TUNICAMYCIN, AN INHIBITOR OF BACILLUS PEPTIDOGLYCAN SYNTHESIS: A NEW SITE OF INHIBITION[1]

George E. Bettinger and Frank E. Young

Department of Microbiology
University of Rochester School of Medicine and Dentistry
Rochester, New York 14642

Received September 2,1975

Summary. Bacillus subtilis membranes can transfer either N-acetylmuramyl-pentapeptide phosphate or N-acetylglucosaminyl phosphate from UMP directly onto undecaprenyl phosphate. Tunicamycin blocks only the latter transfer and inhibits peptidoglycan synthesis by toluenized cells of Bacillus megaterium utilizing added nucleotide sugar precursors or cell wall synthesis by intact cells of B. subtilis. Tunicamycin prevents formation of the cell wall disaccharide lipid intermediate by blocking transfer of N-acetylglucosamine onto undecaprenyl muramyl pentapeptidyl pyrophosphate.

The biosynthesis of the bacterial peptidoglycan involves the transfer of components from soluble nucleotide-linked precursors to the existing cell wall by means of a polyisoprenol lipid intermediate, undecaprenol phosphate (1). There are several antibiotics known to inhibit various stages of this synthetic scheme (2). Phosphonomycin and D-cycloserine interfere with the enzymes responsible for the biosynthesis of the soluble precursors, while penicillin prevents the formation of crosslinked walls. Bacitracin combines with undecaprenyl pyrophosphate to prevent the addition of the UDP muramyl pentapeptide, while vancomycin, ristocetin and ristomycin prevent the transfer of the disaccharide pentapeptide cell wall precursors from the lipid intermediate to the growing cell wall. No antibiotics are known, thus far, which prevent synthesis of the lipid disaccharide intermediate once the undecaprenyl muramyl pentapeptidyl pyrophosphate is formed. A new antibiotic, tunicamycin, has been reported to influence cell surface

[1]Abbreviations: TM, tunicamycin; UDP-GlcNAc, UDP-N-acetyl-D-glucosamine; UDP muramyl pentapeptide, UDP-N-acetyl-D-muramyl-L-alanyl-D-γ-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine.

synthesis in microbial cells (3,4). We have examined the effect of tunicamycin on peptidoglycan synthesis in Bacillus subtilis to determine its possible site of action.

MATERIALS AND METHODS

Bacillus subtilis 168 (strain BR151) and Bacillus megaterium KM-1 (diaminopimelic acid auxotroph, P. E. Reynolds) were cultured in Difco antibiotic medium 3. B. subtilis membranes were prepared by differential centrifugation from cells broken by grinding with alumina (Sigma, type 305), and stored at -70C in 0.1 M Tris, 0.1 M $MgCl_2$, 0.001 M beta-mercaptoethanol buffer, pH 8.0. An equal volume of B. subtilis membranes [15-30 mg protein/ml, (5)] and either UPD-[6-^{3}H]-GlcNAc, 10 mM, 5 µCi/µmole, UDP-[1-^{14}C]-GlcNAc, 2 mM, 1 µCi/µmole (New England Nuclear), or UDP muramyl pentapeptide [U-^{14}C] D-ala-[U-^{14}C]D-ala, 2 mM, 9.2 µCi/µmole were mixed for in vitro synthesis Reactions were stopped after 30 min by the addition of 8 volumes of chloroform methanol (2.1, v/v) or 2 volumes of n-butanol: 6 M pyridinium acetate, pH 4.1 (1:1, v/v) to extract the GlcNAc or muramyl pentapeptide labeled lipids, respectively. The organic phase was withdrawn, backwashed three times with equal volumes of either chloroform:methanol:water (3:47:48, v/v) or n-butanol saturated water, and air dried in a scintillation vial prior to counting. Toluenized cells of B. megaterium were prepared and used for peptidoglycan synthesis as published (6). Incubations were stopped after 1 hr by adding 3 ml of 5% (w/v) trichloroacetic acid, the pellets washed three times and collected on Whatman GF/A filters. The precipitates were given an additional three washes, a final rinse of diethylether, and dried in a scintillation vial for counting. The in vivo incorporation of [U-^{14}C]-D glucosamine into lipid, teichoic acid and peptidoglycan by B. subtilis cells was followed by adding the isotope at 0.5 Ci/ml to log phase cells for 30 min, and fractionating the washed cells by a modified Park-Hancock procedure (7). Radioactivity was estimated using a toluene based commercial scintillant, Omnifluor (New England Nuclear).

TABLE 1. Inhibition of UDP-GlcNAc and UDP-muramyl pentapeptide incorporation by TM.

Additions	Incorporation into lipid[a]	
membranes + UDP-[^{14}C]-GlcNAc	7133	(1.00)
membranes + TM + UDP-[^{14}C]-GlcNAc	1920	(0.26)
boiled membranes + UDP-[^{14}C]-GlcNAc	10	(0)
membranes + UDP-muramyl pentapeptide [^{14}C][b]	3000	(1.00)
membranes + TM + UDP muramyl pentapeptide [^{14}C]	2720	(0.90)

Membranes of B. subtilis were incubated and the lipid extracted as described in Materials and Methods. TM was added to 15 µg/ml 5 min prior to the addition of labeled substrate.

[a]Results presented as disintegrations per min, with relative values shown in brackets.

[b]Carboxypeptidase activity in these membranes is low, removing only 10% of the terminal [^{14}C]-D-ala in 30 min, hence benzylpenicillin was not included in the incubation mixtures.

RESULTS

Bacillus subtilis membranes incorporate UDP-GlcNAc into a chloroform: methanol extractable fraction (Table 1) which consists of 1, 4, or 6 glucosamine units pyrophosphate linked to the lipid, undecaprenol (8, Bettinger, G. E. and F. E. Young, Manuscript in preparation). As shown in the Table, these membranes can also form the undecaprenyl muramyl pentapeptidyl pyrophosphate in the absence of added UDP-GlcNAc. TM inhibits the transfer of GlcNAc from UDP-GlcNAc to the undecaprenyl lipid (74%), but does not prevent the addition of the muramyl pentapeptide to this lipid. In data not presented, TM inhibited the transfer of UDP-GlcNAc by 74% at 11 µg/ml, 58% at 5 µg/ml, and 10% at 0.5 µg/ml.

Since the B. subtilis membranes will not synthesize peptidoglycan, or even the undecaprenyl disaccharide cell wall intermediate (8,9), toluenized

cells of B. megaterium were used for peptidoglycan synthesis, utilizing added nucleotide sugar precursors (6). As shown in Table 2, TM inhibited peptidoglycan synthesis by between 77 and 97%, as compared to control.

The effect of TM and vancomycin on the ability of B. subtilis cells to

TABLE 2. Effect of tunicamycin on peptidoglycan synthesis by toluenized B. megaterium cells.

Additions			Incorporation Exp. 1	Exp. 2
cells + UDP-GlcNAc	+ UDP muramyl pentapeptide	[^{14}C]	1.00 (405)	1.00 (810)
cells + UDP-GlcNAc + TM	+ UDP muramyl pentapeptide	[^{14}C]	0.03	0.23
cells -	+ UDP muramyl pentapeptide	[^{14}C]	-	0.25
boiled cells + UDP-GlcNAc	+ UDP muramyl pentapeptide	[^{14}C]	0	0

Toluenized B. megaterium cells were prepared as published (6) and peptidoglycan synthesis measured as described in Materials and Methods. Synthesis is presented relative to control, and the numbers in brackets represent the ^{14}C disintegrations/min. TM was added at 200 µg/ml.

TABLE 3. Inhibition of glucosamine incorporation in B. subtilis cells by tunicamycin and vancomycin.

Fraction	Control	Tunicamycin	Vancomycin
chloroform:methanol	1.00 (11,000)	0.33	1.50
hot trichloroacetic acid	1.00 (113,310)	0.22	0.03
trypsin	1.00 (5,310)	0.72	0.05
residue	1.00 (549,000)	0.28	0.01

B. subtilis cells were labeled with [^{14}C]-glucosamine in the presence of tunicamycin (10 µg/ml), vancomycin (100 µg/ml) or no antibiotic (control) as described in Materials and Methods, washed with 5% (w/v) trichloroacetic acid and extracted first with chloroform:methanol (2:1, v/v), next with hot 5% trichloroacetic acid (90C, 10 min) to remove teichoic acid, then digested 30 min in bicarbonate buffer with 1 mg/ml trypsin. The residue represents the peptidoglycan. (More than 95% of the residue can be solubilized by lysozyme). The data for each fraction are presented relative to control; the numbers in brackets represent the ^{14}C disintegrations/min.

incorporate glucosamine is compared in Table 3. TM inhibits the formation of teichoic acid and peptidoglycan by 78 and 72% respectively, whereas vancomycin inhibits the synthesis of these polymers by greater than 95%. TM decreased the incorporation of label into lipid by 67%, while vancomycin enhanced it 1.5 fold. TM did not markedly affect the fraction solubilized by trypsin as compared to the 95% decrease caused by vancomycin.

The increased incorporation of label from glucosamine into lipid by vancomycin-treated cells in Table 3 was also observed when D-cycloserine (150 µg/ml, 4.7 fold increase) or bacitracin (300 µg/ml, 3.1 fold increase) were used (data not shown). A similar effect has been noted in Staphylococcus aureus for lysine incorporation (10), and it would appear that the blockage of wall synthesis at various points has a secondary effect on lipid composition.

DISCUSSION

Tunicamycin is a glucosamine-containing antibiotic active against bacteria, yeast, and animal and plant viruses (3,4). Yeast mannan is a polysaccharide which contains protein, covalently attached through an asparaginyl N-acetylglucosaminyl bridge (13). TM inhibits the synthesis of yeast glycoproteins and mannan (11,12), which most likely involve the formation of N-acetylglucosaminyl or mannosyl dolichyl lipid at some point in their synthesis (14-16). TM does not inhibit chitin or glucan synthesis (11,12), polymers which are not covalently attached to a protein. In calf liver microsomes, TM completely inhibits the formation of dolichyl-N-acetyl-glucosaminyl pyrophosphate but does not disturb dolichyl mannosyl phosphate synthesis (17). These data suggest, as mentioned by Tkacz and Lampen (17), that TM acts in the eukaryotic systems by preventing the formation of a polyisoprenyl-N-acetyl-glucosaminyl pyrophosphate intermediate necessary for continued polymer synthesis

TM does not prevent the transfer of muramyl pentapeptide from UDP onto undecaprenyl phosphate (Table 1), but does inhibit peptidoglycan biosynthesis (Tables 2,3). Therefore, the site of action of TM must lie after the formation of the lipid muramyl pentapeptidyl pyrophosphate. TM does inhibit the synthesis

of polyisoprenyl N-acetylglucosaminyl pyrophosphate lipids (Table 1), as in eukaryotes (17). It seems likely, therefore that TM prevents cell wall formation by interfering with the transfer of N-acetyl-glucosamine from UDP-GlcNAc onto undecaprenyl muramyl pentapeptidyl pyrophosphate. If this hypothesis is correct TM would represent the only antibiotic known thus far which acts to prevent completion of the lipid disaccharide intermediate once the first transfer has been effected. In general TM may have a specificity for inhibiting enzymes which transfer N-acetylglucosamine from UDP-GlcNAc directly onto a polyisoprenyl phosphate or to a sugar already attached to a polyisoprenyl lipid.

ACKNOWLEDGEMENTS

Tunicamycin (lot T-11-05) was the kind gift of Dr. G. Tamura, Tokoyo University. UDP muramyl pentapeptide [^{14}C] was prepared by Dr. R. J. Boylan. This work was supported by grant USPHS AI-10141 to FEY.

REFERENCES

1. Osborn, M. J. 1969. Ann. Rev. Biochem. 38: 501-538.
2. Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring 1972. pp. 49-120. The Molecular Basis of Antibiotic Action. J. Wiley & Sons, New York.
3. Takatsuki, A., K. Arima, G. Tamura. 1971. J. Antibiotics, 24: 215-223.
4. Takatsuki, A., K. Shimizu, G. Tamura. 1972. J. Antibiotics, 25: 75-85.
5. Lowry, O., N. Rosebrough, A. Farr, R. Randall. 1951. J. Biol. Chem. 193: 265-275.
6. Schrader, W. P., and D. P. Fan. 1974. J. Biol. Chem. 249: 4815-4818.
7. Park, J. T. and R. Hancock. 1960. J. Gen. Microbiol. 22: 249-258.
8. Bettinger, G. E., A. N. Chatterjee, and F. E. Young. 1975. Abstract, Fed. Proc. 34: 668.
9. Boylan, R. J., A. N. Chatterjee, and F. E. Young. 1973. Abstract, Ann. Meeting, Amer. Soc. Microbiol. p. 153.
10. Hebeler, B., A. N. Chatterjee, and F. E. Young. 1973. Antimicrob. Ag. Chemotherapy, 4: 346-353.
11. Kuo, S. C., and J. O. Lampen. 1974. Biochem. Biophys. Res. Commun. 58: 287-295.
12. Kuo, S. C. and J. O. Lampen. 1975. Abstract, Ann. Meeting, Amer. Soc. Microbiol. p. 1.
13. Sentandreu, R., and D. H. Northcote. 1968. Biochem. J. 109:419-432.
14. Sentandreu, R., and J. O. Lampen. 1972. FEBS Letters, 27: 331-334.
15. Jung, P., and W. Tanner. 1973. Eur. J. Biochem. 37: 1-6.
16. Lennarz, W. J. 1975. Science, 188: 986-991.
17. Tkacz, J. T., and J. O. Lampen. 1975. Biochem. Biophys. Res. Commun. 65: 248-257.