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Uptake of Synthetic Polynucleotides by Competent Cells of *Bacillus subtilis*

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A survey was made of the capacity of competent cells of *Bacillus subtilis* to take up synthetic polynucleotides. Polydeoxyribonucleotides but not polyribonucleotides are taken up by the cells. Both types of polynucleotide failed to compete with transforming deoxyribonucleic acid in the transformation assay whereas both stimulated amino acid incorporation. The latter phenomenon appears to be nonspecific as there is no correlation between the codon composition of the polynucleotides employed and the amino acids whose incorporation was stimulated.

Hurwitz et al. (6) and Evans (2) showed that deoxyribonucleic acid (DNA)-ribonucleic acid (RNA) hybrid complexes are capable of conferring the sulfonamide resistance phenotype upon sensitive recipient cells of *Diplococcus pneumoniae*. More recently Kirtikar and Duerksen (7) reported the induction of penicillinase by treating cultures of *Bacillus cereus* and *Staphylococcus aureus* with RNA prepared from a penicillinase constitutive strain of *B. cereus*. Amos et al. (1) presented evidence favoring the contention that in "soniplasts," prepared from *Escherichia coli*, polyuridilic acid (poly-rU) specifically stimulates the incorporation of phenylalanine but not of leucine.

This paper reports the negative results of diligent efforts to demonstrate the uptake of "synthetic messenger" polynucleotides by competent cells of *B. subtilis*. Rather than discard the present results without mention, we present them to illustrate some potential artifacts and save time for others who may have more ingenious approaches to the problem.

MATERIALS AND METHODS

B. subtilis strain SB893, an arginine and xanthine derivative of the indole-requiring strain 168M, was used. The cells were made competent and were stored and utilized as reported by Stewart (11). DNA was extracted by the Marmur's procedure (9) from the wild-type *B. subtilis* SB850. Uptake of labeled polynucleotides was measured on suspensions of competent cells in Spizizen minimal medium (10) containing 0.5% glucose, at a concentration of 2×10^7 to 4×10^7 cells/ml. After the addition of the labeled polynucleotide, 0.2- to 0.5-ml samples of cell suspension were withdrawn at different time intervals filtered on membrane filters (Millipore Corp., Bedford, Mass.), washed with cold Spizizen medium, dried, and counted.

Incorporation of labeled amino acids into cold acid-insoluble material was determined on cell suspension identical to those reported for the experiments on the uptake of polynucleotides except that samples of the cell suspension were pipetted into 0.2 to 0.5 ml of 10% trichloroacetic acid. The samples were left for at least 30 min at 0 C, filtered through membrane filters, dried, and counted. All experiments were performed on cultures incubated at 37 C on a rotary shaker.

Chemicals. Unlabeled polyadenilic acid (poly-rA), poly-rU, polycytidylic acid (poly-rC), and polyinosinic acid (poly-rI), as well as tritiated, randomly labeled polyadenilic acid, and polyuridilic acid tritiated in the uracil-5-position (specific activity, 64.4 and 29.9 mc/mmole, respectively) were from Miles Laboratories, Inc. Tritiated polydeoxyadenilic-thymidilic (poly-dAdT) and polydeoxycytidylic-polydeoxyguanilic acid (poly-dCdG) (specific activity, 25 mCi per mmole in each case) were from Biopolymers Inc. The molecular weight of both polynucleotides was estimated by sucrose density gradient to be about 2×10^5 to 3×10^5 daltons. ^{14}C-phenylalanine (393 mCi/mmole), ^{14}C-lysine (247 mCi/mmole), and ^{14}C-arginine (173 mCi/mmole) were from Calbiochem, Los Angeles, Calif.

Polyuridilic-polyadenilic acid (poly-rUrA) was prepared by mixing equimolecular amounts ($\simeq$500 μg/ml of each) of the polyribonucleotides in 0.1 M NaCl for 15 min at 70 C and cooling slowly to room temperature. Hybrid formation was ascertained by determining the melting temperature that in 0.1 M phosphate buffer (*p*H 7.8) was estimated to be 52 C. The label was either in the poly-rU or poly-rA moiety.

Poly-rU:DNA was prepared as described by Kubinski et al. (8). Formation of the hybrid was assessed by the decrease in transforming activity as

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compared to that of DNA renaturated in the absence of poly-rU. The label was in the poly-rU. Polyadenilic-polydeoxythymidilic acid (poly-rAdT) was prepared by mixing 0.1 ml of ^{3}H-poly-rA (1 μCi) in 0.4 ml of 0.2 M phosphate buffer (*p*H 7.8) with 0.5 ml of poly-dT at 10 μg/ml in 10^{-2} M tris(hydroxymethyl)amino-methane-hydrochloride buffer (*p*H 7) at 28 C. Label was in the poly-rA. Poly-dT was generously supplied by R. Lehman.

RESULTS

The label from the polyribonucleotides tested (poly-rA, poly-rU, poly-rUrA, poly-rAdT, poly-rU:DNA) was not taken up to a significant extent by the cells under any of our conditions. However, a certain amount of radioactivity was recovered with the cells, but the reaction did not show any time dependency so that it could be ascribed to binding of the polymers to the cell surface or to the uptake of contaminating oligomers, or both. Indeed, in the case of poly-rU, treatment of the cells with ribonuclease after exposure to radioactive poly-rU resulted in almost complete loss of the radioactivity which was bound to the cells. Similar results were obtained in the case of hybrids between one ribopolynucleotide and one deoxyribopolynucleotide such as poly-rAdT or poly-rU:DNA. On the contrary, poly-dAdT and poly-dCdG appear to be taken up significantly with kinetics which are time dependent (Fig. 1).

Attempts were made to alter cell permeability by pretreating the cells with 5% dimethylsulfoxide for 60 min at 37 C or 10^{-2} M ethylenediaminetetraacetic acid (EDTA), or by incubating cells with lysozyme (10 μg/ml). No significant uptake was observed under such conditions, nor in experiments in which protein synthesis was inhibited by pretreating the cells with puromycin or chloramphenicol nor when polysome depolymerization was favored by depriving cells of magnesium or by treating with sodium fluoride.

The same type of experiment employing radioactive poly-rU and poly-rA was performed with equally unsuccessful results on the following bacteria: *E. coli*, *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Proteus vulgaris*, *Alcaligenes foecalis*, and *Corynebacterium xerose*.

Stimulation of amino acid incorporation. Notwithstanding the negative results reported in the previous section, it could be argued that some of the tested polynucleotides were taken up by the cells to a very small extent. This could be tested by using auxotrophic cells of *B. subtilis* in which protein synthesis is blocked by deprivation of the required amino acid. Under such conditions, the incorporation of amino acids into acid-insoluble material might be stimulated specifically by the synthetic polynucleotides acting as messenger RNA. In the case of poly-rU, for instance, stimulation of phenylalanine incorporation should be evident, whereas stimulation of lysine incorporation should be brought about by the addition of poly-rA. The incorporation of both amino acids should be stimulated when poly-rUrA is employed. An obvious control is the study of the incorporation of a different amino acid which is not coded for by the synthetic polynucleotides employed, e.g., arginine, whose codons are rich in cytidine and guanosine and poor in uridine and adenosine.

Addition of poly-rU resulted in stimulation of

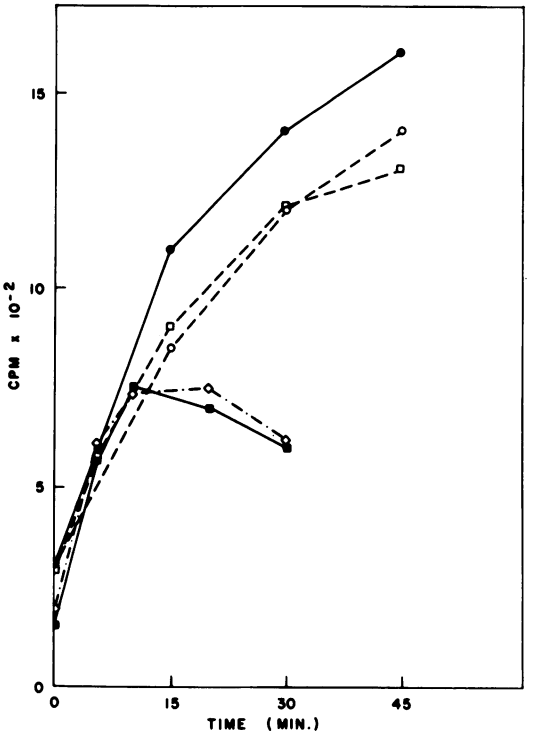

FIG. 1. *Uptake of synthetic deoxyribonucleotides by competent cells of B. subtilis. ^{3}H-poly-dAdT or ^{3}H-poly-dCdG were added at a concentration of 0.1 μg per ml per 8.5 × 10^7 cells to a cell suspension containing: ●, poly-dAT; ○, poly-dAdT plus B. subtilis DNA (1:1); □, poly-dAdT plus adenine plus thymine (each base at a concentration of 20 μg/ml); ■, poly-dCdG; ◇, poly-dCdG plus B. subtilis DNA (1:1). Transforming DNA from wild-type B. subtilis was used at a concentration of 0.1 μg/ml. A 3.5-ml amount of competent cells was added to the mixtures of polynucleotides. At the shown time intervals, 0.4-ml samples were filtered on membrane filters and extensively washed with cold Spizizen medium. The filters were then dried, and the radioactivity was determined.*

the incorporation of phenylalanine in trichloroacetic acid-insoluble material as compared to the control containing no polynucleotides (Fig. 2). However, the incorporation of lysine was also stimulated. The same results were obtained for the incorporation of phenylalanine and arginine in the experiments employing poly-rArU, whereas the incorporation of either lysine or phenylalanine was not stimulated by poly-rA. Such results indicate that the addition of poly-rU brings about a nonspecific stimulation of amino acid incorporation. This could be due to the presence of small amounts of oligomers or mononucleotides present in the polynucleotide preparations or, alternatively, generated upon incubation with the cells. This is substantiated by experiments not reported here, showing that sheared polynucleotides stimulate the incorporation of amino acids much more than intact polynucleotides. [Firshein et al. (3–5) have extensively investigated the stimulation of endogenous synthesis in *Pneumococcus* by DNA and RNA digests as well as by synthetic polynucleotides.]

In the case of the hybrid poly-rAdT, stimulation of amino acid incorporation was observed, but again not of the amino acids that could be coded for by either poly-rA or poly-dT. Similar results were also obtained in the case of the hybrid formed between single-stranded DNA and poly-rU.

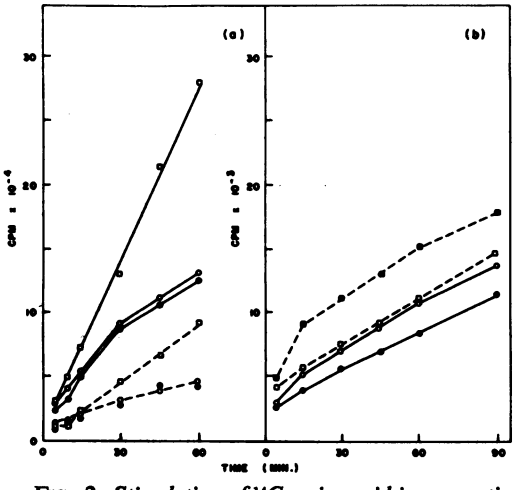

FIG. 2. *Stimulation of ^{14}C-amino acid incorporation in the presence of synthetic polyribonucleotides. (a) Solid lines, ^{14}C-phenylalanine (1.5 μCi/ml); dashed lines, ^{14}C-lysine (1.5 μCi/ml) were added to competent cells previously washed and suspended in Spizizen minimal medium (10) containing 0.5% glucose. The trichloroacetic acid-insoluble counts were determined on 0.2-ml samples of cell suspension withdrawn at the time intervals indicated. ○, No addition; ●, plus 100 μg of poly-rA per ml; □, plus 100 μg of poly-rU per ml. (b) Dashed lines, ^{14}C-phenylalanine (0.25 μCi/ml) as in (a) in the presence (■) or in the absence (□) of 100 μCi of poly-rUrA per ml; solid line, ^{14}C-arginine (0.25 μCi/ml) in the presence (○) or in the absence (●) of 100 μg of poly-rUrA per ml.*

Such results further argue against the hypothesis that the tested polynucleotides are taken up to a significant extent by the cells or that, if taken up, they are utilized as specific messenger RNA for protein synthesis. The stimulation of the incorporation of amino acids brought about by the tested polynucleotides should then be ascribed to an aspecific stimulation of protein synthesis caused by some of these polynucleotides or by the products of their degradation.

In the case of poly-dAdT, a polynucleotide that is taken up by competent cells of *B. subtilis*, no stimulation was observed in the incorporation of tyrosine (one codon of this amino acid is UAU). In this case, however, it may be postulated that transcription of the synthetic deoxypolynucleotide did not take place.

Competition between DNA and synthetic polynucleotides. A further control on the possible uptake of the synthetic polynucleotides was made by studying the competition between these polymers and transforming DNA. If synthetic polynucleotides are taken up by the same mechanism by which DNA is incorporated during transformation, competition should be observed between natural and synthetic polynucleotides. Likewise, competition between synthetic polynucleotides and DNA should be observed if the binding of synthetic polynucleotides to cells takes place at the same sites through which DNA enters the cells.

Addition of poly-rU, poly-rA, and poly-rUrA at the same time as DNA does not decrease transformation, even when the synthetic polynucleotides are employed at a concentration 100-fold higher than that of the transforming DNA (Table 1). Addition of poly-rUrA to the suspension of competent cells before the addition of DNA has little, if any, effect on transformation (Table 2). Indeed, a 1,000-fold excess of hybrid, even when added 10 min before DNA, does not significantly decrease transformation. It may be added that no competition with DNA for transformation was observed by employing the following polynucleotides at concentrations that were, in certain experiments, 1,000-fold higher than that of DNA:poly-rA, poly-rU, poly-rArU, poly-rC, poly-rI, poly-rCrI, poly-rAdT, poly-dCdG, poly-dAdT, soluble RNA, and ribosomal RNA. On the contrary, addition of different concentrations of heterologous DNA (from calf thymus) reduces stoichiometrically the percentage

TABLE 1. *Effect of the addition of synthetic polyribonucleotides on transformation*[a]

Polyribonucleotide concn (μg/ml)	Transformation (‰)		
	poly-rU	poly-rA	poly-rUrA
0	4.0	4.0	4.0
1	4.2	4.5	3.6
10	4.0	4.1	3.9
100	3.9	4.0	4.9

[a] Competent cells were prepared. DNA from wild-type *B. subtilis* was added at a concentration of 1 μg/ml, and the cells incubated at 37 C for 20 min in a rotary shaker. The reaction was stopped by addition of 10 μg of deoxyribonuclease per ml followed by incubation for 10 min at 37 C. Transformation was started by adding the cells to the mixture containing the nucleic acids.

TABLE 2. *Effect of the time of addition of poly-rUrA on transformation*[a]

poly-rUrA concn (μg/ml)	Transformation (‰): poly-rUrA added at		
	−10 min[b]	0 min	+10 min
0	2.28	1.25	1.12
10	2.50	0.93	0.74
100	2.05	1.22	0.67
500	1.84	0.94	0.87
1,000	2.15	0.92	0.92

[a] Experimental conditions reported in Table 1.
[b] Respect to the time of addition of DNA.

of transformants. It remains to be explained why poly-dCdG and poly-dAdT which are taken up by competent cells (Fig. 1) do not compete with DNA for transformation (Fig. 3). The phenomenon may perhaps be explained by the presence of different sites of entrance for the deoxypolyribonucleotides and for DNA or by a difference in the uptake of these polynucleotides by competent and noncompetent cells. Against this last hypothesis is the finding that the uptake of poly-dAdT and poly-dCdG is greater when the competence is higher, indicating that only the competent cells are responsible for their uptake. These observations suggest that natural DNA competes at a level other than the uptake step that is possibly the same for the synthetic deoxyribopolynucleotides. The higher molecular weight of the natural DNA (approximately 100 times) may be one of the discriminating factors.

DISCUSSION

The data reported indicate that synthetic deoxyribopolynucleotides are taken up by competent cells of *B. subtilis* possibly with the same mechanism by which natural DNA is taken up by these cells. This is not the case for synthetic ribopolynucleotides of either single- or double-stranded structure. Such findings indicate the existence of a highly selective uptake system in competent cells of *B. subtilis*. The mechanism for the uptake of deoxyribonucleotides appears to be so selective that a 1,000-fold excess of polyribonucleotides does not interfere with the transformation process.

Although the polyribonucleotides are not significantly taken up by the cells, it has been found that most of the polymers tested, especially poly-rU, stimulate amino acid incorporation. However, this is not a specific effect since no correlation exists between the codon present in the polyribonucleotide used and the amino acids that are incorporated. This result is in contrast with the data reported by Amos et al. (1) for *E. coli* soniplasts in which poly-rU appears to stimulate

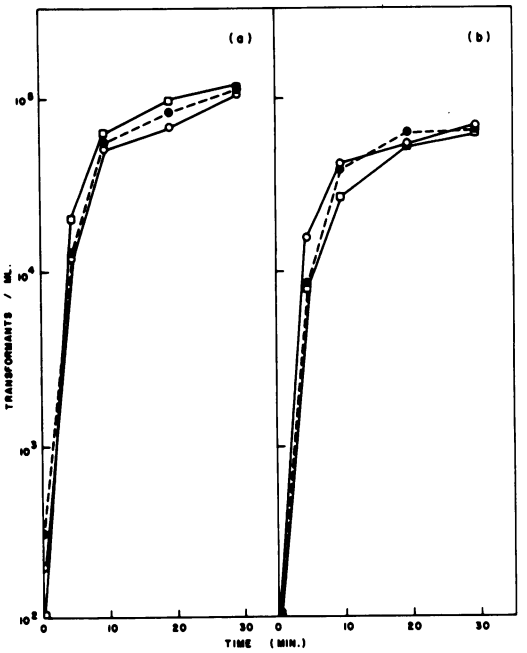

FIG. 3. *Effect of the addition of poly-dCdG and poly-dAT on transformation. Experimental conditions as in Table 1 except that the concentration of transforming DNA was 0.1 μg/ml of cell suspension. At the shown time intervals, 0.1 ml of cell suspension was pipetted into 0.4 ml of Spizizen medium containing 10^{-2} M Mg^{++} and 5 μg of deoxyribonuclease per ml, and incubated for 10 min at 37 C. Tryptophan transformants were scored. (a) ●, DNA at 0.1 μg/ml; ○, 0.1 μg of DNA plus 0.1 μg of poly-dAdT per ml; □, 0.1 μg of DNA plus 1 μg of poly-dAdT per ml. (b) Same as (a) except that poly-dCdG was used in place of poly-dAdT.*

specifically the incorporation of phenylalanine. However, they did not deal with the uptake of poly-rU nor of any other synthetic or natural polynucleotides such as DNA.

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