Inhibition of *Lactobacillus leichmannii* 313 by Purine Nucleotides

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Plate assays for vitamin B_{12}-factors, deoxyribosides, and deoxyribotides as well as bioautography of these growth factors have been carried out in this laboratory using *Lactobacillus leichmannii* 313 ATCC 7830 as the test organism [1,2]. This paper deals with the growth inhibitory activity of several purine nucleotides, notably adenine ribotides, in the *Lb. leichm.* 313 cup plate assay for vitamin B_{12}, deoxyribosides, and deoxyribotides. A report is also given on the usefullness of *Lb. leichm.* 313 for the cup plate assay and bioautography of the growth inhibitory purine nucleotides.

Large plates were used, each containing 150 ml of agar medium inoculated with *Lactobacillus leichmannii* 313 ATCC 7830. The media contained high concentrations of vitamin B_{12}, deoxyribosides, or deoxyribotides, in order to obtain heavy growth in the plates and thereby give a more pronounced appearance of growth inhibition. For the cup plate assays, 0.03 ml aliquots of the test solutions were pipetted into cups, 6 mm in diameter and the plates were incubated over night at 37° C. A detailed report of the preparation of these plates will be published later in this journal.

A series of plate experiments were first made, using *Lb. leichm.* 313 in two types of media which contained: 1) 150 mμg of vitamin B_{12} and 2) 150 μg of thymidine per plate. When tested in the cup plate assay the following purine nucleotides formed clear (or almost clear) inhibition zones: AMP-3'*, adenylic acid (mixture of AMP-2' and AMP-3'), AMP-c2'3' [b], AMP 5' [b], DAMP-5' [c], IMP-5' [d].

Table 1. The inhibitory activity of purine nucleotides in *Lb. leichmannii* 313 ATCC 7830 plates with media containing either vitamin B_{12} (150 mμg per plate) or thymidine (150 μg per plate).

Compound tested	Concentration μg/ml	Inhibition zone diameter in mm [a] *Lb. leichm.* 313 medium containing: Vit. B_{12}	Thymidine
AMP-3' [b]	10	12.0	12.0
	30	14.7	14.8
	100	18.0	18.0
	300	21.5	21.3
	500	23.2	23.0
Adenylic acid [b] (mixture of AMP-2' and AMP-3')	30	13.0	13.3
	100	16.0	16.4
	300	19.0	19.3
	1 000	23.2	23.2
AMP-c2'3' [b]	30	14.0	14.2
	100	17.0	17.2
	300	20.0	20.7
	1 000	25.0	24.8
AMP 5' [b]	10	12.3	13.2
	30	15.4	15.6
	100	18.8	19.0
	300	22.0	22.2
	1 000	25.5	25.7
DAMP-5' [c]	30	14.5	13.9
	100	17.8	17.0
	300	21.0	20.1
	1 000	24.4	23.1
IMP-5' [d]	30	12.7	12.5
	100	14.4	14.0
	300	15.7	15.6
	1 000	16.9	17.2

[a] Each figure for the inhibition zone diameter represents the average reading obtained from twelve cups, evenly distributed in three plates.

[b] Forms sharp edged and completely clear inhibition zones.

[c] Forms well defined and almost clear inhibition zones.

[d] Forms somewhat fainter inhibition zones than those formed by DAMP-5'.

* The following abbreviations are used in this paper: AMP-2', AMP-3' and AMP-c2'3' for the 2'-, 3'-, and the cyclic 2'3'-monophosphates of adenosine; AMP-5', DAMP-5', IMP-5', GMP-5', and DGMP-5' for the 5'-monophosphates of adenosine, deoxyadenosine, inosine, guanosine, and deoxyguanosine; ADP, IDP, ATP, and ITP for the 5'-di- and -tri-phosphates of adenosine and inosine.

and AMP-3′), AMP-c2′3′, AMP-5′, DAMP-5′, and IMP-5′. The sizes of the inhibition zones formed by these compounds are given in Table 1. Bioautography using five different solvent systems showed that the UV-absorbing spot of each of AMP-3′, AMP-c2′3′, AMP-5′, DAMP-5′, and IMP-5′ always corresponded to the growth inhibition spot. Bioautography of adenylic acid revealed that its growth inhibitory activity was due only to AMP-3′; AMP-2′ was inactive.

Very faint and diffuse inhibition zones were formed by DGMP-5′, GMP-5′, and guanylic acid (mixture of the 2- and 3′-phosphates) when these compounds were tested in high concentrations.

When high concentrations of AMP-2′ and ADP were tested in the *Lb. leichm.* 313 plates small inhibition zones were formed, but bioautography revealed that these growth inhibitory effects were due to trace contamination by AMP-3′ and AMP-5′, resp. Similarly the low inhibitory activity of high concentrations of IDP and ITP were probably due to trace contamination by IMP-5′.

No inhibition zones could be detected in the plates when the following compounds were tested separately at concentrations of 1 mg/ml: (a) the ribosides of cytosine, uracil, adenine, guanine, xanthine, and hypoxanthine, (b) uridylic acid and cytidylic acid (mixtures of the 2′- and 3′-phosphates), (c) the 2′- and the 3′-phosphates of cytidine, (d) the 5′-phosphates of uridine and cytidine, (e) the cyclic 2′3′-phosphates of uridine and cytidine, (f) ATP, and (g) yeast nucleic acid (also tested in 10 mg/ml).

As can be seen from Table 1, the two types of *Lb. leichm.* 313 plates gave almost the same size of inhibition zones for each of the strongly growth-inhibitory purine nucleotides. The straight-line relationship between the logarithm of the concentration of the test solutions in the cups and the mean zone diameter normally found in cup plate assays, was obtained for all these compounds. Already after 3—4 h of incubation at 37° C the inhibition zones produced by the purine nucleotides could be seen against the faint background growth in the *Lb. leichm.* 313 plates. These inhibition zones were almost the same size as those formed after the routine incubation time of 16—18 h, when heavy background growth was obtained in the plates. Prolonged incubation of the plates to 48 h did not have any influence on the inhibition zones already formed.

Using these *Lb. leichm.* 313 plates as little as 0.1 μg of either AMP-3′, AMP-c2′3′, or AMP-5′, or 0.5 μg of DAMP-5′ could be detected bioautographically, whereas about 2—3 μg was required for the detection of these compounds by inspection in UV-light. Only amounts larger than 1 μg of IMP-5′ could be detected bioautographically.

In another series of inhibition experiments *Lb. leichm.* 313 plates were made using media containing 150 μg per plate of one of the following nine compounds: deoxyadenosine, DAMP-5′*, deoxyguanosine, DGMP-5′, deoxyinosine, deoxycytidine, deoxycytidylic acid, deoxyuridine, and thymidylic acid. In all these nine different plates the same inhibition patterns for the purine nucleotides were observed as for the two types of plates previously used in which the media contained either vitamin B_{12} or thymidine.

Turbidimetric tube tests with *Lb. leichm.* 313 showed that only early growth of this organism was inhibited by the adenine ribotides. Merely prolonged incubation was sufficient to overcome growth inhibition, in contrast to the plate tests mentioned above.

Inhibition experiments were also made with *Lactobacillus leichmannii* 327 ATCC 7831 plates containing vitamin B_{12} 150 mμg, thymidine 150 μg, or thymidylic acid 150 μg per plate. The same inhibition pattern for the adenine nucleotides was obtained as in the *Lb. leichm.* 313 plates. However, no inhibition zones were formed by IMP-5′ in the *Lb. leichm.* 327 plates.

Some additional inhibition plate experiments were made with other test organisms. However, none of the purine nucleotides which were found to be inhibitory in the plate tests with the two *Lb. leichm.* strains had any inhibitory effect on the growth of the following organisms: *Thermobacterium acidophilus* R-26 ATCC 11506, (grown with large amounts of thymidine or thymidylic acid), *Escherichia coli* 113—3 ATCC 1105 (grown with large quantities of vitamin B_{12} or methionine),

* At the low concentration of 1 μg/ml in the plate medium, DAMP-5′ showed only growth promoting activity. When tested at concentrations of 10 μg/ml and above, DAMP-5′ formed inhibition zones in the cup plate assay.

Streptococcus faecalis ATCC 8043 (grown with large amounts of folic acid or thymidine), and *Pediococcus cerevisiae (Leuconostoc citrovorum)* ATCC 8081 (grown in a high concentration of folinic acid or thymidine).

Some earlier reports in the literature have dealt with the growth inhibitory activity of adenylic acids on certain vitamin B_{12} requiring lactobacilli. Using tube assay procedures Skeggs *et al.* have studied this effect on *Lb. acidophilus (Lb. bifidus)* ATCC 4963 [3-5] and on *Lb. leichm.* 326 ATCC 4797 [6]. The specific growth inhibitory activity of several purine nucleotides in the *Lb. leichm.* 313 plate assay for vitamin B_{12}, deoxyribosides, and deoxyribotides as observed here, needs further investigation before any conclusions can be drawn concerning the mechanism of these inhibitions.

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