Possible Inhibitory Effect of Teichoic Acid on *Bacillus subtilis* Transfer Ribonucleic Acid

By JEAN YOUATT,* ALICE MONTOYA AND E. W. NESTER

Department of Microbiology, University of Washington, Seattle, Wash. 98105, U.S.A.

(Received 1 April 1970)

1. tRNA of *Bacillus subtilis* was found to be variably contaminated with membrane teichoic acid. 2. Samples with high contents of teichoic acid showed no accepting activity for tRNA^{Phe} and tRNA^{Tyr}. 3. Removal of teichoic acid restored accepting activity and fractions containing teichoic acid, separated on Sephadex G-150, inhibited the charging of tRNA^{Tyr}. 4. The presence of teichoic acid did not inhibit the charging of tRNA^{His}.

The presence of contaminating material in tRNA preparations of *Bacillus* species has been reported from two laboratories. Okamoto (1967) described a material separated from the tRNA of *Bacillus stearothermophilus* that inhibited the charging of certain species of tRNA. Migita, Hsu & Doi (1970) described a 'pseudo-inhibitor' in tRNA prepared from *Bacillus subtilis* that prevented normal acid precipitation of the RNA in the aminoacyl-tRNA assay. The latter authors mentioned that there appeared to be inhibition of some other type in their preparations.

Each of the reports described some properties of the inhibitor or pseudo-inhibitor but did not identify the compounds involved.

We have studied the inhibition of the charging of tRNA^{Phe} and tRNA^{Tyr} in *B. subtilis* and have tentatively identified the inhibitor as a polyglycerol phosphate with glucose substituents. This type of teichoic acid is known to occur in or on the membranes of all Gram-positive organisms that have been investigated.

MATERIALS AND METHODS

Reagents. Tritiated amino acids were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.) and Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). Purity was checked by chromatography on Amberlite IR-120 (Na⁺ form)-impregnated paper with 0.1 M sodium phosphate-potassium phosphate buffer, pH 6.8, for histidine and 0.1 M-sodium acetate buffer, pH 4.8, for tyrosine. Strips were scanned with a Vanguard Automatic Chromatogram Scanner. Dr Beverly Dale supplied one

batch of [³H]tyrosine purified by ion exchange on Bio-Rad AG-50W (X8) (Na⁺ form) and eluted by 0.1 M-sodium acetate buffer, pH 5.

Glycerol dehydrogenase and glycerol 1-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and the Glucostat assay kit from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.).

Growth of *B. subtilis* cells. *B. subtilis* strain 746, a prototroph, was grown in a New Brunswick 10-litre Micro Ferm Laboratory Fermentor or the New Brunswick 100-litre Fermacell at 37°C in Spizizen's (1958) medium supplemented with 5 g of Casamino acids (casein hydrolysate)/l. Cells were harvested in the exponential phase of growth and stored at -60°C until required.

tRNA preparation. A 100 g portion of wet cells was shaken for 30 min at room temperature with 50 ml each of phenol and 1 mM-tris-HCl buffer, pH 7.5, containing 0.01 M-MgCl₂. The sedimented cells and phenol layer were re-extracted twice with the same buffer. RNA was precipitated from the aqueous phase by the addition of 0.1 vol. of 20% (w/v) potassium acetate and 2 vol. of ethanol. The RNA, which was soluble in 1 M-NaCl, was reprecipitated by ethanol, and amino acids were discharged by incubation in 1 mM-tris-HCl buffer, pH 8.8, for 60 min at 37°C. RNA was reprecipitated, dialysed and applied to a DEAE-cellulose column at 0.2 M-NaCl and eluted by 1 M-NaCl. This step further decreased the content of rRNA and DNA. The tRNA was finally precipitated, dialysed and stored at -20°C. Preparations taken through to this stage are referred to as crude tRNA.

Aminoacyl-tRNA synthetase. Crude aminoacyl-tRNA synthetase was prepared by streptomycin sulphate precipitation of nucleic acids and (NH₄)₂SO₄ precipitation by the method described by Kelmers, Novelli & Stulberg (1965). The preparation was stored at -60°C in 50% (v/v) glycerol. The charging of the three amino acids, histidine, tyrosine and phenylalanine was maximal after 5 min incubation at a suitable concentration of the crude enzyme preparation. After longer incubation or with a higher concentration of enzyme the extent of charging sometimes

* Present address: Department of Chemistry, Monash University, Clayton, Vic. 3168, Australia.

declined. This effect was not due to ribonuclease activity in the enzyme preparation, since preincubation of enzyme and RNA did not decrease charging.

To select a suitable enzyme concentration a series of twofold dilutions was made covering the range 2–0.05 mg of protein/ml. To these enzyme dilutions was added an equal volume of a reaction mixture that contained twice the normal reagent concentrations defined below.

Assay of tRNA. In the general assay procedure the tRNA was incubated in the presence of 1 mM-ATP, 0.01 M-MgCl₂, 0.1 M-tris-HCl buffer, pH 7.6, and 0.1 mM ³H-labelled amino acids. After 5 min at 30°C the reaction was stopped by the addition of ice-cold 1 M-HCl. All samples were filtered within 1 h on Whatman GF/C glass-fibre filters, washed four times with ice-cold 1 M-HCl and once with ethanol. When *B. subtilis* tRNA was used in a concentration too low to yield a visible precipitate, carrier yeast RNA was added after the addition of 1 M-HCl. The radioactivities of the dried filters were counted in a Beckman liquid-scintillation counter in toluene scintillation fluid with 30% efficiency. Additions of KCl, EDTA, GSH, mercaptoethanol, dithiothreitol or bovine serum albumin, sometimes used by other investigators, were without effect on the 5 min charging reaction. They were, accordingly, omitted.

The extent of charging was the same between pH 7 and pH 8, and lowering the Mg²⁺ concentration to 1 mM had no effect. ATP was always used in large excess. The reaction therefore depended on the concentration of enzyme, already discussed above, and on the concentrations of tRNA and amino acid, which are considered in the Results section.

Chromatography. Benzoylated DEAE-cellulose (BD-cellulose) was obtained from Schwarz BioResearch Inc. and 20–50-mesh size was used for most purposes. Unless otherwise indicated all elutions were carried out with solutions of NaCl containing 0.01 M-MgCl₂.

Columns (50 cm × 1 cm) of Sephadex G-100 or G-150 were packed, equilibrated and run at 4°C with 0.01 M-tris-HCl buffer, pH 7.6, containing 1 mM-MgCl₂. The total column volume was approx. 50 ml and 1 ml fractions were collected.

Paper chromatography was used to detect the glucose, glycerol or glycerol 1-phosphate produced by acid hydrolysis of the teichoic acid. Samples were applied to Whatman no. 1 paper and developed by ascending chromatography in propan-1-ol-acetic acid-water (3:1:1, by vol.) and the spots were revealed by a spray prepared from aq. 5% AgNO₃ with excess of aq. NH₃. Ninhydrin (0.2%) in acetone spray was used to check for amino acids and amino sugars.

Analysis of teichoic acid. Teichoic acid was hydrolysed in several batches at 100°C with either H₂SO₄ or HCl (0.5–2.0 M acid). The hydrolysates were neutralized with NaOH and desalted by addition of ethanol to 80% (v/v). The supernatants were concentrated to dryness and made up to known volume.

Glucose was assayed with Worthington Glucostat kit according to the manufacturer's instructions. Glycerol was determined by using glycerol dehydrogenase and glycerol 1-phosphate by using glycerol 1-phosphate dehydrogenase. Each enzyme was checked with glycerol, glycerol 1-phosphate and glucose to establish the specificity of the reaction.

Bound phosphorus was determined according to the method of Chen, Toribara & Warner (1956).

An anthrone colour test was used to detect the presence of teichoic acid in column fractions. Two vol. of approx. 0.1% anthrone in conc. H₂SO₄ was added to 1 vol. of sample, and colour was developed in a boiling-water bath for 5 min. RNA in the range of concentrations found in the samples did not give a colour under these conditions.

RESULTS

The effect of varying the concentration of tyrosine on the charging of tRNA^{Tyr} depended on the purity of the tRNA. Curve A in Fig. 1 shows the charging of crude tRNA with various concentrations of tyrosine. Each value has been corrected for a blank without RNA at the same amino acid concentration. Curve B in Fig. 1 shows charging at various concentrations of tyrosine of a sample of tRNA^{Tyr} partially purified on BD-cellulose. At high amino acid concentration two processes are apparently being measured, the aminoacylation of tRNA^{Tyr} and a second reaction that continues to increase at higher concentrations of tyrosine and might represent the charging of aggregated tRNA. The sensitivity of the assay for charging tRNA^{Tyr} would therefore be increased at a lower concentration of tyrosine.

The charging of crude *B. subtilis* tRNA was normally linear only to a concentration of 10–20 E₂₆₀ units/ml in the tyrosine assay (Fig. 2). However, in one batch of tRNA, described below, no

Fig. 1. Effect of tyrosine concentration on the charging of tRNA. Curve A (○), charging of tRNA^{Tyr} in crude RNA; curve B (●), charging of tRNA^{Tyr} in a sample of tRNA partially purified on BD-cellulose. All points in curves A and B were corrected for controls containing no RNA.

Fig. 2. Effect of concentration of tRNA on the charging of tRNA^{Tyr}. The maximum charging, expressed in c.p.m., was determined by taking several time-points. [³H]-Tyrosine was at a final concentration of 0.1mM and a specific radioactivity of 100mCi/mmol. Other conditions are described in the Materials and Methods section.

charging of tyrosine or phenylalanine was measurable until the tRNA had been purified further. Non-linear charging with increasing concentrations of tRNA was the first indication of a contaminant that modified the charging of tRNA. The amount of contamination varied from one batch to another, ranging from an estimated 5 to 50% teichoic acid. Migita *et al.* (1970) observed an interfering substance that prevented quantitative precipitation of tRNA in the assay procedure. However, with the assay conditions used in this laboratory, complete precipitation of the RNA was shown to occur in the range of concentration of RNA where charging was non-linear.

Inhibitor of charging of tRNA^{Phe} and tRNA^{Tyr}
Some batches of tRNA prepared by phenol extraction of whole cells had no accepting activity for tyrosine and phenylalanine but normal accepting activity for histidine at 10 E_{260} units/ml. Any one of four procedures listed below purified the tRNA and restored good accepting activity for tyrosine and phenylalanine. The additional procedures are as follows.

(1) Gel filtration of the inactive tRNA through Sephadex G-150.

(2) The inactive tRNA was applied to BD-cellulose at 0.03M-sodium chloride, washed with 0.3M-sodium chloride and active RNA was eluted by 1M-sodium chloride in 10% (v/v) ethanol.

(3) Inactive tRNA was applied to DEAE-cellulose at 0.2M-sodium chloride. The column was eluted first with 0.5M-sodium chloride in 10% ethanol and the salt concentration was then increased to 1M when the E_{260} value began to rise. Active tRNA was collected in the remaining frac-

tions. (The 10% ethanol used in the 0.5M-sodium chloride wash may not be essential.)

(4) tRNA was precipitated at 4°C with 1M-hydrochloric acid. The precipitate was centrifuged, washed with 70% (v/v) ethanol and redissolved in 0.2M-sodium hydroxide to which was immediately added tris-hydrochloric acid buffer, pH 7.6. (The use of dilute sodium hydroxide was essential; tris-hydrochloric acid buffer alone left an insoluble residue that contained most of the tRNA^{Phe} and tRNA^{Tyr}.) Okamoto (1967) and Migita *et al.* (1970) also found that acid precipitation improved the accepting activity of their tRNA preparation.

Each of these four procedures removed the same material. The properties, based on observations to this point, may be summarized. The material was a non-diffusible polymer, soluble in water and dilute acid but insoluble in ethanol. It did not absorb at 260nm. It was not retained by BD-cellulose at 0.3M-sodium chloride. On DEAE-cellulose the polymer was retained at 0.2M-sodium chloride but was eluted at the solvent front with 0.5M-sodium chloride in 10% ethanol. Its presence was detected as an ethanol-insoluble material that did not absorb at 260nm

Identification of teichoic acid. A sample of the polymer was prepared from material separated by DEAE-cellulose and BD-cellulose. The dry weight was determined after dialysis against water. The products of hydrolysis were found to vary with the experimental conditions. With 0.5M acid, glucose and glycerol 1-phosphate were detected, but no other sugar or amino sugar. Hydrolysis by 2M acid destroyed the glucose and gave glycerol but little glycerol 1-phosphate. No amino acids were detected in the hydrolysates but all samples of teichoic acid tested had been exposed to ion exchangers. A complete quantitative analysis was precluded by the difficulty of obtaining complete hydrolysis without some destruction. Nevertheless it was possible to show a minimum of 20% glucose, 35% glycerol+glycerol 1-phosphate and 10% phosphorus. Allowing for the oxygen of the phosphate ester linkage this analysis accounts for 75% of the dry weight of the sample. This is a lower estimate which takes no account of the loss of glucose nor of the possible racemization of glycerol 1-phosphate during acid hydrolysis. The analytical result was consistent with the presence of teichoic acid consisting of polyglycerol phosphate with some glucose substituted in the 2-hydroxyl group. Glaser & Burger (1964) described the isolation of both polyglycerol phosphate and glucosylated polyglycerol phosphate from *B. subtilis*. It is probable that the teichoic acid contaminating the tRNA preparations was not homogeneous. This is suggested both by the analysis and by broad elution bands from Sephadex G-150.

Fig. 3. Decreased charging of $tRNA^{Tyr}$ in the presence of high-molecular-weight fractions separated by Sephadex G-150. Details of the experiment are described in the text. The figure shows corrected extents of charging of added $tRNA^{Tyr}$ in the presence of column fractions of a crude tRNA preparation that contained high concentrations of teichoic acid. The extent of charging in the absence of any column fractions was 990 c.p.m. RNA on the figure indicates the position of RNA excluded by the gel and teichoic acid indicates the range over which teichoic acids were eluted.

The evidence that teichoic acid was responsible for the inactivation of $tRNA^{Phe}$ and $tRNA^{Tyr}$ at this stage was based on the observation that several procedures that removed teichoic acid restored accepting activity to the tRNA. To obtain more direct evidence a crude preparation containing much teichoic acid but no detectable $tRNA^{Tyr}$ was fractionated on Sephadex G-150. Each column fraction was assayed for $tRNA^{Tyr}$, as described in the Materials and Methods section, to provide a series of correction blanks. Samples of an active preparation of $tRNA^{Tyr}$ were then assayed in the presence of the column fractions and the correction blanks were subtracted. In the absence of inhibitor the extent of charging of the tRNA was 990 c.p.m. Fig. 3 shows diminished charging in the presence of high-molecular-weight fractions shown to contain teichoic acid by the anthrone colour method. Fig. 3 also illustrates the width of the elution band of the teichoic acids, which suggests heterogeneity of the material. All fractions exhibited inhibitory action.

The teichoic acid separated by chromatography on BD-cellulose and DEAE-cellulose or by acid precipitation was not inhibitory when added to the assay mixture containing partially purified, active $tRNA^{Tyr}$.

DISCUSSION

There are a number of lines of evidence strongly suggesting that the inhibitor of the charging of $tRNA^{Tyr}$ and $tRNA^{Phe}$ is a membrane teichoic acid: (1) the removal of teichoic acid from an inactive

preparation by any one of four different procedures resulted in an active tRNA preparation; (2) fractions from a Sephadex gel column that contained teichoic acid inhibited the charging of $tRNA^{Tyr}$ when added to active $tRNA^{Tyr}$ from which teichoic acid had been removed; (3) no material other than teichoic acid could be detected in the material separated from the inactive tRNA. From the normal preparative procedure, which yielded the inactive tRNA, we can say that the inhibitor was not diffusible on dialysis and not retained on DEAE-cellulose in 1M-sodium chloride. The subsequent two-stage elution from DEAE-cellulose resulted in a fraction containing an ethanol-insoluble polymer with no u.v. absorption and a second fraction containing active tRNA. The first fraction must contain the material responsible for the previous inactivity of the tRNA. This fraction contained no polynucleotide or protein. No sugar or sugar derivative was found other than glucose, and neutral polysaccharides are not retained by the ion-exchanger. Qualitatively, teichoic acid was the only detectable polymer. Quantitatively, 75% of the dry weight of the fraction containing the inhibitor could be accounted for as teichoic acid. This is a minimal value, since glucose determinations were not corrected for loss during hydrolysis and glycerol diphosphate was not measured.

The results of Okamoto (1967) are also compatible with the presence of polyglycerol phosphate. This author detected no sugar residues, but Glaser & Burger (1964) have described teichoic acids in *B. subtilis* that are free of sugar residues, and *B. stearothermophilus* may also produce teichoic acids of this type.

The one piece of evidence against teichoic acid being the inhibitor is that teichoic acid separated by ion-exchange or acid precipitation failed to react when returned to the reaction mixture. One possible explanation is that a linking agent such as a bivalent cation is required for binding teichoic acid to the RNA. Such an agent may have been lost in the ion-exchange preparations. It has long been known that teichoic acids can combine with RNA. Wicken & Baddiley (1963) describe such complexes, but their nature is not understood. Archibald, Baddiley & Blumson (1968) have suggested that the association of teichoic acids with RNA may be an artifact of preparation.

We have not observed the 'pseudo-inhibitor' effect described by Migita *et al.* (1970). This effect appears to have been highly sensitive to experimental conditions. For example, the authors report normal precipitation with higher protein concentrations.

Further understanding of the inhibitory effects of teichoic acid awaits elucidation of the nature of the association of RNA and teichoic acid, which is

at present unknown. However, as a matter of practical expediency, the removal of membrane teichoic acid by the use of DEAE-cellulose or BD-cellulose restored the accepting activities of tRNA^{Tyr} and tRNA^{Phe} of *B. subtilis*.

This study was performed at the University of Washington, and was supported by U.S. Public Health Service Grant GM09848 from the National Institutes of Health. The capable technical assistance of Miss Judy Day is gratefully acknowledged. We thank Dr Roy Doi for communicating his unpublished results to us, and for helpful discussions.

REFERENCES

- Archibald, A. R., Baddiley, J. & Blumson, N. L. (1968). *Adv. Enzymol.* **30**, 223.
Chen, R. S., Toribara, R. Y. & Warner, M. (1956). *Analyt. Chem.* **28**, 1756.
Glaser, M. M. & Burger, L. (1964). *J. biol. Chem.* **239**, 3168.
Kelmers, A. D., Novelli, G. D. & Stulberg, M. P. (1965). *J. biol. Chem.* **230**, 3979.
Migita, L. K., Hsu, Y. P. & Doi, R. H. (1970). *Biochim. biophys. Acta*, **199**, 248.
Okamoto, T. (1967). *Biochim. biophys. Acta*, **138**, 198.
Spizizen, J. (1958). *Proc. natn. Acad. Sci. U.S.A.* **44**, 1072.
Wicken, A. J. & Baddiley, J. (1963). *Biochem. J.* **87**, 54.