EXTENT AND SIGNIFICANCE OF CONTAMINATION OF DNA BY TEICHOIC ACID IN BACILLUS SUBTILIS*

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In the early studies of genetic transformation in Pneumococcus modification of the capsular polysaccharide was the first genetic marker utilized. Hence it was extremely important to remove polysaccharide from preparations of DNA and to demonstrate that polysaccharide was not responsible for genetic transformation. The classical study of Avery and coworkers (1944) clearly demonstrated that DNA rather than polysaccharide was responsible for genetic transformation. Although investigators such as Kirby (1964) developed numerous techniques to aid in the removal of polysaccharide from DNA, the concern for contamination of DNA with polysaccharide has diminished to the extent that most workers do not use enzymes to remove polysaccharide or employ assays which would detect polysaccharide. A renewed interest in the effect of polysaccharide on transformation in Bacillus subtilis was stimulated by the observations that the major difference in the chemical composition of cell walls of the highly and poorly transformable strains of B. subtilis resided in the content of N-acyl galactosamine isolated with the teichoic acid[1] fraction of the cell wall (Young, et al, 1963 and 1964, Young, 1965). Conceivably the first step in the adsorption of DNA might be a complex mediated by divalent cations between the terminal phosphorus groups of teichoic acid (TA) on the cell wall and DNA. Furthermore, both Pneumococcus (Liu and Gotschlich, 1963) and

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[1] TA, a polymer of either ribitol phosphate (Armstrong et al, 1961) or glycerol phosphate (Burger and Glaser, 1964) constitutes approximately 50-55% of the mass of the cell wall of B. subtilis. The polyol is frequently substituted with D alanine, (Armstrong et al, 1961), and glucose (Armstrong et al, 1961). In addition TA contains amino sugars (Young, et al, 1964).

B. subtilis (Armstrong et al, 1961) contain phosphorylated polysaccharide which could contribute spurious radioactivity to preparations of ^{32}P-labeled DNA. Finally, carbohydrate from TA could produce erroneous conclusions regarding the carbohydrate content of DNA if a significant amount of TA contaminated DNA. Because TA is readily solubilized by lysozyme, centrifugation of protoplasts to remove solubilized TA or purification of the lysate would be essential to remove TA from preparations of DNA.

The following study was initiated to ascertain the extent and significance of contamination of DNA by TA. The data demonstrate that TA is not completely removed from DNA by the usual methods of precipitation with alcohol, by extraction with phenol, or by density gradient centrifugation. TA can be separated from DNA by column chromatography on 4% agarose, or kieselguhr impregnated with methylated serum albumin (MAK). Unlike DNA, TA is soluble in cold perchloric acid. The addition of purified TA does not appear to influence the development of competence or the uptake of DNA by competent cells. TA does not penetrate the cell with DNA during transformation.

Methods: B. subtilis 168 I^-C^+ (Young et al, 1963) and a thymine auxotroph of this strain were utilized. The procedures for genetic transformation (Young and Spizizen, 1961), preparation of DNA (Young and Spizizen, 1961) and preparation of TA (Young, et al, 1964) were in general similar to those employed previously. Cultures of B. subtilis 168 I^-C^+ were grown for 6.5 hours at 37° with vigorous aeration in minimal minus phosphate medium (Young and Spizizen, 1963) containing 0.63 µcurie per ml of ^{32}P, 1 mM potassium phosphate buffer pH 7.0, 24 mM L-tryptophan, 22 mM glucose, 5 mM $MgSO_4$ and 0.02% casein hydrolysate (M-P medium). Half of the culture was used for the preparation of TA. The other half was sequentially treated with lysozyme, RNase, trypsin, and desoxycholate (Young and Spizizen, 1961) and then dialyzed for 48 hours against 100 volumes of 0.15 M NaCl containing 0.015 M sodium citrate. If RNA was still present the process was repeated. DNA labeled with ^3H and ^{32}P was prepared from cultures of the thymine auxotroph grown for 6.5 hours in M-P medium supplemented with 10 µg/ml thymidine and 1.25 µcuries/ml ^3H thymidine (New England Nuclear Company).

Samples of TA and DNA were chromatographed on MAK (Sueoka and Cheng, 1962), P-200 (Bio-Rad Laboratories), and 4% agarose (Pharmacia Uppsula, Sweden). The amount of phosphorus (Lowry et al, 1954), DNA (Burton, 1956), RNA (Young and Spizizen, 1961) and the optical density at 220 and 260 mµ was determined on aliquots of each fraction from the agarose column. The radioactivity was assayed in a Packard scintillation spectrophotometer in vials containg 15 ml of naphthalene-dioxane scintillant (Harrington, 1964) which

was modified to contain 0.073% 1,4-bis-2 (4-methyl- 5 phenyloxazolyl) benzene, 6.25% methanol and 4% cabosil. The distribution of DNA and ^{32}P-labeled TA in a cesium chloride gradient was determined after centrifugation for 60 hours at 37,500 rpm in a SW 39 rotor in a Spinco L-2 ultracentrifuge by puncturing the bottom of the tubes and collecting 3 drop fractions.

The acid precipitability of DNA, TA, and lysates was determined by mixing aliquots of each with 30 μg of carrier DNA, 3 μg of bovine serum albumin and 2.0 ml of 0.6 N $HClO_4$ at 4°. After 5 minutes the samples were centrifuged at 10,000 x g for 10 minutes at 4° and the supernatant liquid decanted. The pellet was resuspended in 2.0 ml of 0.6 N $HClO_4$ at 4° and recentrifuged. After an additional wash the radioactivity of the pooled supernatant liquids and the precipitate resuspended in 1.0 ml of 1 N ammonium hydroxide was determined in a Packard scintillation spectrophotometer.

Results and Discussion: The influence of TA on the development of competence was explored by adding TA to the cells at various intervals prior to the onset of maximal competence. TA did not influence the development of competence or the incorporation of DNA and did not enter the cells during the period of competence (Table 1).

TABLE 1: Effect of Teichoic Acid on Transformation

Additions	Time of Addition		Frequency of Transformation	Uptake of TA
	TA	DNA		
	min	min	% x 10^{-2}	cpm/ml
TA + DNA	0	75	2.8	0
TA + DNA	30	75	2.8	0
TA + DNA	60	75	2.3	0
TA + DNA	75	75	3.2	0.8
DNA	-	75	3.0	-
TA	75	-	-	0.1

Cultures of B. subtilis were grown for 5 hours in growth medium, centrifuged, resuspended in transformation medium containing 15% dimethyl sulfoxide and storred at -60°. The frozen cultures were thawed slowly, centrifuged, and resuspended in 1.0 ml aliquots of transformation medium. The cells were incubated for 75 minutes at 35° with vigorous shaking. Maximal competence develops after 75 minutes of incubation in the transformation medium. At various intervals 100 μg of TA (specific activity 795 cpm/μg) was added. DNA (0.25 μg) was added to the appropriate tubes after 75 minutes of incubation. After an additional incubation of 30 minutes at 35° the reaction was terminated with DNase (50 μg/ml). An aliquot was removed for the assay of transformation. The cells (2 x 10^8/ml) were washed by three successive centrifugations with cold minimal medium containing 22 mM glucose, transferred to a millipore filter disc, and the radioactivity of the disc determined.

In B. subtilis 93 to 99% of the TA is acid soluble. The degree of acid solubility is not influenced by the presence of DNA (Table 2), thus the amount of acid soluble phosphorus in preparations of DNA which are devoid of RNA and nucleotides is a good index of the extent of contamination with TA. Two alcohol precipitations did not separate all of the TA from DNA in either mixtures of purified TA and purified DNA or in lysates containing DNA and TA (Tables 2 and 3). Isopropanol was more effective than ethanol in removing TA, however, greater losses of DNA were encountered (Table 3). The loss of some DNA at the interface during phenol extraction results in a relative enrichment of TA in the aqueous phase as evidenced by a lower acid insolubility (Table 3).

TABLE 2: Co-precipitability of purified ^{32}P-TA with DNA

Sample	Radioactivity	
	Residual	Acid insoluble
	%	%
^{32}P-TA	100	6.4
^{32}P-TA + DNA	100	5.5
1st EP	31.9	7.2
2nd EP	12.3	3.1
1st IP	8.5	8.8
2nd IP	1.3	-

A mixture of ^{32}P-TA (1000 µg) and DNA (2832 µg) was divided into two aliquots. One was precipitated in ethanol (Young and Spizizen, 1961) designated EP, and the other in isopropanol (Marmur, 1961) designated IP. The percent of the initial radioactive TA and the percent of ^{32}P-TA insoluble in perchloric acid were calculated.

TABLE 3: Contamination of ^{32}P-DNA with ^{32}P-TA in preparations of DNA

Sample	Radioactivity	
	Residual	Acid insoluble
	%	%
Standard Lysate	100	57.6
1st EP	54.3	62.5
2nd EP	37.2	77.8
1st IP	26.5	96.0
2nd IP	19.7	99.0
1st PE	49.5	41.2
2nd PE	48.5	35.0

A lysate containing DNA (186 µg/ml) and TA was divided into three aliquots. One aliquot was precipitated in ethanol, another in isopropanol (cf Table 2) and the third extracted with phenol (Massey and Zimm, 1965) designated PE. The percent of the initial radioactivity and the extent of acid precipitability were calculated.

TA can be completely separated from DNA in artificial mixtures and in lysates by chromatography on 4% agarose. The DNA is excluded from the gel while TA which has a molecular weight below 2 million is retarded (Figures 1 and 2). The small peak of radioactive ^{3}H contained in tubes 55-58 (Fig 2) and the asymmetry in the peak of ^{32}P in this region is due to traces of deoxyribonucleotides in the lysate. A partial separation of DNA and TA can be achieved on P-200, however, the descending portion of the DNA peak is usually contamined with TA. Because TA does not absorb at 260 mµ it can be detected only by its optical density at 220 mµ, its high phosphorus content, or radioactivity. The

ratio of specific activity of DNA to TA was determined by comparing the specific activity of the chromatographically pure DNA (cpm/μmole phosphorus) with the specific activity of the phosphorus in purified TA. The specific activity of DNA was usually 4 to 5 fold higher than TA. Chromatography on MAK also completely separates TA, which is voided immediately, from the DNA, which elutes usually with 0.4 to 0.5 M salt.

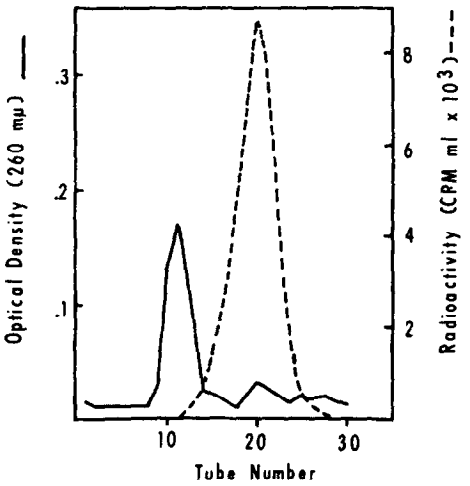

Figure 1. (left) Chromatography of TA and DNA. A 0.6 ml sample containing 71 μg of DNA and 70 μg of ^{32}P-labeled TA was added to a 50 x 1 cm column of 4% agarose and eluted with 0.01 M tris buffer (pH 7.1) at a flow rate of 15.5 ml/hr. The recovery of DNA and TA in the 1.2 ml fractions was 85% and 84% respectively.

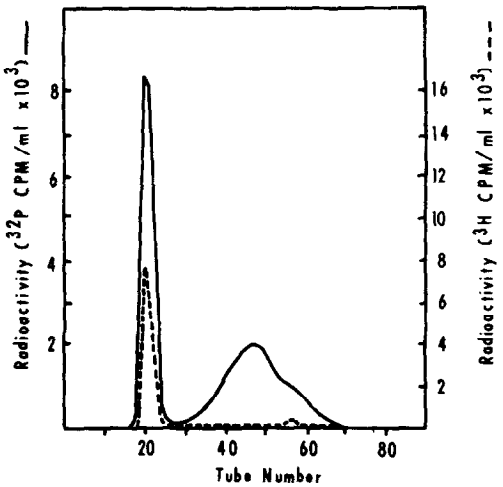

Figure 2. (right) Chromatography of a lysate on agarose. A 2.0 ml aliquot of a lysate containing 141 μg ^{3}H, ^{32}P-labeled DNA and ^{32}P-labeled TA was added to a 20 x 2 cm column containing 4% agarose and eluted with 0.01 M tris buffer (pH 7.1) at a flow rate 38 ml/hr. The acid precipitability of the ^{32}P in the starting material, tube 20 and tube 43 was 42%, 97%, and 7% respectively.

TA does not form a sharp band in density gradients (Figure 3). The extensive heterogeneity prohibits a reliable estimation of buoyant density. Comparison of the buoyant density of DNA, TA and mixtures of DNA and TA demonstrated that the positions of DNA and TA in a cesium chloride gradient are independent of each other.

Contamination of DNA by TA does not influence the transforming activity of DNA nor does TA from B. subtilis penetrate the cells with DNA during transformation of either B. subtilis or Pneumococcus (H. Ephrussi-Taylor personal communication). Furthermore an irreversible complex was not formed between DNA and TA in vitro. The most significant

anomalies would result from the study of the carbohydrate content of DNA which was contamined with TA and the use of lysates of cells labeled with ^{32}P in density gradient studies. The presence of TA in lysates of B. subtilis is readily detected by its acid solubility, is markedly reduced by repeated precipitations with isopropanol and can be completely removed from DNA by chromatography on agarose or MAK.

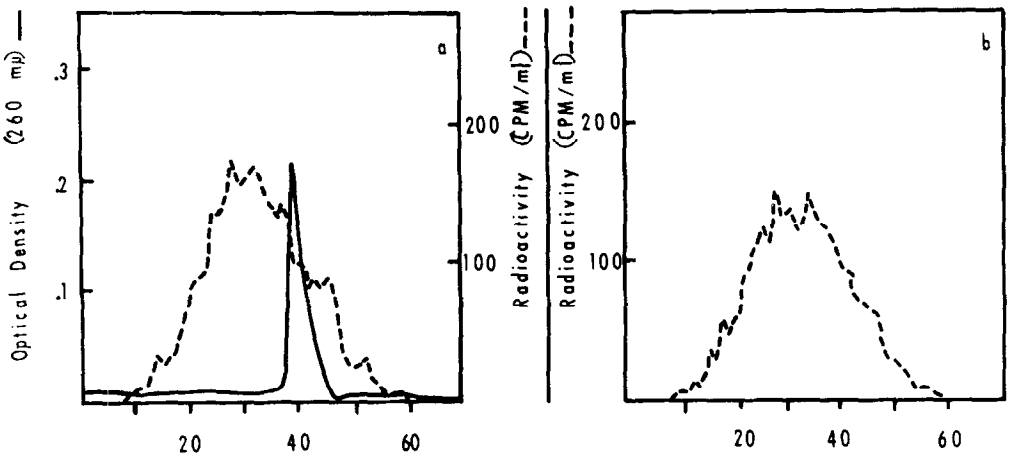

Figure 3. Cesium chloride density gradient centrifugation of TA and a mixture TA and DNA. Samples of DNA, TA and mixtures of TA and DNA were centrifuged and analyzed as described in Methods a. 25 μg DNA + 25 μg TA; b. 25 μg TA.

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