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Polyethyleneglycol-Induced Transformation of *Bacillus subtilis* Protoplasts by Bacterial Chromosomal DNA

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Summary. *Bacillus subtilis* protoplasts, which in the presence of polyethyleneglycol (PEG) are transformed by plasmid DNA (Chang and Cohen 1979) can also be transformed under these conditions by chromosomal DNA. Transformation in this case occurs at a much lower frequency, not fully accounted for by the heterogeneity of this DNA. Another unexpected feature of the transformation studied, which may explain why it previously went unnoticed, is that DNA concentrations higher than 1–2 μg/ml decrease the yield of transformants, without showing signs of general toxicity.

PEG-induced protoplasts (PIP) transformation for chromosomal markers operates normally with protoplasts prepared from a non-transformable bacterial mutant. The evidence indicates that both native linear and plasmid DNAs must somehow be forced into the cells as a result of PEG action. Denatured chromosomal DNA however is almost inactive in PIP transformation. No competition between chromosomal and plasmid DNAs could be detected, when the DNA tested as inhibitor was in tenfold excess.

Introduction

Exposure of bacterial protoplasts to polyethyleneglycol (PEG) is an efficient way of inducing their fusion, which results in the subsequent formation of genetic recombinants (Fodor and Alföldi 1976; Schaeffer et al. 1976; Hopwood et al. 1977). In the presence of plasmid DNA, this exposure may also lead to PEG-induced protoplast (PIP) transformation, a DNase sensitive process recently described by Bibb et al. (1978) and by Chang and Cohen (1979). According to these authors, only supercoiled DNA is fully active in PIP transformation (activity is low or negligible with open-circular or linear plasmid DNA, respectively, and homologous chromosomal DNA is completely inactive), but the recipient protoplasts need not be derived from competent, or even transformable bacteria.

The observation that protoplasts could be transformed in the presence of PEG by chromosomal DNA was first made by K. Fodor, working in this laboratory with DNA that were believed to be from a hybrid made by fusion between *B. subtilis* and *B. megaterium*. It was felt that this observation, at variance with the published work of Chang and Cohen (1979), would be easier to interpret and carry more weight, if it could be repeated with DNA from standard *B. subtilis* strains of the 168 line. With such strains PIP transformation for chromosomal traits could easily be confirmed, and is described in this paper.

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Material and Methods

Bacterial Strains. Protoplasts from three strains have been used as recipients in transformation experiments. Two of them, S3 (Ade$^-$ Ura$^-$ Trp$^-$) and S13 (Met$^-$ Leu$^-$ Thr$^-$) have already been described (Schaeffer et al. 1976; Lévi et al. 1977). The third strain, MO214 (Ade$^-$ Ura$^-$ Spo$^-$), is a Trp$^+$Spo$^-$ derivative of S3 isolated after transformation with excess DNA from MO213 (*spoOA47 acf-1*). (*spoOA5NA* was the initial designation for this *spo* mutation, which simultaneously blocks sporulation at stage zero and prevents the appearance of competent cells, Ionesco et al. 1970).

MO215, another S3 derivative which in this work was used as a source of plasmid DNA, carries in addition to the auxotrophic markers of S3 the chloramphenicol-resistance plasmid pC194. Originating from *Staphylococcus aureus* this plasmid is stable and expressed in *B. subtilis* (Ehrlich 1978).

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Protoplast Preparation. Based on lysozyme action in SMM (an hypertonic buffer, Wyrick and Rogers 1973), the protoplasting treatment has been applied as described (Schaeffer et al. 1976), unless specified otherwise.

Preparation of Plasmid DNA. A cleared lysate was first prepared according to Guillen et al. (1978) from 4×10^{10} MO215 protoplasts suspended in 16 ml of SMM. Pure plasmid DNA was then prepared from it following the method of Humphreys et al. (1975), which includes a concentration of the DNA by precipitation with a 10% PEG solution, and an isopycnic centrifugation followed (after dye removal from the heavier plasmid DNA band) by extensive dialysis against TE buffer (0.05 M Tris-HCl, 0.005 M EDTA, pH 8.2).

Preparation of Chromosomal DNA. Two types of chromosomal DNA preparation have been used in this work. Type I DNA was prepared from plasmid free 168 Trp^+ cells, according to a traditional procedure (Sonenshein et al. 1974). When it was suspected to contain harmful traces of detergent, an alternative procedure producing detergent-free chromosomal DNA from MO215 cells was worked out. In heavy protoplast suspensions such as that described above some protoplasts lyse spontaneously. After centrifugation the detergent-free supernatant can be used as a source of bacterial DNA, which is then concentrated and purified by density gradient centrifugation as described (Humphreys et al. 1975). DNA from the lighter band, after dye removal and final dialysis against TE buffer, is electrophoretically free of plasmid DNA, and is thereafter referred to as Type II chromosomal DNA.

DNA Assay. DNA was assayed either chemically (Burton 1956) or spectrophotometrically (Hotchkiss 1957).

Traditional Transformation. Transformation of competent bacteria was carried out as described by Sonenshein et al. 1974.

Polyethyleneglycol-Induced Protoplast Transformation. Some modifications suggested by our experience with protoplast fusion (Schaeffer et al. 1976) have been introduced in the procedure described by Chang and Cohen (1979). Grown in nutrient broth, bacteria were resuspended in SMM buffer to an O.D. at 570 nm of 2.0 (4×10^8 colony-forming units/ml); treatment by lysozyme (200 µg/ml) was reduced to 30 min at 37° C, with gentle shaking. Chromosomal or plasmid DNA, or their mixture, was added to 0.5 ml of protoplast suspension, at concentrations appearing in the Tables and Figures. This suspension was then exposed for 1 min to 0.5 ml of a 40% (wt/vol) PEG solution in SMM, and immediately plated onto the rich regeneration agar medium of Wyrick and Rogers (1973) containing 5 µg of DNase per ml. Regeneration rate after PEG treatment was calculated from colonies counted after a 3 day incubation at 37° C. Replica plating took place the same day, to either nutrient agar supplemented with chloramphenicol (20 µg/ml), or to variously supplemented minimal medium (Schaeffer et al. 1976). By this modified method, phenotypic expression of the transformants takes place on the regeneration medium. As shown in Fig. 1 the modifications introduced do not significantly affect the response to exposure to plasmid DNA. It has been checked that, as implied in Chang and Cohen's paper, plasmid transformation is completely dependant on PEG treatment.

Denaturation and Renaturation of DNA. Solutions of chromosomal DNA, 10 µg/ml in 2 × SSC (0.3 M NaCl, 0.03 M Na_3 citrate), were exposed to 100° C for 10 min, and then either immediately quenched at 0° C, or annealed at 65° C for 150 min, depending on whether denatured or renatured DNA was being prepared (Marmur et al. 1962).

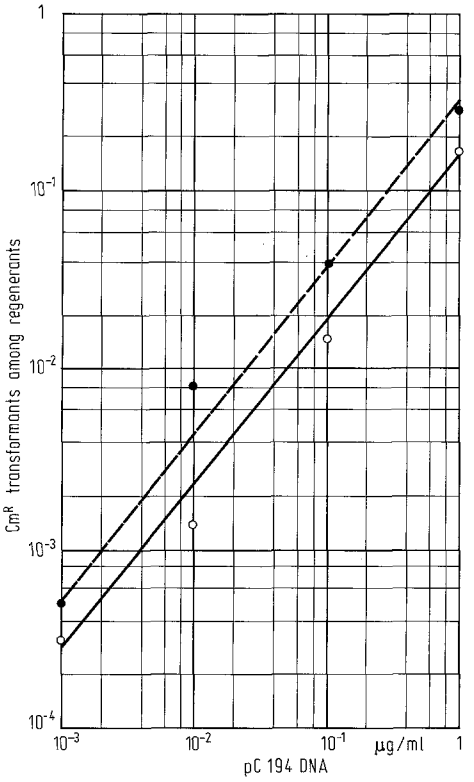

Fig. 1. PIP transformation as a function of pC194 DNA concentration. Protoplasts derived from growing bacteria were treated by varying concentrations of pC194 DNA. Regenerated bacteria were counted, and Cm^R transformants among them. Dotted line: the response published by Chang and Cohen, 1979. Solid line: the response obtained with S3 cells by the modified procedure described under Methods

Results

1. PIP Transformation with Type I Chromosomal DNA Preparations

When transformation of S3 protoplasts to Ade^+, or of S13 protoplasts to Leu^+ was tried, using Type I chromosomal DNA, transformants were formed (Fig. 2, curves 1 and 2, respectively). An unusual feature of these curves is that the yield of transformants (in the protoplast population which successfully returned to the bacterial form) decreased rather abruptly at DNA concentrations higher than 1–2 µg/ml, rather than staying constant as is usual with competent bacteria. With both strains a maximal yield of approximately 5×10^{-5} was observed for DNA concentrations close to 1 µg/ml, suggesting a toxicity of the Type I DNA preparation used.

When the same DNA preparation was used to transform S3 competent cells, however, increasing DNA concentration from 1 to 10 µg/ml did not

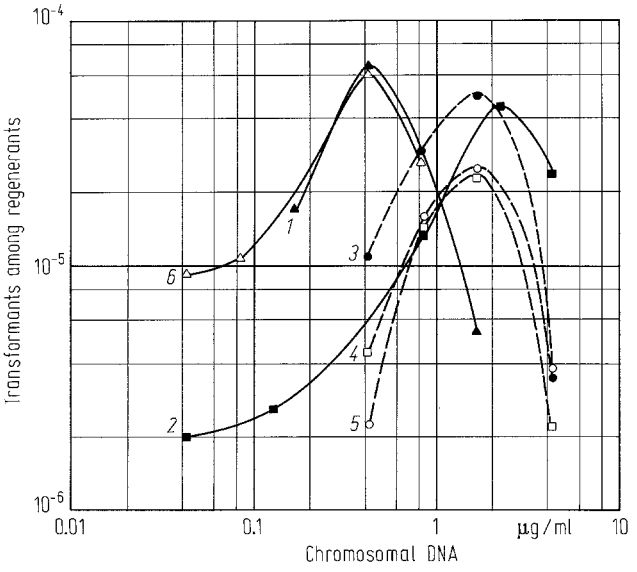

Fig. 2. PIP transformation as a function of chromosomal DNA concentration. Recipient strains and transformation procedure as described under Methods: Solid lines: transformations with Type I DNA. Dotted lines: transformations with Type II DNA. (*1*) ▲ S3 cells transformed to Ade^+; (*2*) ■ S13 cells transformed to Leu^+; (*3*) ● S13 cells transformed to Thr^+; (*4*) □ S13 cells transformed to Leu^+; (*5*) ○ S13 cells transformed to Met^+; (*6*) △ MO214 cells transformed to Ade^+. The protoplast regeneration data from the four experiments appear in Table 1. Curves 3, 4 and 5 are from the same experiment

change the efficiency of transformation (4×10^5 transformants per 1×10^8 bacteria at the competence peak). Thus, the inhibitory effect of DNA concentrations higher than 1 µg/ml is specific of PIP transformation.

In their description of the PIP transformation by plasmid DNA the reminder was given by Chang and Cohen (1979), that residual traces of detergent in the DNA preparation may be toxic to protoplasts. If this were the reason for the apparent toxicity observed here, the wall regeneration rate also should be affected. Since this apparently is not the case (Table 1), interference of excess DNA with some step of the transformation process is indicated. The data in Table 1 illustrate the extent to which regeneration rates fluctuate. Their average values in these experiments (23%, 12% and 17.5% for S3, S13 and MO214, respectively) do not show any clearcut strain-dependance.

2. PIP Transformation with Type II Chromosomal DNA Preparations

To rule out any effect due to detergents, a Type II DNA preparation was made. DNA prepared in this way has at no time been in contact with detergents (see Methods). Bell shaped curves similar to those already observed were obtained, however, when S13 protoplasts were transformed by Type II DNA (Fig. 2, curves 3, 4 and 5). Maximal yield is seen to depend slightly on the particular kind of transformants being scored, but the decrease in yield at supraoptimal concentrations is independant of the type of DNA preparation being used. As expected, wall regeneration is unaffected by Type II DNA concentration either (Table 1, experiments 3, 4 and 5).

3. Transformation for Chromosomal Markers of PEG-Treated Protoplasts Derived from Non-Transformable Bacteria

In order to be transformed by plasmid DNA, PEG-activated protoplasts need not be derived from competent, or even transformable bacteria (Bibb et al. 1978; Chang and Cohen 1979). Whether the same is true when chromosomal DNA is used instead (i.e. when transformation requires recombination) has been checked by using protoplasts from MO214, a non-transformable asporogenous mutant. As can be seen from experiment 6 (Fig. 2 and Table 1) the response with this strain is identical to that given by S3, which is transformable (experiment 1, Fig. 2 and Table 1).

4. Sensitivity of PIP Transformation for Chromosomal Markers to DNA Denaturation

In a classical transformation system, full activity of exogenous DNA requires that it be in its native, double-stranded form. This requirement is not due to unability of single-stranded DNA to carry out genetic transformation, but rather to the fact that under normal conditions competent bacteria do not take up denatured DNA (Marmur and Lane 1960; Lacks 1977). In the PIP transformation studied here, in which competent cells play no role, it seemed worth investigating how denaturation of chromosomal DNA would affect its biological activity.

Prepared as described under Methods, solutions of presumably denatured and renatured chromosomal DNA were made and immediately used, together with native DNA, in PIP transformation experiments at the nearly optimal concentration of 0.43 µg/ml. In two independant experiments, the transforming activity of denatured DNA was 1.3% and 3.5% that of native DNA, and renaturation increased the residual activity by a factor of three. Limited as it was, this reactiva-

Table 1. Regeneration rate of PEG-treated protoplasts used in transformation experiments

Experiment	Recipient strain	DNA µg/ml	Regeneration %
1	S3	0	15
		0.17	12
		0.43	30
		1.7	35
2	S13	0	11
		0.043	4
		0.13	14
		0.86	15
		2.15	34
		4.30	3
3, 4, 5	S13	0	15
		0.42	12.5
		0.85	50.8
		1.7	12
		4.25	11.5
6	MO214	0	12.5
		0.043	16
		0.086	16
		0.43	14
		0.86	29

The transformation data from the four experiments mentioned below are given in Fig. 2

Talbe 2. Lack of inhibition by plasmid DNA of PIP transformation by chromosomal DNA

Chromosomal DNA µg/ml	pC194 DNA µg/ml	Ade^+ transformants among regenerants
0.30	0	2.0×10^{-5}
0.30	3	2.7×10^{-5}

S3 protoplasts were transformed as described to Ade^+ by chromosomal DNA in suboptimal concentration in the presence of a tenfold excess of pC194 DNA

Table 3. Lack of inhibition by chromosomal DNA of PIP transformation by pC194 DNA

pC194 DNA µg/ml	Type I DNA µg/ml	Cm^R transformants among regenerants
0.02	0	0.66×10^{-3}
0.02	0.20	0.77×10^{-3}
0.20	0	5.6×10^{-3}
0.20	2.0	5.0×10^{-3}

pC194 DNA and Type I chromosomal DNA were added to S3 protoplasts. Transformation was tested after PEG treatment as described under Methods

tion insures that the previous inactivation was not (at least entirely) due to degradation. Thus in PIP transformation for chromosomal markers as in transformation of competent bacteria, biological activity requires that DNA be double-stranded.

5. Do Chromosomal and Plasmid DNA compete with One Another in PIP Transformation?

In the absence of PEG treatment protoplasts cannot be transformed by plasmid DNA. We checked that PIP transformation for chromosomal traits also was entirely dependant on exposure to PEG (data not shown). To see whether plasmid DNA competes with chromosomal DNA for transformation by the latter, a suspension of S3 protoplasts containing a suboptimal concentration (0.30 µg/ml) of chromosomal DNA and a tenfold excess of pC 194 DNA was PEG-treated, and Ade^+ transformant bacteria were scored in the usual way. No inhibition could be detected (Table 2). With the same system the same conclusion was reached in the converse experiment, when chloramphenicol-resistant (Cm^R) transformants were scored in the presence of a tenfold excess of chromosomal DNA (Table 3). Thus, in PIP transformation, there seems to be no competition for penetration between CCC DNA and linear native DNA.

Discussion

The PEG-induced transformation of *B. subtilis* protoplasts investigated in this work differs in three ways at least from that described by Chang and Cohen (1979): the transforming DNA used is linear and chromosomal rather than supercoiled and plasmidic, the transformation efficiency is much lower, and the response to varying DNA concentration shows a rather sharp and most unusual optimum (Fig. 2).

Transformation frequency (up to 5×10^{-5} among the regenerated bacteria) is ca. 10^4 times lower than with plasmid DNA. Since with chromosomal DNA only one chromosome segment (out of one hundred liberated by shearing from the bacterial genome) is potentially transforming for any one marker, a frequency reduced by a factor of 10^2 was to be expected. A further reduction of a similar magnitude may indicate either, that the unknown mechanism by which DNA is taken up in the presence of PEG is much more efficient with supercoiled DNA than it is with linear DNA, or that both forms are taken up equally well, but that linear DNA is more sensitive to inactivation within the protoplasts. (Inactivation of incoming transforming DNA has been shown to take place within minutes in *B. subtilis* protoplasts derived from non-competent bacteria, and ascribed to enzymatic activities present in such cells; Hirokawa and Ikeda

1966). Both possibilities are compatible with the observation (Chang and Cohen 1979) that pC194 DNA, once linearized, is from 10 to 100 times less active. Studying the penetration in the presence of PEG of radioactive DNAs, and their biological activity after reextraction, should supply the answers. In spite of its low frequency there is no doubt about the PEG-dependence of the PIP transformation studied in this work, since in agreement with previous work (Tichy and Landman 1969; Wilson and Bott 1970) no transformants are formed at all in the absence of PEG treatment.

The second salient characteristic of the PIP-transformation investigated is the abrupt decrease in transformant yield regularly observed, when DNA concentrations exceeding 1–2 μg/ml are used (Fig. 2). A similar but much smaller decrease was observed by Chang and Cohen (1979) working with pC194 DNA, from which residual detergent had been removed by extensive dialysis. Since the effect in our case was much more pronounced and occured at lower DNA concentration, we were led to prepare chromosomal DNA which at not time had been exposed to detergent (Type II preparation). As shown in Fig. 2, the transformation-inhibiting activity was still there, however. Introducing additional deproteinization steps with proteinase K or additional RNase treatments did not help either. In transformation of competent cells, however, DNA concentration of 10 μg/ml was not inhibitory. Thus, the effect seems to be due to DNA itself, acting specifically on some step (possibly recombination) of the PIP transformation process. It seemed possible that the failure of Chang and Cohen (1979) to detect transformation for a chromosomal marker might be due to their apparently exclusive use of too high a DNA concentration (2 μg/ml), but we also had to consider the possibility that the modifications introduced by these authors in the protoplasting treatment of Schaeffer et al. (1976) might account for the failure. Using the DNA concentration optimal for S3 we found, however, that protoplasts prepared by both methods were transformed, and that in both cases transformation was strictly PEG-dependant.

Recently reported by Vorobjeva et al. (1980), PEG-induced transformation of *Bacillus megaterium* protoplasts by DNA of a megacinogenic plasmid takes place at very high frequency, but no PIP-transformation could be detected when chromosomal *B. megaterium* DNA was used. In the absence of any experimental detail the latter observation cannot be usefully commented upon.

PIP transformation by plasmid DNA takes place normally with protoplasts derived from a non-transformable species of *Streptomyces* (Bibb et al. 1978). Similarly in this work protoplasts from a non-transformable mutant of *B. subtilis* could be transformed for chromosomal traits in the presence of PEG, just as well as protoplasts from the transformable parent strain. This may be valuable information for investigators working with species of bacteria from which protoplasts can easily be made, but in which no genetic transfer is available so far. They also strongly indicate that PEG, a powerful fusogenic agent, is able also to 'squeeze' DNA into cells or membranes, whether this DNA is supercoiled or linear and native. This PEG-dependant mechanism could hardly be identical with the physiological take-up mechanism of competent bacteria, which requires the synthesis of several specific proteins, and results in one strand being degraded while the other strand is taken in (Lacks 1977). It was therefore of interest to know whether, as a result of PEG action, single-stranded DNA also was squeezed in. With chromosomal DNA we found that double-strandedness is required for PIP transformation, as it is for conventional transformation, although it needs not be for the same reason. The data presented do not exclude that duplex DNA is taken in but subsequently degraded. The answer to this question, should be forthcoming when use is made of radioactive DNA.

Inhibition experiments carried out to see whether chromosomal and plasmid DNA compete with one another for transformation failed to detect any such competition. Since these two DNAs could hardly compete for pairing with the chromosome we trust that competition for entry was being tested. Our understanding is that the capacity of the PEG to let DNA, whatever its molecular structure, is so great that it was not limiting in these experiments.

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References

Bibb MJ, Ward JM, Hopwood DA (1978) Transformation of plasmid DNA into *Streptomyces* at high frequency. Nature 274:393–400

Burton K (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochemistry J 62:315–323

Chang S, Cohen SN (1979) High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol Gen Genet 168:111–115

Ehrlich SD (1978) DNA cloning in *Bacillus subtilis*. Proc Natl Acad Sci USA 75:1433–1436

Fodor K, Alföldi L (1976) Fusion of protoplasts of *Bacillus megaterium* Proc Natl Acad Sci USA 73:2147–2150

Guillen N, Le Hégarat F, Fleury AM, Hirschbein L (1978) Folded chromosomes of vegetative *Bacillus subtilis:* composition and properties. Nucl Acid Res 5:475–489

Hirokawa H, Ikeda Y (1966) Genetic recombination of transforming deoxyribonucleic acid molecules with the recipient genome and among themselves in protoplasts of *Bacillus subtilis*. J Bacteriol 92:455–463

Hopwood DA, Wright HM, Bibb MJ, Cohen SN (1977) Genetic recombination through protoplast fusion in *Streptomyces*. Nature 268:171–174

Hotchkiss RD (1957) Methods for Characterization of nucleic acid. In: Colowick SP and Kaplan NO (eds) Methods in Enzymology, vol III. Academic Press Inc, New York, p 708

Humphreys GO, Willishaw GA, Anderson ES (1975) A simple method for the preparation of large quantities of pure plasmid DNA. Biochim Biophys Acta 383:457–463

Ionesco H, Michel J, Cami B, Schaeffer P (1970) Genetics of sporulation in *Bacillus subtilis* Marburg. J Appl Bacteriol 33:13–24

Lacks SA (1977) Binding and entry of DNA in bacterial transformation. In: Reissig JL (ed) Microbial interactions, receptors and recognition, series B, vol 3. Chapman and Hall, London, p 179

Lévi C, Sanchez-Rivas C, Schaeffer P (1977) Further genetic studies on the fusion of bacterial protoplasts. FEMS Microbiol Letters 2:323–326

Marmur J, Lane D (1960) Strand separation and specific recombination in DNA's: biological studies. Proc Natl Acad Sci USA 46:453–461

Marmur J, Schildkraut CL, Doty P (1962) Biological and physical chemical aspects of reversible denaturation of deoxyribonucleic acids. In: The molecular basis of neoplasia. University of Texas Press, Austin, p 9

Schaeffer P, Cami B, Hotchkiss RD (1976) Fusion of bacterial protoplasts. Proc Natl Acad Sci USA 73:2151–2155

Sonenshein AL, Cami B, Brevet J, Cote R (1974) Isolation and characterization of rifampicin-resistant and streptolydigin-resistant mutants of *Bacillus subtilis* with altered sporulation properties. J Bacteriol 120:253–265

Tichy P, Landman OE (1969) Transformation in quasi spheroplasts of *Bacillus subtilis*. J Bacteriol 97:42–51

Vorobjeva IP, Khmel IA, Alföldi L (1980) Transformation of *Bacillus megaterium* protoplasts by plasmid DNA. FEMS Microbiol Letters 7:261–263

Wilson GA, Bott KF (1970) Effects of lysozyme on competence for *Bacillus subtilis* transfection. Biochim Biophys Acta 199:464–475

Wyrick PB, Rogers HJ (1973) Isolation and characterization of cellwall defective variants of *Bacillus subtilis*. J Bacteriol 116:456–465

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