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182. Tadashi Okabayashi and Eitaro Masuo : Occurrence of Nucleotides in the Culture Fluid of Microörganisms.*2 I. Screening of Purine-excreting Bacteria with Purine Auxotrophs of *Escherichia coli*.

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During the past decade a large amount of information has been accumulated on the occurrence of purines, pyrimidines, and their precursors in the culture fluid of microörganisms.

From the published literature, one may conclude that following causes are attributable to these interesting phenomena :

(1) Impairment of *de novo* synthesis of purines and pyrimidines by the presence of antimetabolites,[1~3] lack of nutrient,[4] genetic block,[5~8] etc. (2) Liberation of cellular materials, including nucleosides and nucleotides, after X-ray or ultraviolet-ray irradiation.[9] (3) Release of nucleotides as a result of decomposition of RNA after microörganisms were suspended in appropriate buffer solution containinig chelating agents.[10]

Such information has provided a proof that microörganisms are apt to accumulate or excrete purine and pyrimidine compounds under unusual circumstances as described above.

Occurrence of purine and pyrimidine compounds in the broth of normally growing microörganisms has also been suggested by several experiments.[11,12] It should be noted, however, that no systematic study has been made on this subject.

This series of investigations was designed to study purine and pyrimidine compounds that occur in the culture broth of microörganisms grown in usual conditions. The first screening program of purine-excreting bacteria was carried out with purine auxotrophs of *Escherichia coli*.

Materials and Methods

Purine Auxotrophs of *E. coli*—*E. coli*** strain ATCC 9637 was irradiated with ultraviolet lamp and the resultant purine auxotrophs were isolated with the aid of penicillin mutant technique.[13] A strain, Pb-4, which was found to be a nonexacting purine-auxotrophic mutant was used in this work. Strain B-96, which was reported to be nondiscriminative in its requirement for purine[6],*3 and to accumulate 5-amino-4-imidazolecarboxamide riboside in its culture fluid, was also used. Both strains

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*2 A preliminary account of this work appeared in This Bulletin, 8, 370(1960).

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were found to be suitable as analytical tools for primary screening of purine-excreting microörganisms.

Composition of Media—*E. coli* purine-auxotrophs and all other bacterial strains were maintained on bouillon agar slant. Measurement of response of purine-auxotrophs to various purines and shake culture of purine-excreting bacteria were carried out in the medium listed in Table I. For primary screening the same medium was solidified by the addition of 1.5% of washed agar.

Table I. Composition of the Medium (1 L.)

K_2HPO_4	7.0 g.	*p*-Aminobenzoic acid	1 γ
KH_2PO_4	3.0 g.	Ca-pantothenate	20 γ
Na-citrate	0.5 g.	Pyridoxine HCl	200 γ
$MgSO_4$ aq.	0.1 g.	Nicotinic acid	20 γ
$(NH_4)_2SO_4$	1.0 g.	Biotin	2.5 γ
Vitamin-free casamino acid	2~5 g.	Riboflavin	100 γ
		Thiamine	100 γ

Analytical Procedure—Measurement of growth of purine-requiring mutants was made in the liquid medium listed in Table I. One drop of washed-cell suspension of the mutant was inoculated in each of the test tubes containing the medium and various levels of the purine compound. After incubation at 37° for 24~40 hr., turbidity of broth was measured with a Hitachi spectrophotometer. The growth was expressed as optical density at 500 mμ.

Paper chromatography was carried out with iso-PrOH-H_2O (7:3; NH_3 as vapor phase)[14] as the solvent. The spots of purines and pyrimidines were located with the aid of ultraviolet lamp equipped with ultraviolet filter.*4 In cases where the determination of base component was desired each spot was eluted with H_2O, hydrolyzed with 1*N* HCl at 100° for 1 hr., and the hydrolyzed products were examined on paper chromatogram with 70% *tert*-BuOH and 0.8*N* HCl[15] as the solvent.

Measurement of absorption spectrum was made with a Hitachi spectrophotometer.

Screening of Purine-excreting Bacteria—Agar medium listed in Table I was heated in a water bath to melt the agar and cooled to 40~50°. Washed cells of purine-auxotrophs were added and mixed well by shaking. Every 20 cc. of medium was dispensed in petri dishes of 9 cm. in diameter and cooled. After the medium solidified, one loopful of bacteria was streaked. After incubation for 48 hr. at 28° and additional 48 hr. incubation at 37°, each bacterial strain was inspected for halos due to the satellite growth of purine auxotrophs of *E. coli*. The halo indicates that microörganisms are excreting purine compounds that support growth of purine-requiring mutants.

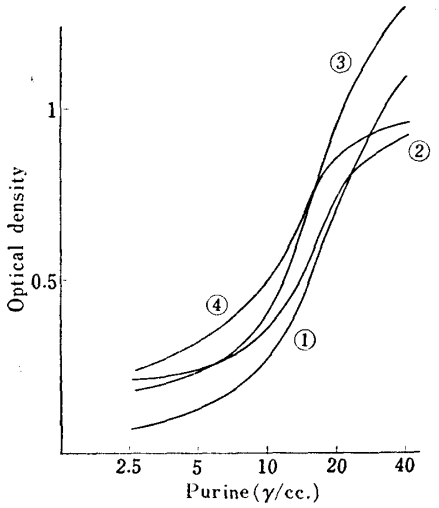

Fig. 1. Growth of Pb-4 with Purines (1)
① Adenine ③ Xanthine
② Guanine ④ Hypoxanthine

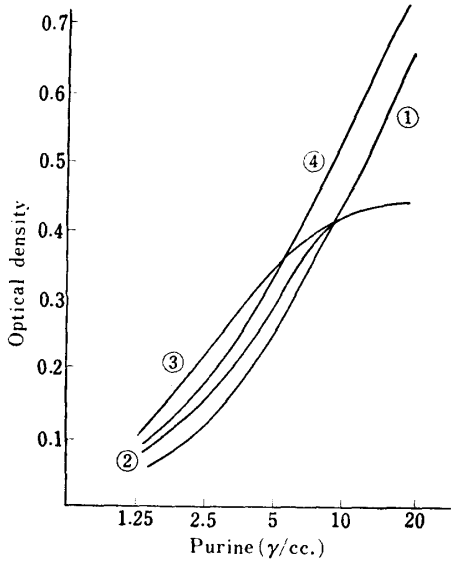

Fig. 2. Response of B-96 to Purines (1)
① Adenine ③ Xanthine
② Guanine ④ Hypoxanthine

*4 The authors wish to express their gratitude to Dr. Eiichi Iwase of the Institute of Physical and Chemical Research for his kind gift of the ultraviolet filter.

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Results

Growth of Purine Auxotrophs with Purines

Prior to carrying out the primary screening program, the response of purine auxotrophs to purines was measured. Figs. 1 and 2 show that both Pb-4 and B-96 can grow well with any one of adenine, guanine, xanthine, and hypoxanthine. Figs. 3 and 4 indicate that not only free purines

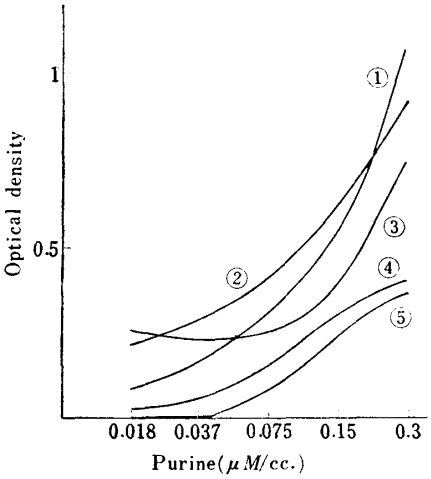

Fig. 3. Growth of Pb-4 with Purines (2)
Adenosine, 5′-AMP and ATP were sterilized by filtration and added to the medium aseptically.
① Adenine ③ Adenosine ⑤ ATP
② Guanine ④ 5′-AMP

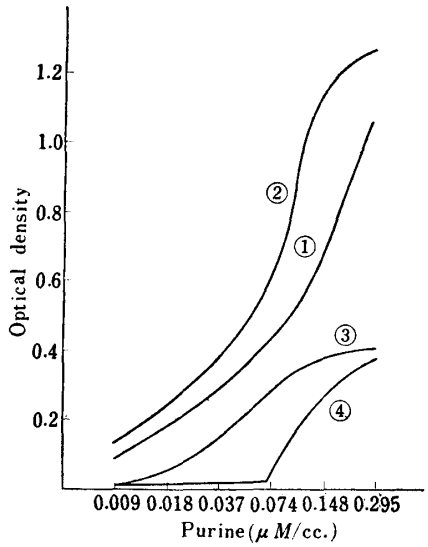

Fig. 4. Response of B-96 to Purines (2)
① Adenine ③ 5′-AMP
② Adenosine ④ ATP

but also nucleosides and nucleotides such as adenosine, 5′-AMP (adenosine 5′-monophosphate), and ATP (adenosine 5′-triphosphate) can serve as growth factor for both mutants. The results obtained with B-96 are consistent with the data of Gots who reported that almost all purines, their nucleosides, and nucleotides can support growth of B-96 in a salt-glucose-amino acid medium.

Screening of Purine-excreting Bacteria with Purine Auxotrophs

Seventy strains of type culture including *Bacillus*, *Micrococcus*, *Escherichia*, *Proteus*, *Pseudomonas*, *Serratia*, *Protaminobacter*, *Alcaligenes*, *Aerobacter*, *Corynebacterium*, *Brevibacterium*, *Arthrobacter*, and *Erwinia* as well as more than 300 strains isolated from drainage were examined for their properties to make halos on agar plate including washed cells of purine auxotrophs. Table II shows the results

Table II. Primary Screening with Purine Auxotroph[a)]

Strain	Halo[b)]	Strain	Halo[b)]
B. subtilis	##	*Brev. helvoleum.* ATCC 11822	±
Pr. vulgalis[d)]	±	*Cory. equii*[c)] IFO 3730[c)]	±
B. circulans	±	*Cory. sepednicum* IFO 3306[c)]	+
B. pumilis IFO 3028[c)]	±	*Alc. metaalcaligenes*	#
B. macerans IFO 3483[c)]	±	*Alc. viscasus* AN-14[d)]	#
B. firmus	±	*A. cloacae*[d)]	±
B. brevis	#	*Erw. carotovora*	±
Se. plymuthicum	##	*Protami. alboflavum* ATCC 8458	±
E. coli S-16[d)]	+	*Ar. ureafaciens* ATCC 7562	±
E. freundii S-89[d)]	±	*Ar. simplex* ATCC 6946	±
M. pyogenes	±	*Bact. ketoglutaricum*	#
Brev. acetylicum ATCC 953	±		

a) *E. coli* B-96 was used in this experiment, strain Pb-4 giving almost the same results.
b) ##, #, +, and ± show the size of halos formed.
c) Kindly supplied by Dr. Takeji Hasegawa of the Institute for Fermentation.*5
d) Obtained from Prof. Kazuyoshi Aiiso and Fumiyoshi Yanagisawa of the Institute for Food Microbiology, Chiba University.*5

*5 The authors are deeply grateful for the supply of the strains.

obtained with these type culture strains. As shown in the table, a considerably large number of bacterial strains could make halos under the conditions employed. The form of the halos varied depending on bacterial strains : Some strains gave typical halos with distinct margins while others gave faint halos with vague margins. Some strains gave growth inhibition zone of purine auxotrophs between their colony and the halo.

Some strains gave faint halos even in the first incubation period at 28°, but it was helpful to carry out the second incubation at 37°, as described in the Experimental, because this procedure gave clear halos with distinct margins.

Screening of Purine-excreting Bacteria with Shake Culture

About 30 strains selected by primary screening as described above were examined for their properties to excrete purine compounds in liquid culture. The bacterial strains were cultured in the liquid medium listed in Table I with shaking at 28° for 40 hr. The broth was centrifuged to sediment bacterial cells and UV absorption spectrum of the clear supernatant was measured in 0.1*N* HCl. Culture fluid not inoculated with the bacteria but treated similarly was used as the blank solution. If the bacteria excrete appreciable amount of purine compounds one would expect that the culture fluid would show UV spectrum with a maximum in the vicinity of 260 mμ and a minimum near 230 mμ. Fig. 5 gives the most typical example, in which it is shown that the UV absorption spectrum of the culture fluid of strain 123-3 (later, the strain was designated as *Brevibacterium liquefaciens novo sp.*) is consistent with this assumption.

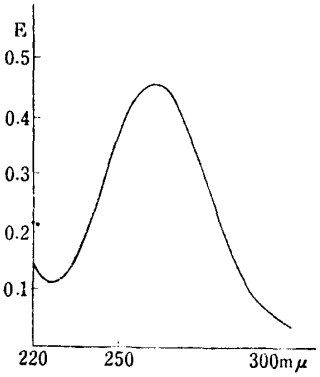

Fig. 5.

Ultraviolet Absorption Spectrum of the Culture Fluid of 123-3 (*Brevibacterium liquefaciens novo sp.*)

Strain 123-3 was cultured for 48 hr. as described in the text, and the absorption spectrum of 1:50 diluted supernatant of broth was measured in 0.1*N* HCl. In the blank solution 1/50 volume of uninoculated broth was added.

Table III. Screening of Purine-excreting Bacteria by Shake Culture

Strain	Primary[b] screening	Absorption of 1 cc. of the broth at		
		280 mμ	260 mμ	250 mμ
154-G-8[a]	++	4.5	6.5	6.0
116-G-8[a]	++	4.5	8.0	7.2
118-G-20[a]	++	9.0	13.2	13.5
119-G-10[a]	++	2.7	5	4.6
123-3[a]	+++	12.5	22	16.2
123-5[a]	++	7.5	10.2	11.2
123-9[a]	+++	4.0	7.6	8.0
155-G-10[a]	+++	4.0	7.5	9.0
116-G-10[a]	++	7.5	12	10
Se. marcescens	++	1.2	2.5	1.5
Erw. carotovora	+	0	2.5	1.2
E. coli S-16	+	0	1.2	0.7
E. freundii	+++	0.7	2.2	1.1
Pr. morganii	++	1.2	2.4	2

a) These strains were isolated from drainage.
b) Same as *b*) in Table II.

Table III shows some results of this screening. The data presented in the table show that some strains isolated from drainage excrete considerably large amount of UV-absorbing substance, while most of the type culture strains failed to produce UV-absorbing substances under the conditions employed. Appropriate amount of charcoal was added to the supernatant and stirred vigorously for 30 min. The suspension was filtered through a bed of washed Supercell, and washed with H_2O, followed by elution with 50% EtOH containing 2% conc. NH_4OH. Without delay the ammoniacal

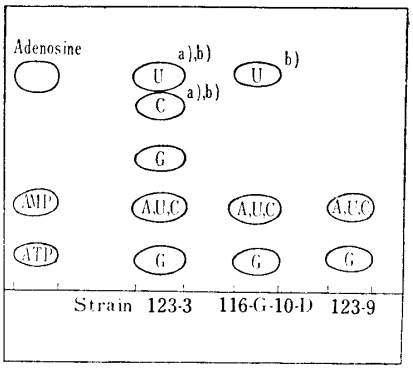

Fig. 6.
Examination of Ultraviolet-Absorbing Substances by Paper Chromatography with Isopropanol-water (Ammonia as Vapor Phase)

a) A, G, C, and U denote the base component determined as in the text. A, adenine; G, guanine; C, cytosine; U, uracil.
b) Faint spots.

solution was evaporated to a small volume under a reduced pressure and the residue was submitted to paper chromatography. As is shown in Fig. 6 the culture fluids of 123-3, 116-G-10-D, and 123-9 gave distinct spots that migrated at approximately the same rate as 5′-AMP or ATP. From the Rf values and the fact that they all contained organic phosphorus (detected by the method of Hans and Isherwood[16]), they were assumed to be nucleotide. Each spot was eluted with H_2O, hydrolyzed with 1*N* HCl at 100° for 1 hr., and the base component was determined. Besides purine compounds such as adenine and guanine, pyrimidine compounds such as cytidylic acid and uridylic acid were found on paper chromatogram with the solvent of *tert*-BuOH-HCl-H_2O and by measurement of absorption spectra. Faint spots that moved as fast as adenosine were also found to contain uracil and cytosine.

Discussion

In spite of the fact that many efforts have been made to determine pyrimidines or DNA components by microbiological methods, only a little work has been done on microbiological assay method for purine compounds. The primary screening method for purine-excreting bacteria described here does not give quantitative results and thus needs further tests using chemical methods. With the microbiological method, however, one can afford to examine more than 200 bacterial strains in one experiment and thus eliminate the complexity of chemical methods which take a long time.

As was indicated in the experiment, several strains that were selected by the primary screening method could produce considerable amount of ultraviolet-absorbing substances in their culture fluid. The isolation of these strains would undoubtedly facilitate further study of purine and pyrimidine compounds in the broth of microörganisms. Examination of ultraviolet-absorbing substances by paper chromatography revealed the occurrence of a variety of nucleotide including not only purine compounds but also pyrimidine nucleotides. The isolation and identification of these nucleotides will be given in the following paper.

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Summary

Seventy strains of type culture and more than 300 bacterial strains isolated from drainage were tested for their properties to excrete purine compounds in their culture fluid using nonexacting purine auxotrophs of *E. coli*. Several strains thus selected excreted a considerable amount of ultraviolet-absorbing substances in their culture fluid. Examination by paper chromatography revealed that ultraviolet-absorbing substances consisted mainly of nucleotides having adenine, guanine, uracil, or cytosine as the base.

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