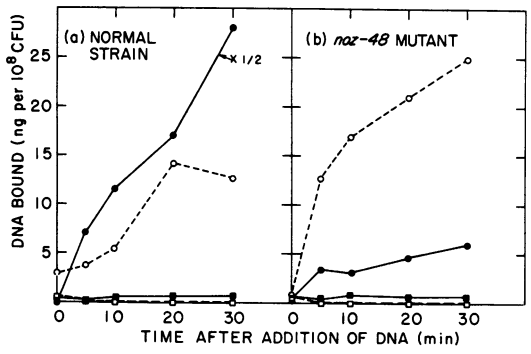

FIG. 5. DNA binding and uptake in normal and mutant strains grown in the presence and absence of trypsin. (*a*) Strain Moend1exo2; (*b*) strain Moend1exo2ntr48, which carries the *noz-48* mutation. After growth at 37° in the absence (○,●) or presence (□,■) of trypsin, cultures were held 40 min at 30°, treated with [^{32}P]DNA, and sampled at intervals. (○,□) DNase-susceptible binding; (●,■) DNase-resistant uptake. Note reduction of ordinate for uptake in *a*.

Mutants of the *ntr* class show no detectable transformation. Neither do they bind or take up DNA (Table 1). In the DNase plate assay they give a normal response for *end,exo* strains, whereas *noz* mutants give either no zones or colorless zones of reduced size (indicated by ± in Table 1).

Both *noz* and *ntr* mutations can be transferred by trans-

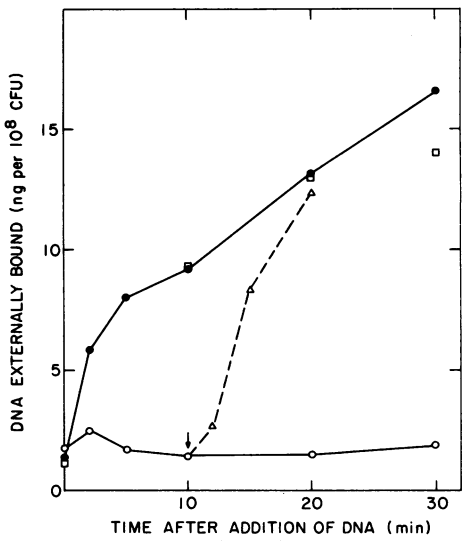

FIG. 6. Requirements for DNA binding. A culture of Moend-1exo2ntr48 was held 30 min at 30° and washed. Portions were suspended in minimal medium with and without sucrose or $MgCl_2$. After 10 min at 24°, [^{32}P]DNA was added and sampling begun. (●) Full minimal medium; (□) no $MgCl_2$; (○) no sucrose; (△) sucrose added at 10 min.

formation, by the techniques for selecting mutants. The mutant alleles confer similar properties on their new hosts (Table 1), and the frequencies of such transfer are consistent with introduction of a single gene. In one case, *noz* and *ntr* mutations appeared in the same isolate, R6end1exo2ntr9. Using this isolate as donor of DNA, we obtained transformants containing one or both of these mutations (Table 1).

DNase Content of Transformation-Defective Strains. Various transformation-defective mutants and their derivatives were compared to their normally transformable (but *end-1,exo-2*) progenitors with respect to DNase content (Table 2). All three DNases are unchanged in amount in the *ntr* mutants. In the *noz* mutants, however, the endonuclease is virtually absent. This finding supports a role for this enzyme in normal DNA entry. Exonuclease activity may also be somewhat reduced in the *noz* mutants. Growth of a normal culture with trypsin, which inhibits transformation by destroying the competence factor, does not alter the amount of residual endonuclease in the cell. Thus, activation for competence, which was shown above to be essential for binding of DNA, is not essential for formation of the DNase, which presumably acts at a later step in the uptake process.

DISCUSSION

The results indicate that DNA uptake by *D. pneumoniae* involves two steps. In the initial step DNA is bound to the outside of the cell. This process requires prior activation of the cell by competence factor; cells grown with trypsin, which destroys the competence factor, cannot bind DNA. The binding step appears to be energy dependent since it requires the presence of sugar. This step is blocked in *ntr* mutants.

In the second step bound DNA enters the cell. Entry requires the action of a particular DNase, which may be called a DNA translocase, since *noz* mutants, which lack the enzyme, are deficient in this step. Either magnesium or manganous ions are essential for entry as they are for the enzyme activity *in vitro*, and the *in vitro* products consist of oligonucleotides similar in size to those appearing outside cells during DNA uptake. Activation by competence factor may only affect uptake secondarily, by preventing the binding of DNA, while the mechanism of entry itself may be independent of activation since the formation of the essential DNase is not blocked by growth of cells in the presence of trypsin.

Some time ago a model for DNA entry was suggested in which one strand of DNA was pulled into the cell while the strand of opposite polarity was attacked and degraded (5). The possibility that the energy for pulling in the intact strand was derived from hydrolysis of the opposite strand appeared to be contradicted by the requirement of an additional source of energy for uptake (9, 15). However, this energy may be required only to obtain binding of DNA, and entry may proceed as originally envisioned. It has been suggested that fragments from the degraded strand are released only into the medium (10). An earlier finding—that the *noz-1* mutant forms less degraded DNA externally than it apparently takes up—seemed inconsistent with this proposal (9). (The *noz-1* mutant is only partly defective in entry, perhaps because *in vivo* it retains some DNA translocase activity.) However, since the amount of DNA bound by *noz-1* is large compared to the amount that enters and since some bound DNA may adhere to cells even after DNase treatment, the amount of external degraded DNA may equal the amount of DNA that has in fact entered. Thus, both the nature and amount of degradation products in the medium are consistent with their formation by the action of the DNase on one strand during the entry of the other strand into the cell.

It is not known whether the residual endonuclease implicated in DNA entry is a mutant form of the major endonuclease or a distinct enzyme present in *end*$^+$ cells. Both endonucleases fractionate similarly and produce similar fragments. Thus, *noz* mutations are possibly additional, more deleterious mutations in the *end* gene. Alternatively, they may be mutations in a distinct structural gene. Differences in the levels of transformability resulting from different *noz* mutations may arise either from structural gene mutations rendering the essential DNase defective to different degrees or from regulatory gene mutations affecting the amount of enzyme made. The latter could perhaps explain the reduced exonuclease activity in *noz* mutants.

Uptake of DNA by other transformable bacteria also requires energy (16–18), but it is not known whether the energy is required for the initial binding reaction. Donor DNA is also converted to single strands (7, 8) and external fragments (3) in *B. subtilis*, so its entry mechanism may also involve DNase action. *Haemophilus influenzae* presents a different pattern: there is a rapid, energy-dependent uptake of DNA into a DNase-resistant form (18), but this DNA remains double stranded (19, 20). At a much slower rate donor DNA fragments appear outside the cell (19) and single-stranded segments are inserted into recipient DNA (20). However, if it is assumed that the surface of this gram-negative species is such that DNA bound in the initial binding step is already protected from external agents, then the mechanism of entry into the interior of the cell may also resemble that of *D. pneumoniae*.

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