DEFINED MEDIUM FOR GROWTH OF TWO TRANSFORMABLE STRAINS OF *DIPLOCOCCUS PNEUMONIAE*

H. P. RAPPAPORT AND W. R. GUILD

Department of Biophysics, Yale University,[1] *New Haven, Connecticut*

Received for publication January 26, 1959

The classic bacterial transformation system, and one of the two most thoroughly studied systems, is *Diplococcus pneumoniae*. Transformation systems seem to offer a unique opportunity to study, at the molecular level, the hereditary material, deoxyribonucleate, and the molecular units of the organism it determines. Unfortunately this study has been retarded by a lack of well defined biochemical mutants. In pneumococcus the hereditary markers which have been chiefly studied are capsule type, drug resistance, and sugar utilization. The small number of well defined biochemical mutants has resulted from the lack of a chemically defined medium for growth of the organism. We report here a defined medium which supports the growth of two different transformable strains of pneumococcus and which has allowed us to isolate biochemical mutants.

MATERIALS AND METHODS

Two transformable strains of rough pneumococcus were used, Hotchkiss's R-6 and a strain derived from his R-19, designated by us R-19X. For transformation studies the complex medium of Marmur and Hotchkiss (1955) was used. It consists of casitone (Difco) 1.0 per cent; tryptone (Difco), 0.5 per cent; yeast extract (Difco), 0.1 per cent; NaCl, 0.5 per cent; glucose, 0.1 per cent; bovine albumin powder (Armour), 0.2 per cent; $CaCl_2$, 0.01 per cent; and K_2HPO_4, 0.015 M. To test for transformation, cells were usually subcultured in this medium for 15 generations before transformation was attempted. The transformation marker used was high level streptomycin resistance, isolated by Hotchkiss (1955).

For growth studies of pneumococcus in synthetic medium, the cells were seeded into 1 ml of medium in 13 by 100 mm tubes. The cultures were not shaken or aerated. The incubation temperature was 37 C. Cell counts were made with a Petroff-Hausser counter using dark phase contrast optics at a magnification of 970 or more.

With the exception of tris(hydroxymethyl)-aminomethane (Tris, Fisher Purified), the inorganic chemicals used in the defined medium were either Fisher's Certified Reagent grade or Mallinckrodt's Analytical Reagent grade. The amino acids and vitamins of the medium were of the highest purity obtainable from the California Foundation for Biochemical Research. The purity is listed in table 1. Glucose was Fisher Certified Reagent grade glucose. Pyruvate was obtained either from the California Foundation or Nutritional Biochemical Corporation.

Sterilization was done by filtration, using the Morton (1944) ultrafine fritted disc filter apparatus. Glassware was routinely cleaned by boiling in an aqueous solution of the detergent 7X (Linbro Chemical Company, Inc., New Haven, Connecticut), rinsed 10 to 12 times in tap water, and 5 times in distilled water.

Agar plates contained 15 g/L of agar (Difco) for the complex medium and 10 g/L of purified agar (Difco) for the defined medium. Plating was done either by pouring soft agar containing the cells or by smearing the cells on the surface with a glass rake. Plates were incubated at 37 C.

RESULTS AND DISCUSSION

The medium listed in table 1 was found to support growth of both R-6 and R-19X. Because the cells of both strains grow in chains of varying number, it was not possible to obtain accurate generation times by plating on agar. Instead, cells were counted in a Petroff-Hausser counter. Cultures were diluted (10^1 to 10^6 cells) and the time required to grow to the original concentration (10^7 cells/ml) was observed. No indication of any lag due to concentration effects was observed for cultures inoculated as low as 10 cells/ml. In this way we obtained generation

[1] Aided by grants from the American Cancer Society and the National Science Foundation.

TABLE 1

Composition of defined medium for growth of pneumococcus

Salts		Amt*
Tris(hydroxymethyl)amino-methane		4.84 g
NH_4Cl		2 g
NaCl		5 g
KCl		0.4 g
$MgSO_4 \cdot 7H_2O$ (or 5×10^{-3} g $MgSO_4 \cdot 7H_2O$ plus 0.25 g Na_2SO_4)		0.44 g
$CaCl_2$		0.026 g
Na_2HPO_4		0.18 g
$Fe(NO_3)_3 \cdot 6H_2O$		0.7×10^{-3} g
Amino Acids	**Purity**	
L-Aspartic acid	(CfP)	260 mg
L-Threonine	(CfP)	60 mg
L-Cysteine-HCl	(CfP)	100 mg
L-Histidine-HCl·H_2O	(CfP)	10 mg
L-Arginine-HCl	(CfP)	60 mg
L-Glutamine	(CfP)	300 mg
L-Valine	(CfP)	200 mg
L-Leucine	(CfP)	200 mg
L-Isoleucine	(CfP)	300 mg
Vitamins		
Ascorbic acid	(USP)	50 mg
Choline chloride	(Purified)	1 mg
d-Pantothenate, calcium	(USP)	100 μg
Riboflavin	(USP)	150 μg
Nicotinic acid	(USP)	15 μg
Thiamin-HCl	(USP)	15 μg
Vitamin B_{12} (cyanocobalamin)	(USP)	1.5 μg
i-Inositol	(Purified)	350 μg
d-Biotin	(USP)	15 μg
Pyridoxine-HCl	(USP)	10 μg
Other		
Glucose		2 g
Pyruvate, sodium		0.8 g

* The amounts are for 1 L of distilled water. The pH is adjusted to 7.3 to 7.4 with HCl.

times of 90 to 100 min for R-6 and 200 to 220 min for R-19 at 37 C.

The R-6 strain has been grown over 500 generations in the defined medium with dilution to approximately 10 to 100 cells/ml after every factor of 10^5 to 10^6 in cell multiplication. The property to be transformed to streptomycin resistance was still present after 500 generations. In a similar way, R-19X has been grown over 1000 generations in the medium. However, the latter cultures eventually lost the ability to be transformed to streptomycin resistance. The longest time the culture retained this property was 200 generations.

Both strains clump rather badly and grow in widely varying chain lengths in the defined medium at the higher concentration of $MgSO_4$ listed, but both these phenomena are considerably reduced at the lower $MgSO_4$ concentration. The maximal concentration for both strains is 3×10^8 cells/ml. Under dark phase contrast the cells of R-6 average about 0.6 μ in diameter and the R-19X about 0.8 μ. The latter strain shows a very large distribution in cell size.

Plating efficiency for both strains in the defined medium compared to the complex medium of Marmur and Hotchkiss (1955) minus bovine albumin and $CaCl_2$ is 100 ± 15 per cent. Colonies are visible to the eye after 48 hr in the defined medium. It is necessary to emphasize that the efficiency measured is not per cell but per chain.

Both the amino acids threonine and aspartic acid can be eliminated by growing the cells in a bicarbonate buffer (pH 7.3 to 7.4; 0.19 per cent $NaHCO_3$) and stoppering the growth tubes. The concentration of choline, riboflavin, ascorbic acid, and pantothenate can be reduced a factor of 10 without appreciably affecting the growth rate. The higher levels are listed in table 1 because most of the studies were performed at the higher concentrations. The presence of bovine albumin at 0.2 per cent, necessary for transformation, does not appear to have any effect on the rate of growth in this medium.

Using ultraviolet irradiation and the penicillin concentration method of Davis (1948), Lederberg and Zinder (1948), we have obtained 30 per cent yields of mutants unable to grow on the defined medium. Cells from log phase cultures in complex medium were centrifuged and resuspended in a buffer solution consisting of 0.04 M Tris (pH 7.3), 0.5 per cent NaCl, and 0.2 per cent bovine albumin. Ultraviolet irradiation, 2537 A, was given at a dose leaving 2 to 5 per cent survival on the complex medium. The cells were centrifuged and resuspended in complex medium at a concentration of 10^5 viable cells/ml. The culture

was allowed to grow until it reached 10^8 cells/ml. The cells were centrifuged and washed twice with the above buffer solution and resuspended at 10^5 cells/ml in defined medium to which was added bovine albumin (0.2 per cent) and penicillin (0.1 u/ml). The culture was incubated for 6 to 8 hr at 37 C. At the end of this time the cells were plated on the complex medium. After 18 to 24 hr, colonies were picked and tested for growth on defined and complex medium. Those that did not grow on defined medium were tested twice more. The necessity of 0.2 per cent bovine albumin during penicillin treatment has been investigated in model experiments. R-6 cells were placed in the defined medium minus one amino acid. Colony forming ability on complex medium fell very rapidly with time. The addition of bovine albumin prevented this fall in colony count for many hours. Auxotrophs requiring pyrimidines and specific amino acids have been isolated.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Julius Marmur and Dr. Maurice Fox for strains of transformable pneumococcus.

SUMMARY

A chemically defined medium which supports growth of two transformable strains of *Diplococcus pneumoniae* is reported. Characteristics of growth for the strains are given. A method for the isolation of biochemical mutants is presented.

REFERENCES

DAVIS, B. D. 1948 Isolation of biochemically deficient mutants of bacteria by penicillin. J. Am. Chem. Soc., **70**, 4267.

HOTCHKISS, R. D. 1955 *The Harvey lectures (1953–1954)*. Academic Press, Inc., New York.

LEDERBERG, J. AND ZINDER, N. 1948 Concentration of biochemical mutants of bacteria with penicillin. J. Am. Chem. Soc., **70**, 4267–4268.

MARMUR, J. AND HOTCHKISS, R. D. 1955 Mannitol metabolism, a transformable property of pneumococcus. J. Biol. Chem., **214**, 383–396.

MORTON, H. E. 1944 A new style assembly for fritted filters. J. Bacteriol., **47**, 379–380.