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INDUCTION OF NORMAL LEVELS OF GENETIC TRANSFORMATION IN A CLASS OF ENDONUCLEASE-DEFECTIVE MUTANTS OF PNEUMOCOCCI.

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Summary. Pneumococcal mutants were isolated that showed no zones of DNA hydrolysis around the bacterial colonies, even after prolonged incubation on the surface of agar plates containing DNA and methylgreen. Lysates of such mutants contained only very low levels of endonuclease activity; the mutants were also defective in genetic transformation even though they could still bind DNA to their surface. However, the same mutants could be made to undergo normal, high frequency genetic transformation by treatment with the activator protein, under appropriate conditions. The same treatment caused no detectable increase in the endonuclease level. Poor transformability of these mutants seems to be related to an inhibitory factor(s) released into the growth medium. Activation in the presence of this factor(s) leads to an abnormal (non-productive) DNA adsorption - both in mutant and in wild type pneumococci.

INTRODUCTION. In genetic transformation large polynucleotides cross cellular boundaries without loss of their genetic activity. The mechanism of this important process is not understood. In wild type pneumococci, cellular ability to adsorb and take-in exogenous DNA seems to be controlled by a protein ("activator") factor that is produced by growing cultures at a critical cell concentration (1). It is also known that DNA molecules taken up by such activated pneumococci are exposed to the action of several types of nucleases during the process of transformation and it has been proposed that surface-located nucleases are actually components of a DNA recognition and transport system in these bacteria (2-6). In an attempt to obtain genetic evidence for this attractive model, Lacks and his colleagues have isolated mutants that showed no zones of DNA hydrolysis when plated on methylgreen-DNA agar ("noz" mutants, 7). Lysates of such mutants were shown to have very low levels (less than 1%

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of wild type) of endonuclease activity and mutant cultures would only transform poorly, while still capable of adsorbing DNA to the cell surface (7). Endonuclease defect and poor transformability were found to co-transform in transformants selected for the noz phenotype. It has been suggested that endonuclease functions as a "DNA translocase" in genetic transformation (6); in this model endonuclease-defective mutants would be capable of attaching DNA to normal receptors while the subsequent (transport) stage would be defective.

In an independent and parallel line of investigations using direct biochemical techniques, putative DNA receptors have been isolated from competent pneumococci (9,10). It seemed that the use of mutants blocked at a stage beyond DNA adsorption might offer important technical advantages for the purification of such, hypothetical DNA receptors. For this reason, we isolated several noz-type mutants, following the technique described by Lacks and Greenberg (11). Upon closer examination of 7 of these mutants, (isolated as clones showing no zones of DNA hydorlysis on methylgreen-DNA agar) we have come to the conclusion that at least in this set of mutants the correlation between endonuclease defect and low transformability may be fortuitous and in this communication we describe the observations upon which this conclusion is based.

Materials and Methods. Bacterial strains: the wild type (R6) pneumococci used are derived from the Rockefeller University strain of Streptococcus pneumoniae (R36A). A nuclease-defective mutant (end1 exo2) (11) was kindly provided by Dr. Sanford Lacks (Brookhaven National Laboratories, Upton, N.Y.). This mutant was the parent of the noz mutants, isolated and selected according to the methods of Lacks and Greenberg (11). Bacteria heavily mutagenized by ten consecutive treatments with nitrosoguanidine - were plated on agar medium containing DNA and methylgreen and seven clones that showed no detectable zones of decolorization after 4 days of incubation (at 37°C) (noz mutants 2 through 8) were picked and used in the experiments. All the mutants grew with normal generation times in the synthetic and semi-synthetic media (12,13). Published procedures were used for the assays of bacterial growth, viability and transformants (13,14) and for the preparations of activator and DNA (15). Spent growth media were separated from the bacteria by centrifugation at 10000 x g when the cell concentration reached 7.5×10^7 viable bacteria per ml; the supernatants were stored at -70°C. Activator treatment was done in the following manner: 0.8 ml of

fresh or spent medium was mixed with 0.1 ml of bacterial suspension (containing a total of 10^6 to 10^7 viable cells) plus 0.1 ml of activator (100 to 500 units; ref. 15). After incubation at 30°C for 20 min, DNA was added (usually 0.1 µg per ml) and the incubation continued for 20 min more. At this time, 0.2 ml of the suspension was diluted into 6 ml fresh C-medium containing yeast extract (Difco; 0.2% final concentration) and pancreatic DNase (Worthington, 2x crystallized) and the suspension was incubated at 37°C for 90 min; the number of viable cells and the number of streptomycin resistant transformants was determined.

Determination of spontaneous DNA binding, uptake and transformability. The method of Lacks and Greenberg was used. Mutant and wild type cultures (10 ml) were grown in C-medium supplemented with albumin or with albumin plus yeast extract (12). At a cell concentration of $5 - 7 \times 10^7$ colony forming units (cfu) per ml, the entire culture was transferred to a 30°C bath for 20 to 40 min to allow optimal expression of competence (6). After this period, 0.1 ml portions were diluted into 1.0 ml fresh, 30°C medium containing transforming DNA (0.1 µg per ml, streptomycin resistance marker) and after a further incubation of 10 min, deoxyribonuclease (DNase) was added to terminate transformation. Another portion of the 30°C preincubated culture was used to determine DNA binding and uptake: 4 ml of bacteria were incubated with DNA (1.4 µg and 2.4×10^5 cpm per ml) at 30°C for 20 min. The suspension was divided into two portions (2 ml each); one was treated for 5 min at 30°C with 5 µg/ml DNase and then washed 3 times with medium (C pH 8 + alb) (by centrifugation at 8000 rpm for 5 min in the cold). Final pellet was suspended in 1 ml medium and 1 ml portion was pipetted into cold 10% CCl_3COOH (2 ml). The precipitate (after 20 min at 0°C) was collected on a glass-fibre filter and washed 3 times with CCl_3COOH to determine "DNA uptake".

The other portion was immediately centrifuged, washed, and precipitated with cold CCl_3COOH to determine "Total DNA bound". Medium containing 10 mM EDTA was used during washings. Total nuclease content of bacteria was determined using the method described by Lacks (7) - 10 ml cultures at cell concentration of 7.5×10^7 viable bacteria per ml - were harvested by centrifugation; the cells were lysed by incubation in 0.2 ml of 0.1% Triton X-100 solution (0.1 M Tris (pH 7.6) - 0.001 M $MgCl_2$ - 0.0015 M 2-mercaptoethanol) at 30°C for 15 min. The lysates were diluted by the addition of 1.8 ml Triton-free buffer and -if necessary- stored at -20°C before use. Nuclease assays were carried out at 30°C in total volumes of 2.0 ml, containing 900 µl buffer (0.05 M Tris HCl pH 7.6 - 0.04 M NaCl - 0.0015 M $MgCl_2$ - 0.04% Triton X-100); 100 µl bacterial extracts and 100 µl ^{3}H-DNA (1.4 µg); in order to determine the RNA-inhibited endonuclease activity, parallel samples also recieved 20 µg per ml RNase A. At sampling times, 200 µl portions were pipetted into 200 µl cold, 10% trichloroacetic acid and 20 µl of 4% albumin was added as carrier. The precipitated DNA was removed by centrifugation (10000 x g, 20 min) and 100 µl portions of the supernatants were assayed for soluble radioactivity - in 5 ml of Biofluor solution (New England Nuclear Corp., Boston, Mass.), using a scintillation spectrometer (Searle, Mark II). A unit of nuclease was defined as the activity capable of hydrolyzing 1 µg DNA to an acid soluble form in 1 hour at 30°C. Protein concentration of the bacterial extracts was determined by the method of Lowry (16).

Results. Table 1 summarizes the properties of 7 pneumococcal mutants isolated on the basis of their inability to form zones of DNA hydrolysis around the bacterial colonies plated on methylgreen-DNA agar plates (11).

Table 1. Properties of endonuclease-defective (noz) mutants in pneumococci[1]

Strain	Endonuclease activity (units per mg protein)	Transformability (number of transformants per 100 viable cells)	Total ^{3}H-DNA bound (associated with 1.4×10^7 cells)	^{3}H-DNA taken up (by 1.4×10^7 cells)	Transformability after activator treatment (number of transformants per 100 viable cells)
wild type (R6)	59	1.0	20,114 cpm (114ng)	7200 cpm (41ng)	1.0
nuclease-defective mutant (end1 exo2)	6	1.0	22,705 (134ng)	6874 (39ng)	1.2
noz 2	0.5	0.008	16,359 (97ng)	187 (1.1ng)	0.78
noz 3	0.4	0.003	29,870 (176ng)	265 (1.6ng)	0.81
noz 4	0.5	0.002	17,515 (103ng)	109 (0.6ng)	0.78
noz 5	0.4	0.5	21,205 (125ng)	3313 (19.5ng)	1.1
noz 6	0.3	0.0007	16,293 (96ng)	37 (0.2ng)	0.9
noz 7	0.5	0.6	36,660 (215ng)	4486 (27ng)	0.78
noz 8	1.2	1.0	36,378 (213ng)	6530 (39ng)	0.88

[1] Transformability of cultures was assayed at cell concentrations of 7.5×10^7 viable bacteria per ml, by the assay described in the Methods.

Table 2. Nuclease content of Triton-lysates of noz mutants before and after activation to competence[1]

	Source of Triton X-100 lysate	Acid soluble fragments produced (cpm)	
		in presence of RNase	in absence of RNase
exp. 1 (1 hour incubation during the nuclease assay)	incompetent noz 3	4900 (1.22 units)	———
	noz 3 activated with noz 4 activator	4950 (1.23 units)	———
	noz 3 activated with wild type activator	4750 (1.18 units)	———
exp. 2 (3 hours incubation during the nuclease assay)	incompetent noz 3	10,270	7970
	noz 3 activated with noz 4 activator	9500	7950

[1] Activation was performed in fresh growth medium. Nuclease activities were assayed either after 1 hour (exp. 1) or after 3 hours (exp. 2) of incubation.

In confirmation of the findings of Lacks and his colleagues, all the mutants exhibited low levels of endonuclease activity in deoxycholate (DOC) lysates; during growth in liquid cultures, mutant cultures developed the capacity to bind radioactive DNA, but genetic transformation by the mutants was only a fraction (0.1 to 0.0001) of that of parental cultures grown under the same conditions.

Our novel finding documented in column e of Table 1 is that the defective transformability of each one of the mutants could be corrected by treating the bacteria in fresh medium with activator prepared either from wild type - or, from noz mutant bacteria. The same findings were also reproduced in a transformant (NN 33) obtained by treating competent parental cells with DNA prepared from a noz mutant (noz 3) and selecting for noz transformants (not documented). Activation to normal (wild type) levels of transformability was also demonstrated using electrophoretically homogeneous wild type activator.

Table 2 demonstrates that the treatment with activator in fresh medium did not cause a detectable increase in the endonuclease activity of lysates of the activated bacteria.

These observations suggested that noz mutants may liberate into their growth media (an) inhibitory factor(s) that interferes with normal activation. Results illustrated in Figure 1 indicate that this is the case: culture media of noz mutants collected at the time when the cells exhibit maximal DNA binding capacity inhibit activation of both noz and wild type (endonuclease positive) pneumococci to competence. The inhibitory factor(s) may be neutralized by dilution and this factor(s) has no effect on the genetic transformation of already competent cells (i.e. bacteria - either noz or wild type - that have been pre-treated with activator in fresh medium and then transferred to spent medium during exposure to DNA) (Table 3). Table 3 documents several additional observations. The abortive activation in the spent medium of noz cells

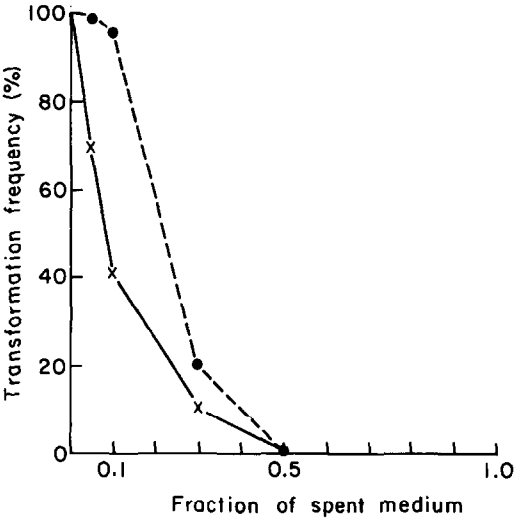

Figure 1. Titration of the inhibitory activity of spent media from cultures of noz 3 and wild type pneumococci.

Ten ml cultures of the noz and the R6 strains were grown in C pH 8 medium at 37°C. At the cell concentration of 7.5×10^7 viable bacteria per ml, cells were removed by centrifugation and the supernatant spent media were used as suspending media for the activation of physiologically incompetent R6 cells to competence. Incompetent R6 culture was grown in the C medium at pH 6.6; 100 µl of cells (2.5×10^6 viable bacteria) were pipetted into 900 µl of spent media - or into spent media prediluted to various degrees with fresh C pH 8 medium -. Each tube recieved 100 µl of activator and was assayed for the induction of transformability, as described in the Methods. Spent medium of wild type (R6): dashed line; spent medium of mutant: solid line.

leads to the development of DNA binding capacity of both noz and the parental (end1 exo2) pneumococci in a process that appears to have the same requirements as activation to "full" competence, i.e. low pH and trypsin inhibit the appearance of binding capacity. DNA adsorption without DNA uptake could also be demonstrated by treating physiologically incompetent wild type (R6) pneumococci with activator in the spent medium of noz cells (Table 4). Cells activated in spent medium may develop incorrectly located receptors. Alternatively, DNA attached to such receptors may be damaged in such a manner that it can no longer be transported.

The chemical nature of the inhibitory factor(s) is unknown. At

Table 3. Effect of growth conditions and activator treatment on the DNA binding capacity of mutants[1]

Growth conditions:	Strain	Total ^{3}H-DNA bound (associated with 1.4×10^7 cells)	^{3}H-DNA taken up (by 1.4×10^7 cells)	Transformability (number of transformants per 100 viable cells)
1) optimal for spontaneous competence	parent (end1 exo2)	24,226 cpm (142ng)	7690 cmp (45ng)	1.0
	noz 3	26,122 (154ng)	364 (2.1ng)	0.002
2) inhibitory for spontaneous competence:				
i) trypsin	parent (end1 exo2)	3010 (18ng)	240 (1.4ng)	0.0001
	noz 3	2395 (14ng)	266 (1.6ng)	0.0001
ii) pH 6.6	parent (end1 exo2)	3120 (18ng)	150 (1.0ng)	0.002
	noz 3	5013 (29ng)	220 (1.3ng)	0.003
3) pH 6.6 followed by treatment with activator from wild type	parent (end1 exo2)	26,040 (153ng)	8010 (47ng)	0.9
	noz 3	30,015 (176ng)	7535 (44ng)	1.1
4) pH 6.6 followed by treatment with activator from noz 4	parent (end1 exo2)	20,253 (114ng)	6454 (36.5ng)	0.98
	noz 3	17,712 (100ng)	6549 (37ng	1,0

[1] a) Cultures were grown in C pH 8 medium supplemented with albumin (40 µg per ml; Armour fraction 5) and tested at a cell concentration of 7.5×10^7 viable bacteria per ml. b) Trypsin (2 µg per ml; crystallized; Worthington) was added to the C pH 8 medium. c) Before activator treatment the cells were diluted 10 fold into C pH 8 medium. d) activator was prepared by the published procedure (15) from the pellet of a 400 ml culture of noz 4 harvested at the cell concentration of 7.5×10^7 viable cells per ml. The third extract of activator (15) was used.

Table 4. Effect of the spent culture medium of noz mutants on the activation of wild type pneumococci to competence[1]

Bacterial strain		Medium used during activation and transformation	Total ^{3}H-DNA bound (cpm) (associated with 1×10^8 cells)	^{3}H-DNA taken up (cpm) (by 1×10^8 cells)
Physiologically incompetent	wild type (R6)	fresh medium	41,000 (100%)	6000 (14%)
		spent medium of noz 3	42,000	400 (1%)
Physiologically incompetent	parent (end1 exo2)	fresh medium	23,000	9000
		spent medium of noz 3	9600	200
Physiologically incompetent		fresh medium	42,600	4800
		spent medium	17,000	340

[1] All bacterial strains were used at the cell concentration of 7.5×10^7 viable cells per ml. Physiologically incompetent cultures were grown in C medium at pH 6.6 to a cell concentration of 2.5×10^7 per ml; these bacteria were transferred to the C pH 8 medium by centrifugation before treatment with activator. Spent medium was prepared from the noz 3 culture at the density of 7.5×10^7 cells per ml.

least part of the inhibitory activity could be neutralized by dialysis against fresh medium and readjustment of the somewhat acidic pH to a value around 7 - 7.2 (not documented). Figure 1 suggests that the inhibitor(s) is also produced by wild type bacteria but at cell concentrations higher than that of noz cultures. Wild type pneumococci have been known to liberate (an) activation inhibitory factor(s) into the growth medium at a time past the peak of spontaneous competence of such cultures (17). It is concievable that the same inhibitor(s) is released prematurely in the mutant bacteria.

It is important to emphasize that all the aberrant features of the transformability in noz mutants could be reproduced using wild type cells resuspended in the spent culture medium of the mutant bacteria. Thus, defective transformability of the noz mutants seems to be related to an abnormal, abortive activation in the presence of inhibitory factor(s) rather than to the low endonuclease levels.

Further studies on the chemical nature and mechanism of action of the filtrate inhibitor(s) may give important insights concerning the mechanism of action of the activator protein.

Literature cited

1. Tomasz, A. (1966). J. Bacteriol. 91, 1050-1061.
2. Lacks, S. and Greenberg, B. (1973). J. Bacteriol. 114, 152-163.
3. Morrison, D. and Guild, W.R. (1972). J. Bacteriol. 112, 1157-1168.
4. Dubnau, D. and Cirigliano, C. (1972). J. Mol. Biol. 64, 31-46.
5. Morrison, D. (1971). J. Bacteriol. 108, 38-44.
6. Lacks, S., Greenberg, B. and Neuberger, M. (1974). Proc. Natl. Acad. Sci. U.S. 71, 2305-2309.
7. Lacks, S., Greenberg, B. and Neuberger, M. (1975). J. Bacteriol. 123, 222-232.
8. Lacks, S. and Greenberg, B. (1976). J. Mol. Biol. 101, 255-275.
9. Seto, H. and Tomasz, A. (1975). J. Bacteriol. 122, 1339-1350.
10. Seto, H. and Tomasz, A. (1975). J. Bacteriol. 124, 969-976.
11. Lacks, S. (1970). J. Bacteriol. 101, 373-383.
12. Lacks, S. and Hotchkiss, R.D. (1960). Biochim. Biophys. Acta 39, 508-517.
13. Tomasz, A. (1964). Bact. Proc., p.29.
14. Hotchkiss, R.D. (1957). p.692 in S.P. Colowick and N.O. Kaplan (ed.), Methods in Enzymology, Vol. 3. Academic Press Inc. New York.
15. Tomasz, A. and Mosser, J.L. (1966). Proc. Natl. Acad. Sci. U.S. 55, 58-66.
16. Oyama, V.I. and Eagle, H. (1956). Proc. Soc. Exp. Biol. Med. 91, 305-307.
17. Tomasz, A. and Hotchkiss, R.D. (1964). Proc. Natl. Acad. Sci. U.S. 57, 480-487.