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Competence for Deoxyribonucleic Acid Uptake and Deoxyribonuclease Action External to Cells in the Genetic Transformation of *Diplococcus pneumoniae*

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A mutant of *Diplococcus pneumoniae* that apparently does not require activator can become competent for uptake of deoxyribonucleic acid (DNA) when grown in dilute cultures or in the presence of trypsin. Development of competence in both mutant and wild strains is temperature dependent, being 10-fold greater at 30 C than at 37 C. Induction of competence on a shift from 37 to 30 C requires protein synthesis and the presence of Mg^{2+} and Ca^{2+}; uptake of DNA does not require protein synthesis. Competence decays exponentially at higher temperatures. As well as taking up DNA, competent cells release oligonucleotide fragments of donor DNA in the medium external to the cells. Normal strains release fragments comparable in amount to the DNA taken up; but, in a mutant selected for inability to degrade DNA in agar, the amount of fragments formed external to the cells is only 40% of DNA uptake. Requirements for external deoxyribonuclease action are identical to those for DNA uptake: prior development of competence and the presence during treatment with DNA of Mg^{2+} ions and a source of energy.

Competence in bacterial transformation refers to the ability of cells to take up deoxyribonucleic acid (DNA) so that they can be genetically transformed. Competence is generally a transient property of bacteria; in cultures of *Diplococcus pneumoniae*, high levels of competence usually occur only during late logarithmic growth. Tomasz and Hotchkiss (18) found that dense cultures of pneumococci contain a diffusible activator able to elicit competence in physiologically noncompetent cells. Pakula and Walczak (12) found a similar substance in streptococcal filtrates. Interaction with activator, however, is only a first step; a second step that requires protein synthesis is necessary for the development of competence (17). The activator is destroyed by proteolytic enzymes (19); hence, pneumococci grown in the presence of trypsin are poorly transformable. In this investigation we have found a mutant that appears to dispense with the activation step. It can develop competence in dilute cultures and when grown in the presence of trypsin.

It has been known for some time that DNA uptake by pneumococci is dependent on temperature in an unusual manner: uptake is considerably faster at 30 C than at 37 C (1, 8). This observation has not been, hitherto, related to the level of cellular competence, although Hotchkiss (2) showed that temperature shifts cause fluctuations of transformability. We have found that a step in the development of competence subsequent to activation is temperature dependent. The level of competence increases 10-fold on transfer of a culture from 37 C to 30 C. Development of this high level of competence requires protein synthesis. Competence is rapidly lost on incubation at higher temperatures.

The competent state is characterized not only by ability to take up DNA but also by ability to degrade DNA external to the cell. Morrison and Guild (Biochim. Biophys. Acta, in press) have also found acid-soluble fragments of DNA external to cells in amounts comparable to the amounts of DNA taken up. Some of this deoxyribonuclease activity may be intimately connected to the process of DNA uptake.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains of *D.*

pneumoniae that were used in this investigation. Genetic markers were introduced by transformation. Strain Moendlexo2 was derived from R6endlexo2, which lacks the two major deoxyribonucleases of *D. pneumoniae* (6), by introduction of *mal-515*, a maltose-negative mutation previously called Mo (5). Strains nozl and ntr48 were mutants of Moendlexo2. The *noz* mutant does not give a colorless zone in agar containing DNA and methyl green. *ntr* mutants are either not transformable or poorly transformable. Strain RF46SKNE containing several markers for drug resistance was obtained from M. Fox. The *thy-7* mutation, which confers a requirement for thymidine at 50 μg/ml, was isolated by a procedure similar to that of Okada et al. (11). Mutations *ltr-1* and *vlt-3* reduced the thymidine requirement to 5 and 0.5 μg/ml, respectively. The *trt* marker refers to transformability in the presence of trypsin; *trt-1* was obtained together with the *hex-4* mutation, which integrates all markers at high efficiency (6) from a culture of Moendlexo2 by the procedure described below. *Noz-1* was transferred into this strain.

Media. The growth medium was previously described (5). It contained 0.2% sucrose and was buffered at pH 7.6. The minimal medium for uptake of DNA, also at pH 7.6, contained 0.05 M potassium phosphate, 0.15 M NaCl, 0.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.4 mg of bovine albumin (Armour, fraction V) per ml, 3 units of catalase (Worthington) per ml, and 0.2% sucrose. For induction of competence 0.1% enzymatic casein hydrolysate (Nutritional Biochemicals Corp.) was added to minimal medium.

Preparation of DNA. Transforming DNA was prepared from strains of *D. pneumoniae* by the method of Hotchkiss (3). Preparation of ^{32}P DNA from *Escherichia coli* was described previously (7).

Transformation. The typical procedure for transformation is illustrated by the experiment summarized in Table 2. Moendlexo2 and trtlhex4 were grown at 37 C to an absorbancy at 650 nm of 0.16, equivalent to ~8 × 10^7 colony-forming units (CFU) per ml. These cultures were diluted 2,000-fold into growth medium with or without trypsin (Calbiochem) at 2 μg/ml. In other experiments either Pronase (Calbiochem), chymotrypsin (Worthington), or subtilisin (Nutritional Biochemicals), each at 2 μg/ml, was added to the medium. A sample containing 2 ml was removed immediately, and the rest of the culture was incubated at 37 C. Further samples were removed at 40-min intervals. One-tenth milliliter of each sample was used to determine the culture density. The rest of the sample was held for 15 min at 30 C and then treated with 2 μg of DNA from RF46SKNE for 15 min. The reaction was terminated by addition of 2 μg of pancreatic deoxyribonuclease (Worthington), and the samples were transferred to 37 C for 90 min to allow phenotypic expression of streptomycin resistance. Transformants were scored by the procedure of Hotchkiss in which transformed clones appear as countable colonies on the bottom of tubes containing low concentrations of antibody in liquid selective medium (2). Total CFU were determined similarly or by plating in agar. Transformation frequencies are expressed as the ratio of streptomycin-resistant transformants to total CFU after the period of phenotypic expression.

TABLE 1. *Strains of D. pneumoniae used*

Strain	Genotype
Moendlexo2	*end-1, exo-2, mal-515*
nozl	*end-1, exo-2, mal-515, noz-1*
ntr48	*end-1, exo-2, mal-515, ntr-48*
RF46SKNE	*sul-a, sul-d, str, bry, nov, ery*
thy7LSN	*thy-7, ltr-1, vlt-3, str, nov*
trtlhex4	*end-1, exo-2, hex-4, trt-1*
trtlhex4nozl	*end-1, exo-2, hex-4, trt-1, noz-1*
trtlthy7hex4	*end-1, exo-2, hex-4, trt-1, thy-7*

TABLE 2. *Competence in dilute cultures of normal D. pneumoniae and the trt mutant in the presence and absence of trypsin*

Time at 37 C after 1:2000 dilution (min)	Presence of trypsin at 2 μg/ml	Moendlexo2			trtlhex4		
		Culture density[a] (CFU/ml)	*str* transformants[b] (CFU/ml)	Transformation frequency[c] (× 10^3)	Culture density[a] (CFU/ml)	*str* transformants[b] (CFU/ml)	Transformation frequency[c] (× 10^3)
0	−	4.3×10^4	1.9×10^3	2.5	2.1×10^4	3.1×10^3	16.3
	+				2.6×10^4	3.0×10^3	11.5
40	−	1.1×10^5	<10	<0.003	4.3×10^4	1.9×10^3	3.3
	+				3.9×10^4	1.7×10^3	2.5
80	−	5.2×10^5	6.0×10	0.006	8.7×10^4	—[d]	—
	+				1.1×10^5	4.9×10^3	5.0
120	−	2.1×10^6	2.9×10^2	0.011	3.4×10^5	3.8×10^4	10.3
	+				2.4×10^5	3.3×10^4	11.8
160	−	4.4×10^6	5.6×10^4	1.5	8.2×10^5	9.3×10^4	11.9
	+				5.8×10^5	2.9×10^4	4.0

[a] At time of sampling for transformation.
[b] After 90 min at 37 C for phenotypic expression.
[c] Ratio of *str* to total CFU after phenotypic expression.
[d] Experimental tube was broken.

Normal and *trt* strains were grown in mixed culture in the experiment shown in Table 4. Each component of the population was selectively scored: Moendlexo2 in medium containing sucrose and lacking thymidine and trt1thy7hex4 in medium containing maltose and thymidine. Streptomycin-resistant transformants scored in the former medium included a contribution of *thy*$^+$, *str* double transformants of trt1thy7hex4. This contribution was calculated by multiplying the concentration of *str* CFU scored in medium with maltose and thymidine by the trt1thy7hex4 transformation frequency. Since these values never amounted to more than 20% of the observed *str* transformants in sucrose medium, the contribution of the double transformants was ignored.

Selection of trt. A culture of Moendlexo2 was treated once with the mutagen 1-methyl-3-nitro-1-nitrosoguanidine as previously described (6). An inoculum of 10^7 CFU was added to 10 ml of medium containing trypsin at 1 μg/ml and grown to an absorbancy at 650 nm (A_{650}) of 0.1. The culture was treated with DNA from RF46SKNE and scored for maltose-positive transformants. These appeared at a frequency of only 0.02%; a control without trypsin gave 3% transformation. A 5-ml amount of the transformed culture was centrifuged, and the cells were incubated in 100 ml of medium containing trypsin and maltose instead of sucrose. When the culture reached A_{650} of 0.1, 1 ml was treated with DNA from thy7LSN and scored for thymidine-requiring transformants in medium containing thymidine and trimethoprim (Burroughs-Wellcome). *thy*$^-$ colonies were found at a frequency of 0.05%. A single colony isolate behaved as *thy*, *trt*, *hex*. This strain was transformed by DNA from RF46SKNE to *thy*$^+$ to give strain trt1hex4.

Selection of noz. Strains of *D. pneumoniae* that lack both the major endonuclease and exonuclease still form small, colorless zones due to deoxyribonuclease action around colonies in agar containing DNA and methyl green after incubation for 4 days at 37 C (6). A culture of Moendlexo2 treated with mutagen three times was plated to give ~1,000 colonies distributed in 20 dishes. One colony failed to give a colorless zone on repeated testing. The mutation in this strain, *noz-1*, was introduced into trt1hex4 with a transformation frequency expected for single genetic markers.

Selection of ntr. Nontransformable mutants were obtained by screening a mutagen-treated population of Moendlexo2 for transformation within agar containing transforming DNA. Three agar layers were poured into 10-cm petri dishes. All contained growth medium with a high concentration of maltose (0.3%) and a low concentration of sucrose (0.02%). The bottom layer consisted of 10 ml of medium with 1% agar. The middle layer contained 50 to 100 CFU, 50 μg of DNA from RF46SKNE, and 0.3% agar in 5 ml of medium. The covering layer consisted of 7 ml of medium with 0.7% agar. During incubation for 40 h at 37 C, cells within wild-type colonies underwent transformation to *mal*$^+$ and gave rise to large colonies. Nontransformable clones able to use only the limiting amounts of sucrose in the medium gave small colonies. About 30 *ntr* isolates were obtained, most of which appeared defective in DNA uptake.

Uptake of ^{32}P-DNA and release of acid-soluble ^{32}P in medium. Cultures were grown at 37 C to A_{650} = 0.15 and either processed directly or centrifuged, washed, and suspended at 0 C in simpler media. Details are given in the figure legends. Cultures were rendered competent by incubation for 20 to 40 min at 30 C. They were then either used directly or resuspended prior to addition of ^{32}P-DNA to a final concentration of 0.3 μg/ml. Shifts in temperature were accomplished instantaneously by adding sufficient medium at a higher or lower temperature to bring the culture to the temperature desired.

Samples of 3 ml were taken at different times after addition of ^{32}P-DNA and chilled at 0 C. Cells were removed by centrifugation. The amount of acid-soluble ^{32}P in the medium was determined by adding 0.4 ml of 3.5% perchloric acid to 0.4 ml of medium, holding 10 min at 0 C, centrifuging, and plating 0.4 ml of the acid supernatant fluid on copper planchets. The cells were suspended in medium containing pancreatic deoxyribonuclease at 2 μg/ml, incubated for 10 min at 30 C, centrifuged, washed once with medium, and suspended finally in 0.6 ml. Of this, 0.4 ml was plated to determine uptake of ^{32}P-DNA. Samples were counted on a Nuclear-Chicago low-background planchet counter. About 1% of the donor ^{32}P-DNA was acid soluble; either this amount or the value given by a 0-time control was subtracted from the experimental values to give the amount of acid-soluble DNA released by cellular action.

Analysis of external DNA fragments. After removal of cells from cultures treated with ^{32}P-DNA, oligonucleotide fragments in the medium were analyzed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose (16) and by fractionation on dextran gels (15).

RESULTS

A mutant strain of D. pneumoniae that does not depend on activator. To facilitate investigation of later steps in the development of competence, we sought a strain independent of the activation step by selecting for ability to undergo transformation in the presence of trypsin. Strain trt1hex4 was obtained from a mutagen-treated culture of Moendlexo2 after two successive selective transformations in the presence of trypsin. Table 2 shows that unlike its progenitor, cells of the mutant strain can become highly competent both when taken from young, dilute cultures and when grown in the presence of trypsin. On dilution of a dense culture of the normal strain Moendlexo2 2,000-fold, its cells were initially highly competent, but within 40 min at 37 C competence dropped by a factor greater than 10^3. High

levels of competence returned only when the culture reached a density >2 × 10^8 CFU/ml. When the same culture was diluted only fivefold (Table 3), competence remained high, as expected. But in the presence of trypsin, competence fell by two to three orders of magnitude, which shows that this strain requires continuous activation to remain competent. Strain trt1hex4, however, showed only slight reduction in its manifestation of competence on dilution or in the presence of trypsin.

Figure 1 shows that three other proteolytic enzymes, with specificities different from trypsin, had similar effects. Pronase, chymotrypsin, and subtilisin all prevented the establishment of high levels of competence in Moend1exo2 but not in trt1hex4. Residual competence of Moend1exo2 was ~10^{-3} that of the *trt* mutant in the presence of all four proteolytic enzymes and appeared to be proportional to the total cell count. These facts suggest that the residual competence depends on the absorption of activator by cells before it enters the medium where it is subject to degradation.

To further analyze the difference between the normal and *trt* strains, we compared the development of competence in cells of these strains grown separately and in mixed culture (Table 4). The genetic makeup of the strains used, Moend1exo2 and trt1thy7hex4, allowed them to be individually scored for total cell count and *str* transformants. Competent cultures of these strains, at ~5 × 10^7 CFU/ml, were each diluted 1:200, separately and together, into medium with and without trypsin. The *trt* strain was as highly competent in mixed culture as it was alone; this excludes a destabilizing effect on activator by normal cells as an explanation of the difference between the

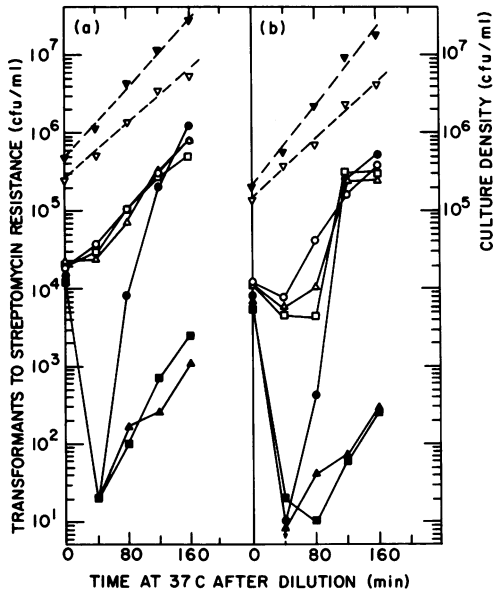

FIG. 1. *Competence in cultures of normal D. pneumoniae and the trt mutant in the presence and absence of proteolytic enzymes: (a) experiment with trypsin and Pronase; (b) experiment with chymotrypsin and subtilisin. Competent cultures of Moend1exo2 and trt1hex4 at 5 × 10^7 to 8 × 10^7 CFU/ml were diluted 1:200 in (a) and 1:300 in (b) in medium with and without the proteolytic enzyme. Culture density was measured by colony counts of samples (taken from the cultures without added enzyme) before transformation. Samples were tested for transformation by holding at 30 C for 15 min, treating with DNA for 15 min, and incubating 90 min at 37 C for phenotypic expression. Symbols: ▼, ▽, culture density; ●, ○, transformants without added enzyme; or with in (a) ▲, △, trypsin; ■, □, Pronase; and in (b) ▲, △, chymotrypsin; ■, □, subtilisin. Filled symbols, Moend1exo2; open symbols, trt1hex4.*

TABLE 3. *Competence in dense cultures of normal D. pneumoniae and the trt mutant in the presence and absence of trypsin*

Time at 37 C after 1:5 dilution (min)	Presence of trypsin at 2 μg/ml	Moend1exo2		trt1hex4	
		Culture density[a] (CFU/ml)	*str* transformants[b] (CFU/ml)	Culture density[a] (CFU/ml)	*str* transformants[b] (CFU/ml)
0	−	1.4 × 10^7	7.0 × 10^5	1.7 × 10^7	1.2 × 10^6
	+	1.4 × 10^7	5.4 × 10^5	1.7 × 10^7	1.1 × 10^6
40	−	3.7 × 10^7	3.0 × 10^6	3.7 × 10^7	1.8 × 10^6
	+	3.7 × 10^7	1.8 × 10^3	3.4 × 10^7	6.3 × 10^5
80	−	7.2 × 10^7	4.5 × 10^6	6.9 × 10^7	2.4 × 10^6
	+	6.5 × 10^7	9.0 × 10^2	7.4 × 10^7	1.3 × 10^6

[a] At time of sampling for transformation; calculated from absorbancy at 650 nm, equating an absorbancy of 0.2 to 10^8 CFU/ml.

[b] After 90 min at 37 C for phenotypic expression.

TABLE 4. *Development of competence in normal D. pneumoniae and in the trt mutant grown alone and in mixed culture*

Time at 37 C after dilution (min)	Presence of trypsin at 2 μg/ml	Moendlexo2				trtlthy7hex4			
		Grown alone		Mixed culture		Grown alone		Mixed culture	
		Culture density (CFU/ml)	Transformation frequency (× 10^3)	Strain density (CFU/ml)	Transformation frequency (× 10^3)	Culture density (CFU/ml)	Transformation frequency (× 10^3)	Strain density (CFU/ml)	Transformation frequency (× 10^3)
40	−	6.8×10^5	0.001	6.8×10^5	0.020	4.0×10^5	4.3	3.0×10^5	3.5
	+	6.2×10^5	0.001	6.8×10^5	0.004	4.0×10^5	2.3	2.6×10^5	2.3
80	−	2.1×10^6	0.015	2.2×10^6	1.3	1.1×10^6	5.7	8.6×10^5	6.9
	+	2.0×10^6	<0.001	3.6×10^6	0.013	8.2×10^5	3.7	1.3×10^6	3.7
160	−	1.5×10^7	4.7	1.5×10^7	3.2	6.5×10^6	9.6	4.1×10^6	8.7
	+	3.3×10^7	0.002	4.6×10^7	0.074	4.6×10^6	2.1	5.8×10^6	2.4

strains. Similarly, the poor transformation of Moendlexo2 after dilution, even in the mixed culture, precludes a stabilization of activator by the *trt* strain. The low transformability of Moendlexo2 in mixed culture in the presence of trypsin shows that the *trt* strain elaborates neither a trypsin inhibitor nor a trypsin-insensitive activator. Competence of Moendlexo2 did appear sooner in the mixed culture, as can be seen in the 80-min samples. The culture density at this time was at the threshold of the usual upsurge of competence (Table 2), and a contribution of activator from trtlthy7hex4 cells in the mixed culture could explain this observation. The production of activator by the *trt* cells is also suggested by the generally higher (but still rather low) levels of competence of Moendlexo2 in the mixed culture at 40 min and at later times in the presence of trypsin. Although *trt* cells appear to elaborate extracellular activator, it is of the normal variety, and it is not essential for maintenance of the activated state of these cells.

The simplest explanation for the behavior of the *trt* mutant is that it dispense with activation. However, it may be internally activated; that is, it may respond to intracellular activator. The mutant may also show a partial response to extracellular activator; this is suggested by the small reduction in competence after dilution or in the presence of proteolytic enzymes. It is conceivable that the mutant responds to very low concentrations of activator. It is also conceivable that activation in the mutant is much more stable than in the wild type. Although exponentially growing cultures of *trt* strains always manifest high levels of competence, cultures in lag phase—when inoculated from cultures frozen for long periods or from cultures in stationary phase—do not. At all stages of the growth cycle both normal and *trt* strains give at least 10 times more transformation at 30 C than at 37 C. Therefore, strain trtlhex4 still requires a temperature-dependent step involving protein synthesis for the development of competence.

Temperature dependence of the formation of competence. When cultures are preincubated in growth medium at 30 and 37 C and then maintained at these temperatures after addition of DNA, uptake is linear in both cases, but the rate of uptake is 10-fold greater at the lower temperature (Fig. 2). When a culture preincubated at 37 C is shifted to 30 C on addition of DNA, the rate of uptake gradually increases, so that after an induction period of 10 to 20 min it reaches the maximal rate observed at 30 C. Conversely, when a culture at 30 C is shifted to 37 C, the rate of uptake is initially high, and only after 8 min is it reduced to the rate observed in a 37 C culture. These results indicate that the rate of DNA uptake per se is not greater at 30 C, but that a higher level of competence is built up and maintained at 30 C than at 37 C.

The induction of competence on shifting a culture to 30 C appears to be a process distinct from both activation and uptake and temporally intermediate. This view is supported by the effect of treatment with trypsin at different stages on uptake of DNA by strain Moendlexo2 (Table 5). Trypsin does not interfere appreciably with uptake itself, and when the enzyme is present only during the period of induction about half the level of competence is attained. (No increase in competence could occur during the uptake period which was carried out in the absence of amino acids.) Only when trypsin was present prior to the induction of competence was DNA uptake abolished, presumably due to proteolytic destruction of the activator.

Nutritional requirements for formation

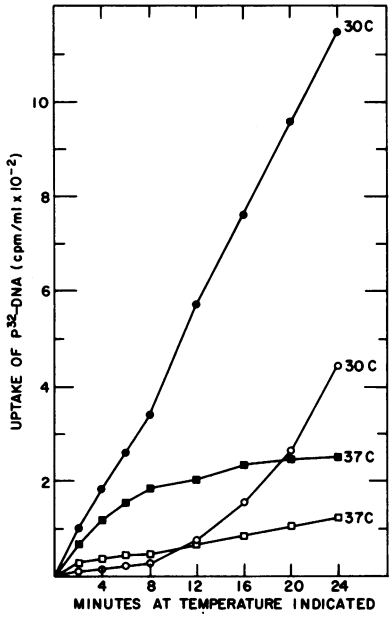

FIG. 2. *Effect of preincubation temperature on uptake of DNA. Portions of a culture of Moendlexo2 grown at 37 C were preincubated for 20 min at 30 and 37 C. On addition of ^{32}P-DNA at zero time, samples at each temperature were shifted instantaneously to 30 and 37 C. Experimental points represent uptake of DNA by cells in growth medium versus time of exposure to DNA at the temperature indicated. Symbols: ●, 30 C throughout; ■, 30 C, then 37 C; ○, 37 C, then 30 C; □, 37 C throughout.*

TABLE 5. *Effect of trypsin on uptake of ^{32}P-DNA by D. pneumoniae strain Moendlexo2*

Presence of trypsin at 10 μg/ml during			Uptake of ^{32}P-DNA (counts per min per ml)	Percentage of untreated culture
Growth of culture at 37 C	Induction of competence 30 min at 30 C	Uptake of DNA 20 min at 25 C		
No	No	No	315	(100)
No	No	Yes	287	91
No	Yes	No[a]	163	52
Final 30 min	Yes	No[a]	11	3
6 h	Yes	No[a]	15	5

[a] Trypsin removed by centrifuging and washing cells at 0 C.

of competence and DNA uptake. Protein synthesis is essential for the induction of competence but not for the uptake of DNA. Figure 3 shows the uptake of DNA by cells grown at 37 C and suspended at 30 C in a minimal medium containing albumin, catalase, sucrose, NaCl, $CaCl_2$, and $MgCl_2$ in potassium phosphate buffer with and without the addition of amino acids in the form of an enzymatic casein hydrolysate. Induction of competence occurs only in the presence of amino acids. Formation of competence is also abolished by the addition of chloramphenicol at 1 mg/ml or by the absence of sugar in the medium. When cells were preinduced by incubation for 40 min at 30 C in minimal medium containing amino acids prior to suspension in fresh medium and addition of DNA, the absence of amino acids had little effect on uptake over a 20-min period (Fig. 3).

That the divalent cations Ca^{2+} and Mg^{2+} are also required for the formation of competence is demonstrated in Fig. 4. With both ions present uptake was rapid and linear after 20 min. Extrapolation of this linear uptake (short dashes) to the abscissa shows a latency period of 11 min. No increase in uptake occurred in the absence of either ion, but as will be seen below, these ions are necessary for uptake itself. However, if the competent state were induced in the absence of the ions, on addition of the missing ion at 20 min, uptake should

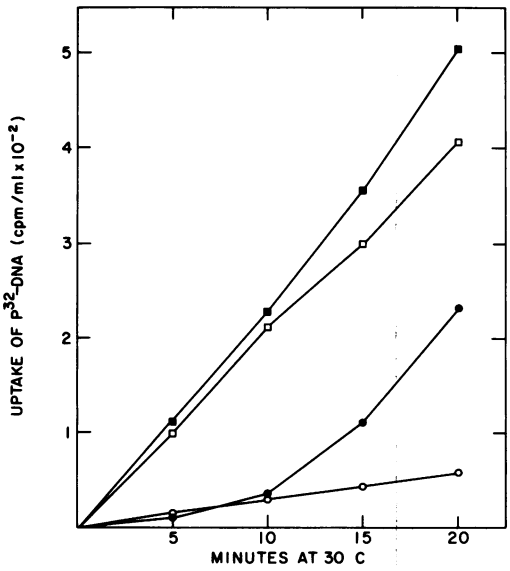

FIG. 3. *Effect of amino acids on formation of competence and uptake of DNA. Portions of a culture of Moendlexo2 grown at 37 C were suspended directly in minimal medium at 30 C with (●) and without (○) enzymatic casein hydrolysate. Another portion was incubated for 40 min at 30 C in minimal medium plus enzymatic casein hydrolysate before suspension in minimal medium with (■) and without (□) casein hydrolysate. ^{32}P-DNA was added at 0 min.*

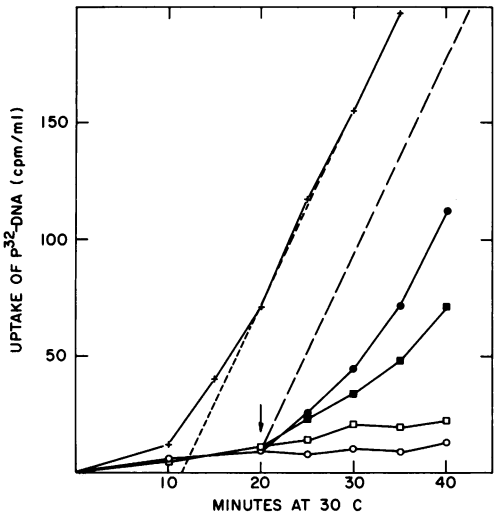

FIG. 4. *Effect of calcium and magnesium ions on the development of competence. Portions of a culture of Moendlexo2 grown at 37 C were suspended in minimal medium supplemented with enzymatic casein hydrolysate and containing both Ca^{2+} and Mg^{2+} or lacking one of these ions. ^{32}P-DNA was added, and the cultures were incubated at 30 C. At 20 min, indicated by the arrow, the missing ion was added to samples of the cultures. Symbols: +, Ca^{2+} and Mg^{2+} present throughout; ○, no Mg^{2+}; □, no Ca^{2+}; ●, Mg^{2+} at 20 min; ■, Ca^{2+} at 20 min; -----, extrapolation of maximal rate of uptake; - - -, expected maximal rate of uptake beginning at 20 min.*

occur at the fully induced rate (long dashes). Instead there is a delay of 8 min when Mg^{2+} is restored and of 13 min when Ca^{2+} is restored which indicates that these ions are essential at some stage in the development of competence prior to DNA uptake. When cells that are already competent are incubated in the absence of divalent cations, there is no delay in uptake on addition of the missing ion.

Requirements for DNA uptake are evident from Fig. 5. Here, cells were rendered competent by incubation for 30 min at 30 C in the presence of amino acids and then were washed and suspended in minimal medium lacking amino acids and various other constituents. Very little uptake was observed in the absence of sugar; thus, uptake appears to be an energy-dependent process. Mg^{2+} was quite essential for uptake; Ca^{2+} was somewhat less so, but uptake was greatly enhanced in the presence of both ions. Chloramphenicol only moderately inhibited DNA uptake as opposed to its strong effect on formation of competence.

Deoxyribonuclease action external to cells. Competent cells are not only able to take up DNA from the medium, but they also degrade donor DNA to acid-soluble fragments that are released into the medium external to the cells. This deoxyribonuclease action seems to occur on the cell surface since culture supernatant fluids do not show any activity. The major endonuclease and exonuclease of *D. pneumoniae* are missing in the strains studied (6), therefore these enzymes are not responsible for the activity. Figure 6 shows the dependence on competence of the release of acid-soluble DNA external to the cell. Without amino acids present during the induction period neither the ability to take up nor to degrade DNA is generated. It is evident that comparable amounts of DNA are taken up and rendered acid soluble in the medium.

Requirements for the deoxyribonuclease action are similar to those for uptake of DNA (Fig. 7). Sugar and Mg^{2+} are both essential, whereas amino acids are not. Adenosine triphosphate (ATP) cannot substitute for sugar in the medium for either the uptake reaction or the release of acid-soluble material.

External release of acid-soluble radioactivity was compared to ^{32}P-DNA taken up in repli-

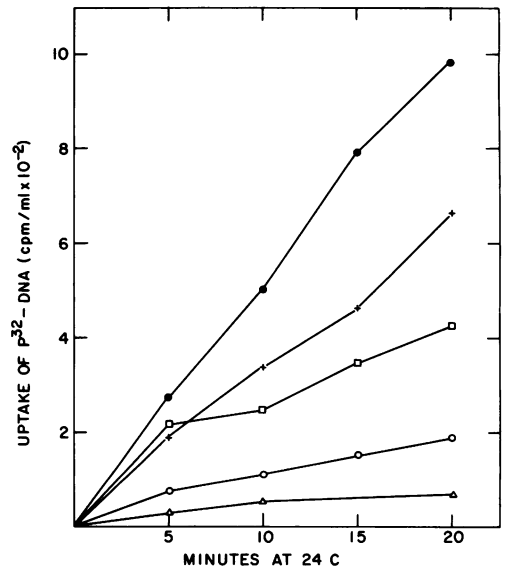

FIG. 5. *Requirements for DNA uptake. A culture of Moendlexo2 grown at 37 C was held for 30 min at 30 C. The cells were washed and suspended in minimal medium, either complete or lacking various ingredients, and preincubated at 24 C for 20 min. Chloramphenicol at 1 mg/ml was added to one sample 2.5 min before DNA. ^{32}P-DNA was added at 0 min. Symbols: ●, complete; +, complete plus chloramphenicol; □, no Ca^{++}; ○, no Mg^{++}; △, no sucrose.*

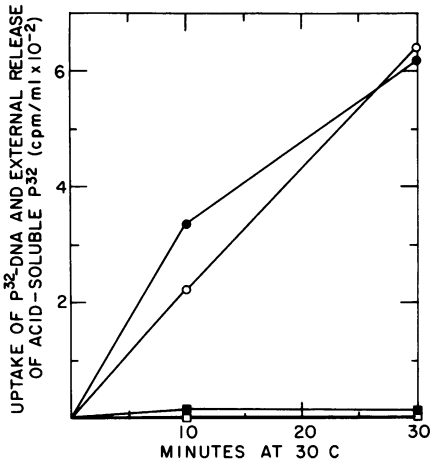

FIG. 6. *Dependence of external deoxyribonuclease action on cellular competence. Portions of a culture of trt1hex4 grown at 37 C were suspended in minimal medium with* (○, ●) *and without* (□, ■) *enzymatic casein hydrolysate for 30 min at 30 C.* ^{32}*P-DNA was added, and incubation was continued at 30 C. Samples were tested for DNA uptake (solid symbols) and release of acid-soluble DNA in the medium (open symbols).*

cate cultures of several different strains of *D. pneumoniae* (Fig. 8). A nontransformable mutant, ntr48, showed neither uptake nor degradative activity; this strain appears to be genetically incapable of achieving competence. Replicate cultures of trt1hex4 all showed about equal amounts of external acid-soluble ^{32}P and internal ^{32}P. However, cells containing the *noz-1* mutation, while taking up substantial amounts of DNA, produced acid-soluble material external to the cell equivalent to only 40% of the amount of DNA taken up. The *noz-1* mutation was originally isolated in a strain selected for defective deoxyribonuclease activity as manifested by inability to form a colorless zone around colonies in agar containing DNA and methyl green.

We are currently investigating the nature of the DNA fragments formed external to the cell in both normal and *noz* strains. Preliminary analyses by chromatography on dextran gels and DEAE-cellulose show that for both strains the fragments consist of oligonucleotides ranging in length from 1 to 20 residues. The mean length, however, appears to be greater in the normal strain. In one experiment the mean length for the normal strain was ~10, and for the *noz* strain ~3. With both strains the mean length and the proportion of mononucleotides also seem to depend on the duration of incubation.

Inactivation of the competent state at high temperatures. Figure 2 showed that competence is lost when a culture induced at 30 C is transferred to a higher temperature. We have analyzed the rates of this inactivation at various temperatures after first inducing competence and then transferring the cells to minimal medium in which no further formation of competence could occur. The cells were then held at various temperatures after which they were shifted to 24 C for measurement of the residual rate of DNA uptake. Rates of inactivation of the ability to take up DNA were generally exponential (Fig. 9), and reduction in the ability to form acid-soluble fragments external to the cell followed similar kinetics. In the experiment depicted in Fig. 10, however, a short lag preceded exponential decline. This may be due to an excess buildup of the thermolabile component essential for competence in the cells used in this particular experiment. This explanation is supported by the fact that the rapidly declining portions of the curves extrapolate back to the ordinate at approximately the same value—about 70% greater than the initial rate of uptake.

Table 6 expresses the data of Fig. 10 in terms

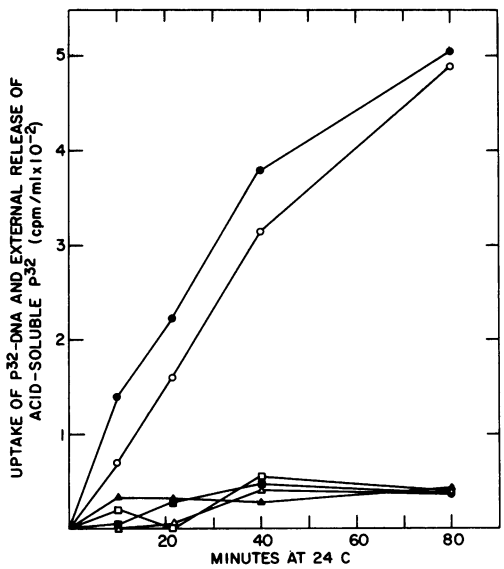

FIG. 7. *Requirements for external deoxyribonuclease action. A culture of trt1hex4 grown at 37 C was held for 30 min at 30 C. The cells were washed and suspended in minimal medium: complete* (○, ●), *without* Mg^{2+} (□, ■), *or without sucrose* (△, ▲). ^{32}*P-DNA was added immediately on transfer to 24 C. Open symbols represent release of acid-soluble DNA in the medium; solid symbols represent DNA uptake by the cells.*

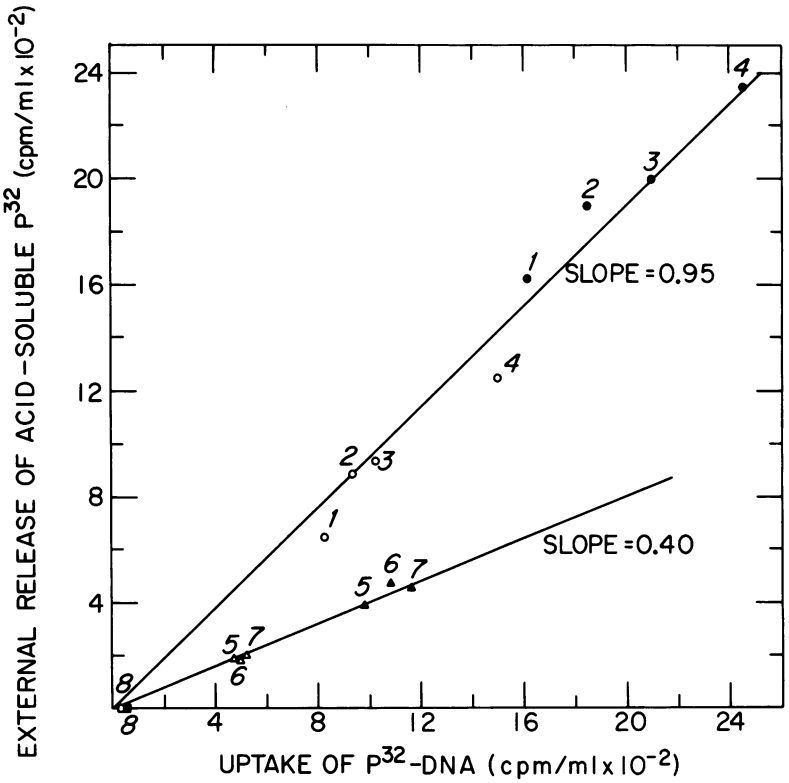

FIG. 8. *Relationship between external release of acid-soluble DNA fragments and uptake of DNA in normal, noz, and ntr strains. Independent cultures of strains trt1hex4 (1–4, circles), trt1hex4nozl (5–7, triangles), and ntr 48 (8, squares) were grown at 37 C. The cultures were held 20 min at 30 C. Then* ^{32}*P-DNA was added, and incubation was continued at 30 C. Samples were tested for DNA uptake and external release of fragments at 20 min (open symbols) and 60 min (solid symbols) after addition of DNA.*

of inactivation rates. Over the 10 C range of temperature, there is an 18-fold increase in the rate of inactivation. This increase is considerably greater than the expectation for a simple enzymatic reaction and corresponds more to a reaction with a higher activation energy such as protein denaturation. In support of a simple denaturation hypothesis, we found that the absence of sugar or amino acids does not affect the rate of decay of competence. Neither does the presence of DNA during the treatment at high temperature protect the uptake mechanism from inactivation.

DISCUSSION

Our results demonstrate the importance of temperature in the development and maintenance of competence in *D. pneumoniae*. Development of competence on a shift-down in temperature from 37 to 30 C requires protein synthesis and the presence of Mg^{2+} and Ca^{2+} ions. Protein synthesis was previously shown to be required for the appearance of competence by Fox and Hotchkiss (1) in frozen cultures that had been stored for long periods and by Tomasz (17) in noncompetent cultures grown at pH 6.8 after a shift to pH 8 and treatment with activator. Raising the temperature causes an irreversible loss of competence. The rate of destruction depends sharply on temperature, but contrary to the report of Tomasz (18), who also observed a gradual inactivation at 30 C, the rate was not influenced by the absence of amino acids or sugar. At a given temperature, it appears that the level of competence represents a steady state of formation and destruction.

We confirmed the finding by Tomasz (17) that the uptake of DNA itself does not require protein synthesis but does depend on a source of energy. The enhancement of uptake by calcium ions has already been reported (1). Demonstration of a requirement, also, for Mg^{2+} in uptake may be significant in that this ion is

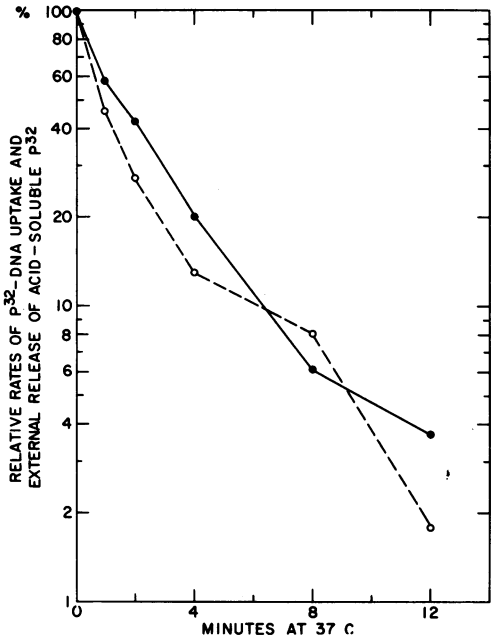

FIG. 9. *Loss of competence on incubation at 37 C. A culture of trt1hex4 was grown at 37 C, suspended in minimal medium plus enzymatic casein hydrolysate for 30 min at 30 C, centrifuged, and suspended in minimal medium. Portions were held various times at 37 C before shifting to 24 C and addition of ^{32}P-DNA. Samples were tested after 10, 20, and 80 min. Rates of uptake and external degradation of DNA were essentially linear. Symbols: ●, relative rate of ^{32}P-DNA uptake; ○, relative rate of release of acid-soluble ^{32}P external to cells.*

a requisite cofactor for several deoxyribonucleases of *D. pneumoniae*.

It is not yet possible to decide between two alternative explanations for the thermolabile nature of competence. One view is that competence represents a modified state of the cell surface which requires new synthesis of nonspecific surface proteins at lower temperatures and which is unstable at higher temperatures. If the competent surface structure were more porous, the various manifestations of competence—specific antigenic behavior (10), agglutination at low pH (20), uptake of DNA, and deoxyribonuclease action external to the cells—could all be explained by greater accessibility of preexisting cellular proteins to the outside. The other view is that induction of competence represents the synthesis of a specific thermolabile surface protein. This single protein could conceivably serve as the competence antigen, the acid agglutinin, and the critical part of the uptake mechanism. If the protein itself is not responsible for both DNA uptake and external deoxyribonuclease action, it could be a shared component of both pathways, for example by binding DNA to the cell surface so that it is accessible to both the uptake mechanism and deoxyribonucleases located on the surface.

Although external deoxyribonuclease action occurs only with competent cells, it is not clear to what extent this action is related to the act of DNA uptake. For equal amounts of uptake the *noz* strain produces only 40% as much acid-soluble DNA external to the cells as does

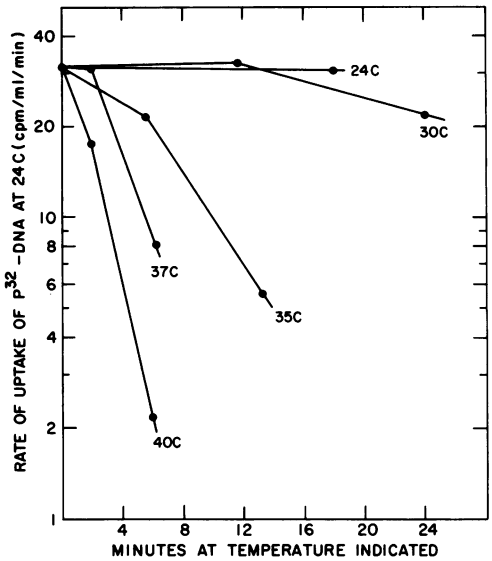

FIG. 10. *Temperature dependence of the inactivation of competence. A culture of trt1hex4 was grown at 37 C, suspended in minimal medium plus enzymatic casein hydrolysate for 30 min at 30 C, centrifuged, and suspended in minimal medium. Portions were held at the indicated temperatures for various times before shifting to 24 C and addition of ^{32}P-DNA. Samples were tested for uptake after 5, 10, and 20 min, and the rate of uptake is indicated for each exposure.*

TABLE 6. *Rates of inactivation of competence at different temperatures*

Inactivating temperature (C)	Slope of exponential inactivation expressed as t_{37} = time for 37% survival (min)	Exponential inactivation rate constant $k = 1/t_{37}$ (per min)
30	31.0	0.03
35	5.7	0.18
37	3.1	0.32
40	1.9	0.53

the normal strain. This difference could result from an altered uptake mechanism in *noz* cells. Another possibility is that the normal strain contains two enzymes that give rise to acid-soluble products externally, but that only one of the two enzymes, the one remaining in *noz* mutants, is connected with DNA uptake. Interestingly, both the oligonucleotide nature of the products and the requirement for a source of energy resemble properties of the ATP-dependent deoxyribonuclease found in *D. pneumoniae* by Vovis and Buttin (22).

Earlier studies suggested a mechanism of DNA uptake in which one strand of a donor duplex enters intact while the complementary strand is degraded by a cellular deoxyribonuclease (4). Morrison and Guild (9) recently found that with highly intact DNA the proportion of introduced DNA in the form of single strands is as high as 80%. They suggest that the fragments appearing outside the cell may represent the degraded strand (D. A. Morrison and W. R. Guild, Biochim. Biophys. Acta, in press). However, the paucity of external fragments formed by the *noz* strain would, at least in this strain, require fragments of the degraded strand to appear inside as well as outside the cells. In the normal strain fewer fragments may be retained within the cell, or else the additional external fragments may not derive from the strand complementary to the one taken up.

Tomasz and co-workers (21) have emphasized the importance of cellular incorporation of choline in the development of competence. Although the bulk of choline in *D. pneumoniae* is found in a teichoic acid and none has been detected in phospholipids, it is still possible that a minor phospholipid containing choline is crucial for transformation. Such a choline requirement could be related to the action of a deoxyribonuclease at the cell membrane during uptake. Membrane-bound enzymes often require specific phospholipids for their activity (14). The galactosyltransferase of *E. coli* for example, requires a phospholipid containing ethanolamine but is inactive with a phospholipid containing choline (13). The converse situation may occur in the case of DNA uptake by *D. pneumoniae*, since cells grown with ethanolamine in place of choline cannot be rendered competent (21).

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