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A DEFINED AGAR MEDIUM FOR GENETIC TRANSFORMATION OF *NEISSERIA MENINGITIDIS*

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ABSTRACT

CATLIN, B. WESLEY (Marquette University, Milwaukee, Wis.) AND GERTRUDE M. SCHLOER. A defined agar medium for genetic transformation of *Neisseria meningitidis*. J. Bacteriol. **83**:470–474. 1962.—An agar medium was developed for use in quantitative genetic studies of *Neisseria meningitidis* strain 15. It contains eight inorganic salts, sodium citrate, sodium lactate, arginine, cysteine, glycine, sodium glutamate, and purified agar. Abundant surface growth in the absence of supplemental carbon dioxide was obtained during 50 serial subcultures. A close correspondence was found between numbers of parental type colonies developing on the defined medium and on a complex medium. Cells subcultured serially three or four times on defined agar medium and placed directly into a solution of transforming deoxyribonucleic acid in defined liquid medium were susceptible to transformation without additional supplements. Of the treated population, 0.1 to 0.3% of the cells were transformed to streptomycin resistance.

Nutritional studies of *Neisseria meningitidis* have been carried out by a number of investigators (see Scherp, 1955), who used the defined liquid medium of Frantz (1942) or modifications of it (Grossowicz, 1945). The Frantz medium contains glutamic acid, cystine, glucose, and five inorganic salts. That the medium is not completely adequate, however, is suggested by requirements for relatively large inoculum sizes and for supplemental carbon dioxide (Grossowicz, 1945; Tuttle and Scherp, 1952), and by indications that growth involved the selection of variants arising from the parent strains (Scherp and Fitting, 1949). Moreover, the medium solidified with agar did not support colonial growth (Frantz, 1942). The supplemental CO_2 required for growth of one strain could be replaced by 0.01% yeast extract (Tuttle and Scherp, 1952). A minimal agar medium with ammonium chloride as the only nitrogen source was developed by Jyssum (1959) for use in metabolic studies. However, isolated colonies did not develop unless more than 1000 cells were plated.

N. meningitidis cultivated in complex media undergoes genetic transformation (Alexander and Redman, 1953; Catlin, 1960). A defined agar medium suitable for growth of transformable strain *N. meningitidis* 15 was sought for use in projected genetic studies. Though the liquid media of Frantz (1942) and of Grossowicz (1945) supported moderate growth of strain 15, colonies did not develop on the surfaces of the corresponding media supplemented with agar. Accordingly, we examined the possible growth-promoting effects of various compounds added singly and in combination, together with agar, to the medium of Grossowicz (1945). The basic formula was altered as the investigation progressed. A defined agar medium was finally obtained which gave essentially 100% recovery of colony-forming cells of strain 15, compared with identical platings on a complex agar medium.

MATERIALS AND METHODS

N. meningitidis strain 15 and transformation test procedures have been described (Catlin, 1960; Catlin and Cunningham, 1961). The strain has been maintained relatively unchanged by preparing 30 tubes of the same culture and keeping them at −60 C. A thawed culture was streaked on the complex medium, subcultured daily, and discarded at the end of 1 week. All cultures were incubated at 37 C in a water-jacketed incubator.

The complex medium (HIY-1 agar; Catlin, 1960) was composed of heart infusion broth (Difco) with 0.3% (w/v) yeast extract (Difco)

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and Bacto-agar (1.4%, w/v, for hard agar and 0.7% for soft agar). After the medium was autoclaved, separately autoclaved supplements were added aseptically to give the following final concentrations: ribonucleic acid (Nutritional Biochemicals Corp.), 250 μg/ml; sodium glutamate, 0.00005 M; and calcium chloride, 0.0005 M.

Cells taken from young cultures on complex or on defined agar medium produced smooth suspensions in defined liquid medium. Microscopic observation showed single and paired cells, rarely groups of three or four cells. Total numbers of colony-forming units were assayed in both complex and defined media by incorporating measured volumes of an appropriate dilution (in defined liquid) in soft agar media (liquefied and held at 44 C). Soft agar, which contains half the agar concentration of the corresponding hard agar medium, was used in volumes of 4 ml for the complex and 5 ml for the defined medium. Immediately after inoculation, the agar was poured over supporting layers of the corresponding hard medium. Colonies were counted after incubation at 37 C for 3 days. The values reported were calculated from the mean number of colonies on five plates. This agar overlay method yielded higher and more uniform colony counts than the surface spreading method, in confirmation of earlier findings with strain 15 on complex agar (Catlin, 1960).

The number of streptomycin-resistant transformants/ml was determined by adding a sample of the transformation reaction mixture (described in footnote to Table 2) to 40 ml (complex medium) or 50 ml (defined medium) of streptomycin-free soft agar (at 44 C). The entire volume was immediately dispensed in 4-ml or 5-ml samples on the surface of a set of 10 plates of the corresponding hard agar medium (20 ± 0.5 ml volumes dispensed several days earlier); 5 min later the plates were placed without stacking in an incubator at 37 C. Phenotypic expression (change from streptomycin susceptibility to resistance) was essentially complete 7 hr after the time of initial exposure of cells to deoxyribonucleic acid (DNA). Plates were removed to room temperature, and each was overlaid with a top layer (4 ml of complex medium or 5 ml of defined medium) of the corresponding soft agar, which was supplemented with a quantity of crystalline dihydrostreptomycin sulfate (Squibb) sufficient to give 500 μg/ml after diffusion of the antibiotic throughout the underlying agar. After an additional period of 45 min (to allow diffusion), plates were returned to 37 C and were not stacked until each had warmed uniformly. Colonies were counted after incubation for 4 days. The oxidase reaction was used to check the identity of colonies on selected plates.

All chemicals used were of the highest purity obtainable from the particular company. The defined medium was developed using Nutritional Biochemical Corp. amino acids and other chemicals obtained from Mallinckrodt Chemical Works or from Merck and Co. In view of the possibility that a trace factor present in these components might be essential for growth of strain 15, further experiments were carried out with fresh supplies of chemicals. Amino acids, except sodium glutamate, were purchased from the California Corporation for Biochemical Research; all other chemicals were purchased from the Fisher Scientific Co. All solutions were prepared in redistilled water; the second distillation was carried out in a Yoe Pyrex glass apparatus (Fisher Scientific Co.).

All glassware was cleaned in detergent, rinsed, and immersed for 24 hr in concentrated sulfuric acid-dichromate solution. (For petri dishes, a fresh 2.5% solution of sulfuric acid was used.) Thereafter, glassware was rinsed 15 times in tap water and 3 times in distilled water.

Sterilization by filtration was carried out either with ultrafine Pyrex fritted-disc filter apparatus, or with Millipore type HA filters mounted in Pyrex holders.

RESULTS

The composition of the defined medium is given in Table 1. Most of the solutions were prepared in quantities sufficient for 8 to 10 liters of medium. They were sterilized separately by filtration (solutions A, C, and D) or by autoclaving (121 to 123 C for 15 to 18 min depending on the volume; solutions B, E, and F). Alternatively, solution A was sterilized by autoclaving; this lowered the pH and necessitated adjustment to pH 7.4 by aseptic addition of 5 N NaOH. Solution C in sterile tubes containing quantities suitable for a single use was stored at −20 C. Solution D was refrigerated; other solutions were stored at room temperature in air-tight Pyrex containers. The double-strength purified agar (solution E) was prepared the same day it was used. To avoid unnecessary heating, it was melted and sterilized

in one step; after thorough mixing, it was placed in a 50-C water bath. Solutions A, B, C, and D were mixed aseptically; the "other" solution (water, carbohydrate, or other supplement being investigated) was added, and the pH of the mixture was determined. If necessary, the pH was adjusted to 7.4 ± 0.05 by aseptic addition dropwise of 1 N NaOH (prepared in sterile redistilled water). The mixture was warmed to 50 C, and was added to the agar. Lastly, the calcium solution (F) was added while the container was being rotated. The well-mixed medium was dispensed into sterile dishes.

The soft agar medium for use in overlays was prepared similarly, except for the final concentration of agar (0.5%). The defined liquid medium used in transformation tests, and for preparing all cellular dilutions, was the same except that sterile redistilled water was substituted for the agar solution.

TABLE 1. *Composition of defined medium for Neisseria meningitidis strain 15**

Solution	Components	Amount	Final concn
		g	*mM*
A	NaCl	5.85	100
	KCl	0.186	2.5
	NH_4Cl	0.401	7.5
	Na_2HPO_4	1.065	7.5
	KH_2PO_4	0.170	1.25
	$Na_3C_6H_5O_7 \cdot 2H_2O$	0.647	2.2
	Water, redistilled	350 ml	
B	$MgSO_4 \cdot 7H_2O$	0.616	2.5
	$MnSO_4 \cdot H_2O$, 0.15 M solution	0.05 ml	0.0075
	Water, redistilled	50 ml	
C	L-Arginine-HCl	0.300	1.4
	L-Cysteine · HCl · H_2O	0.010	0.057
	Glycine	0.100	1.3
	Mono - Na glutamate	1.100	6.5
	Water, redistilled	25.0 ml	
D	Na lactate†	25.0 ml	55.5
Other	Water, redistilled (or other supplement)	50 ml	
E	Purified agar	10.0‡	
	Water, redistilled	500 ml	
F	$CaCl_2 \cdot 2H_2O$, 1.0 M solution	0.5 ml	0.5

* Final pH: 7.4 ± 0.05.

† Prepared from reagent lactic acid (87%): to 23.0 g chilled in an ice bath, 5.0 N NaOH was slowly added with stirring until pH 7.4 was reached (about 40.5 ml); redistilled water was added to a final volume of 100 ml.

‡ To make the same volume of soft agar, 5.0 g was used.

N. meningitidis strain 15 grew abundantly on the surface of the defined agar medium without supplemental carbon dioxide, and its growth was unimpaired after 50 serial subcultures. Isolated colonies were visible, though very small, after incubation at 37 C for 15 hr, provided the agar surface was properly moist and the inoculum was from a 20-hr to 24-hr culture. Diameters of isolated colonies were 2 to 3 mm after incubation for 48 hr.

The adequacy of the defined medium for quantitative transformation studies was investigated, with specific reference to three questions. (i) Does *N. meningitidis* strain 15 undergo transformation after being serially subcultured on defined medium? (ii) Do the numbers of isolated colonies on a series of plates inoculated with samples of a cellular suspension correspond to the dilution plated? (iii) Is there a reasonable correspondence between numbers of colonies of streptomycin-resistant transformants recovered on defined and on complex media?

The experiments reported in Table 2 were carried out with cells which had been subcultured 3 to 16 times serially on defined agar at intervals of 18 to 24 hr. Cells from punctiform surface colonies on defined agar incubated 15 to 18 hr were suspended in warm (36 C) defined liquid medium to give a slight visible turbidity (about 10^7 cells/ml); 10-fold dilutions (expt. 1, 2, 4) or 20-fold dilutions (expt. 3) were mixed with DNA solution (in defined liquid medium at 36 C). The DNA, extracted from *N. meningitidis* strain 15 *str-r* 51, was a purified preparation (ribonuclease-treated, deproteinized; Catlin, 1960) bearing a high-level streptomycin-resistance genetic marker. Control tests, identical except that the DNA was treated with deoxyribonuclease before addition of cells, were included in all experiments, and were typically negative. After a period of exposure to the DNA, samples were taken for assays (Table 2) of total number of cells/ml (E), and for number of streptomycin-resistant transformants/ml (T).

Colony counts for assays of E showed satisfactory correlations between plates inoculated

TABLE 2. *Transformation of Neisseria meningitidis strain 15 cultivated on defined agar; streptomycin-susceptible recipients and streptomycin-resistant transformants assayed in duplicate on defined and on complex media**

Expt.	No. of serial transfers on defined agar	No. of recipients exposed/ml (E) assayed on:		No. of transformants/ml (T) assayed on:		(T/E) × 10^5 determined on:	
		Defined agar	Complex agar	Defined agar	Complex agar	Defined agar	Complex agar
4a	3	1.2×10^7	1.2×10^7	26,494	34,632	221	289
4b	3	1.2×10^6	1.2×10^6	3,683	4,398	307	367
4c	3	1.2×10^5	1.2×10^5	252	382	210	318
1	4	1.8×10^6	1.6×10^6	2,368	2,782	132	174
2a	7	8.2×10^5	8.0×10^5	186	273	23	34
2b	7	8.7×10^4	7.7×10^4	25	33	29	43
3a	16	5.0×10^6	3.8×10^6	620	634	12	17
3b	16	2.4×10^5	2.0×10^5	33	32	14	16

* Cells, taken from defined agar and suspended in defined fluid medium, were mixed at 36 C with solutions of transforming DNA; final concentrations were 4.0 μg DNA/ml (expt. 1, 2, 3) or 10.0 μg/ml (expt. 4). After an incubation period (at 36 C) of 15 min (expt. 1, 2, 3) or 30 min (expt. 4), unbound transforming DNA was inactivated by added crystalline deoxyribonuclease (Worthington; final concentration: 10 μg/ml).

with different volumes (0.05, 0.1, and 0.5 ml) of a single dilution. Also, assays of test suspensions (e.g., 4a, 4b, and 4c of Table 2) accurately reflected the reduction of population size obtained by serial dilution, provided that the tests were sampled promptly.

The numbers of cells plated for assays of T were varied over a 25-fold range in experiment 4 (Table 2); the highest counts were not expected to be accurate, inasmuch as they involved more than 1,000 colonies per plate. Undiluted samples were plated in volumes of 0.5 ml for 4a, 1.0 ml for 4b, and 2.0 ml for 4c. In both defined and complex agar media, maximal yields of transformant colonies were found with 4b. This biasing effect of differences of cellular concentration was apparently independent of the composition of the medium. On the other hand, the yield of streptomycin-resistant transformants was greater by 20 to 30% on complex agar than on defined agar.

DISCUSSION

The defined agar medium satisfies minimal requirements for use in quantitative studies of *N. meningitidis* transformation. Development of colonies in defined medium plated with fewer than 10 to more than 500 cells occurred with equal facility, and numbers of parental-type colonies corresponded to those found on complex media plated in duplicate. The defined agar was intended to be optimal, not minimal (Wilson and Miles, 1955). On a minimal medium with ammonium chloride as the only nitrogen source, 1,000 to 1,500 cells had to be plated to obtain any *N. meningitidis* colonies (Jyssum, 1959). Jyssum obtained a more favorable ratio (about 11:1) of cells plated to colonies developed by increasing the inoculum to 6,000. Some of the components listed in Table 1 are not strictly essential; glycine, for example, was included because it slightly increased the total colony counts. The amino acids are useful as buffers as well as nutrients. Since deviation of the initial pH of the medium beyond the range 7.3 to 7.5 resulted in marked reduction in colony yields, several additional buffers were investigated. Tris(hydroxymethyl)aminomethane and imidazole appeared to be toxic for *N. meningitidis* strain 15.

Defined media are available for genetic transformation studies of *Bacillus subtilis* (Spizizen, 1958), *Diplococcus pneumoniae* (Rappaport and Guild, 1959), and *Haemophilus influenzae* (Talmadge and Herriott, 1960). The defined medium for *N. meningitidis* increases the variety of bacteria which can be investigated in transformation tests employing nutritional markers. Furthermore, additional insight into transformation processes may be gained in the future by comparative studies of several bacterial systems under defined conditions. Similarities and differences can be discerned even now.

In contrast to other systems (e.g., see Anag-

nostopoulos and Spizizen, 1961), susceptibility to transformation develops in *N. meningitidis* without supplementary factors. Cells grown overnight on defined agar medium and placed directly into defined liquid medium with DNA are transformable at frequencies of the same order of magnitude as those observed in studies using complex media (compare results of expt. 1 and 4, Table 2, with those reported earlier: Catlin, 1960; Catlin and Cunningham, 1961).

Both *N. meningitidis* and *D. pneumoniae* (Rappaport and Guild, 1959) apparently undergo population changes which lead to reduced transformation ratios. For the experiments reported in Table 2, serial transfers were made by selecting cells from 10 to 20 typical colonies to minimize population shifts. Nevertheless, the results indicate a tendency toward a lower transformation ratio with repeated serial subculture. Investigation of the ability of *N. meningitidis* to be transformed (Catlin, 1960) will be aided by use of this simple defined medium.

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LITERATURE CITED

ALEXANDER, H. E., AND W. REDMAN. 1953. Transformation of type specificity of meningococci. J. Exptl. Med. **97**:797–806.

ANAGNOSTOPOULOS, C., AND J. SPIZIZEN. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.

CATLIN, B. W. 1960. Transformation of *Neisseria meningitidis* by deoxyribonucleates from cells and from culture slime. J. Bacteriol. **79**:579–590.

CATLIN, B. W., AND L. CUNNINGHAM. 1961. Transforming activities and base contents of deoxyribonucleate preparations from various neisseriae. J. Gen. Microbiol. **26**:303–313.

FRANTZ, I. D., JR. 1942. Growth requirements of the meningococcus. J. Bacteriol. **43**:757–761.

GROSSOWICZ, N. 1945. Growth requirements and metabolism of *Neisseria intracellularis*. J. Bacteriol. **50**:109–115.

JYSSUM, K. 1959. Assimilation of nitrogen in meningococci grown with the ammonium ion as sole nitrogen source. Acta Pathol. Microbiol. Scand. **46**:320–332.

RAPPAPORT, H. P., AND W. R. GUILD. 1959. Defined medium for growth of two transformable strains of *Diplococcus pneumoniae*. J. Bacteriol. **78**:203–205.

SCHERP, H. W. 1955. *Neisseria* and neisserial infections. Ann. Rev. Microbiol. **9**:319–334.

SCHERP, H. W., AND C. FITTING. 1949. The growth of *Neisseria meningitidis* in simple chemically defined media. J. Bacteriol. **58**:1–9.

SPIZIZEN, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Natl. Acad. Sci. U. S. **44**:1072–1078.

TALMADGE, M. B., AND R. M. HERRIOTT. 1960. A chemically defined medium for growth, transformation, and isolation of nutritional mutants of *Hemophilus influenzae*. Biochem. Biophys. Research Communs. **2**:203–206.

TUTTLE, D. M., AND H. W. SCHERP. 1952. Studies on the carbon dioxide requirement of *Neisseria meningitidis*. J. Bacteriol. **64**:171–182.

WILSON, G. S., AND A. A. MILES. 1955. Topley and Wilson's principles of bacteriology and immunology. The Williams and Wilkins Co., Baltimore, vol. 1, p. 75.