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Production of Exotoxin A by *Pseudomonas aeruginosa* in a Chemically Defined Medium

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A defined medium was developed in which easily measured quantities of exotoxin A (PE) were produced by *Pseudomonas aeruginosa* PA-103. The medium contained three L-amino acids (arginine, aspartic acid, and alanine), basal and trace salts including 14 mM K_2HPO_4, 14 mM glucose, and 140 mM glycerol. The concentrations of amino acids which yielded most satisfactory results were 6 mM alanine, 13 mM aspartic acid, and 16 mM arginine. The identity of PE in the culture supernatant fluid was demonstrated by adenosine diphosphate-ribosyl transferase activity and by immunodiffusion with sheep antitoxin elicited with purified PE and with PE produced in Trypticase soy broth dialysate and pure PE as controls. PE production was also demonstrated by mouse lethality and passive hemagglutination. As compared to Trypticase soy broth dialysate, *P. aeruginosa* produced 25 to 50% PE in the defined medium. Different strains of *P. aeruginosa* produced PE in the defined medium in proportions relative to those in Trypticase soy broth dialysate.

The importance of exotoxin A (PE) as one of the most potent virulence factors produced by *Pseudomonas aeruginosa* has been well established. The purified toxin has a characteristic median lethal dose of 0.1 μg or less in laboratory mice (4, 9), is cytotoxic for a variety of cell lines (15, 18), and specifically inhibits protein synthesis both in vivo and in vitro by adenosine diphosphate (ADP)-ribosyl transferase activity on elongation factor 2 (8, 16). Toxic factors in experimental models can be neutralized by specific antitoxin, and antibodies to toxin have been detected in sera from patients with infections caused by *P. aeruginosa* (13, 17, 19).

The medium of choice for the cultivation of *P. aeruginosa* and production of PE for these and other studies as well is the dialyzable portion of Trypticase soy broth (TSBD) described originally by Liu (12). Using proper conditions for growth and medium supplements such as glycerol and monosodium glutamate, substantial quantities of PE can be recovered from the supernatant fluid. Bjorn et al. (2) showed recently that when the iron concentration was increased from 0.05 to 1.5 μg/ml of TSBD, there was a 90% decrease in PE production.

Aside from these data, however, little is known about the substances in TSBD or culture conditions that are essential for the production of PE. The purpose of this study was to develop a chemically defined medium easily prepared from low-molecular-weight materials and from which measurable amounts of PE are produced by *P. aeruginosa*. Although the organism can be easily grown in different media and with varied growth conditions, the requirements for PE production have been shown to be much more specific (2, 12). Therefore, the development of this medium should provide a more thorough understanding of materials required and/or most beneficial for toxin biosynthesis.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strain PA-103, isolated by Liu (11), was used for all experiments except where otherwise indicated. This organism produces PE but negligible amounts of protease (3, 11, 21). *P. aeruginosa* strain WR-5, a nontoxigenic, protease-producing strain (3), was kindly provided by J. C. Sadoff, Walter Reed Army Institute of Research, Washington, D.C.; *P. aeruginosa* strain PAKS-1, which produces both PE and protease, and a spontaneous mutant of the organism, strain PAKS-10, which produces low levels of protease (23), by B. Wretlind, Karolinska Hospital, Stockholm, Sweden; *P. aeruginosa* strain M-2, which produces protease and moderate amounts of PE, by I. Holder, Shriners Burns Institute, Cincinnati, Ohio; *P. aeruginosa* strain 388, which produces protease and negligible amounts of PE, by B. Iglewski, University of Oregon Medical School, Portland.

Culture conditions and media. Undefined culture medium (TSBD) was the dialysate of Trypticase soy broth (Baltimore Biological Laboratory, Cockeysville, Md.) with 1% glycerol and 50 mM monosodium glutamate (12). Synthetic culture media were prepared from the following stock solutions: 25 ml of a twofold L-amino acid concentrate in 0.01 N HCl adjusted to

pH 7.5 with 6 N NaOH; 12.5 ml of a fourfold basal salt solution, pH 7.5; 12.5 ml of distilled water or a fourfold solution of glucose or K_2HPO_4, or both; and 0.1 ml of a trace salt solution containing nitrilotriacetate as a chelator to avoid formation of insoluble ion complexes. Final medium concentrations of salt solutions are shown in Table 1. Amino acids and basal salt solutions were sterilized by autoclaving, and the other solutions were sterilized by filtration.

Shake cultures were grown at 32°C and 250 rpm for 20 to 24 h in an environmental shaker incubator (New Brunswick Scientific Co., Inc., New Brunswick, N. J.). To achieve maximum aeration, each culture was prepared with 50 ml in a 300-ml Nephalo flask with baffles (Bellco Glass, Inc., Vineland, N.J.). Cell densities were measured by removing 1 ml or less from a culture, making an appropriate dilution with normal saline, and reading the absorbance at 620 nm. Each culture inoculum was prepred from Trypticase soy agar slants grown overnight at 32°C. The growth on the slants was suspended in normal saline and diluted so that 1 ml was used for the inoculation of each flask with an experimental medium. Starter cultures, containing media identical to those in the experimental flasks, were inoculated to obtain an initial absorbance of 0.05 and were grown to an absorbance of 0.5. Five milliliters was used to inoculate the corresponding experimental flasks.

After incubation, the cells were removed by centrifugation at 12,000 × *g* for 20 min at 4°C and filtration of the supernatant fluid (spent medium) through a membrane filter (0.45-μm porosity). Spent media were kept cold throughout all procedures, and samples were stored at −20 or −70°C. When necessary, medium samples were concentrated by ultrafiltration with YM-10 membranes (Amicon Corp., Lexington, Mass.), which have a low protein-binding capacity.

Biochemical assays. Protein was measured according to the manufacturer's instructions, using a protein dye reagent (Bio-Rad Laboratories, Inc., Richmond, Calif.); 0.2 ml of the reagent was added to a 1.2-ml sample and mixed immediately. Pure bovine gamma globulin (Bio-Rad Laboratories, Inc.) was used as the standard, and the absorbance of each sample was determined at 595 nm. Protease activity was measured according to the method of Wretlind and Wadstrom (24). The substrate was 1% electrophoretically pure casein (Difco Laboratories, Inc., Detroit, Mich.) in 0.05 M sodium phosphate buffer, pH 7.4.

Hemolysin assays were performed by adding 0.2-ml samples to 1-ml volumes of a 0.5% prewashed erythrocyte suspension. Potassium phosphate buffer, pH 7.0, 0.01 M in normal saline, was used to prewash blood cells three times and prepare dilutions of samples. The reactants were incubated for 2 h at 37°C, mixed at each 0.5-h interval, and centrifuged for 5 min to remove unlysed cells. The absorbance of each supernatant fluid was determined at 540 nm.

TABLE 1. *Salt composition of synthetic medium*

Component	Concn ($M \times 10^{-3}$)
Basal salts	
NaCl	42.7
KCl	33.5
Na_2HPO_4	1.4
Trace salts	
$MgSO_4$	0.415
$MnCl_2 \cdot 4H_2O$	0.025
$FeCl_3$	0.001
NTA[a]	0.021

[a] NTA, Nitrilotriacetic acid.

Toxin assays. Hemagglutination inhibition (HI) assays were performed according to the method of Pollack and Taylor (20). The Veronal-buffered saline used in this assay consisted of normal saline with the following added components: 0.1% bovine serum albumin, 4 mM barbituric acid, 1.8 mM sodium barbital, 1 mM $MgCl_2 \cdot 6H_2O$, and 0.15 mM $CaCl_2 \cdot 2H_2O$. HI assays were read within 4 h to prevent interference with possible hemolytic activity.

ADP-ribosyl transferase activity as described by Iglewski and Kabat (8) was measured with wheat germ as the source of elongation factor-2 (6). The reaction mixture contained 50 μl of wheat germ extract, 25 μl of a twofold dilution with water of nicotinamide [^{14}C]-adenine dinucleotide (302 mCi/mmol; Amersham/Searle, Arlington Heights, Ill.), 10 μl of toxin preparation, and 50 μl of buffer consisting of 125 mM tris(hydroxymethyl)aminomethane (pH 8.2), 0.2 mM ethylenediaminetetraacetic acid, and 100 mM dithiothreitol. Toxin was activated with 4 M urea and 1% dithiothreitol for 15 min at 23°C. The reactions were initiated with the addition of the isotope, incubated for 10 min at 23°C, and stopped with 10% trichloroacetic acid. Precipitates were collected, washed, and counted (5).

Swiss white female mice (NIH/Nmri CV strain) weighing 20 ± 2 g were injected intravenously with 0.2-ml samples to compare lethal effects of spent media. Ouchterlony assays were used to compare antigenic identity of toxin preparations.

Amino acid uptake. Uniformly labeled L-^{14}C-amino acids (Amersham/Searle) were each dissolved in 10 ml of normal saline and sterilized by filtration. Each amino acid (7.5 μCi in 1.5 ml) was added to an individual culture flask before inoculation with the organism. These cultures were incubated, and 0.2-ml samples were taken at 0, 1, 4, and 8 h. With a 1-ml syringe, each sample was injected into a 25-ml Erlenmeyer "reaction" flask. Each flask contained 2 ml of 15% trichloroacetic acid and was fitted with a rubber cap from which was suspended a small plastic cup containing filter paper soaked with 0.2 ml of 1 M Hyamine hydroxide in methanol to trap CO_2 (22). CO_2 was released from the alkaline spent media when acidified with the trichloroacetic acid and was absorbed by the CO_2 trap. The flasks with samples were allowed to shake in a water bath for 1 h at 25°C. CO_2 production was determined from counts of the filter paper-cup combination, and protein incorporation was determined on trichloroacetic acid precipitates, which were collected and washed with 10% trichloroacetic acid on membrane filters (0.45-μm porosity) presoaked in uninoculated medium to avoid nonspecific absorption. Counts per minute were determined using a liquid scintillation counter (Beckman Instrument Co., Inc., Irvine, Calif.) after putting each filter in 7.5 ml of Hydromix (Yorktown Research, S. Hackensack, N.J.).

RESULTS

The systematic approach described by Callahan et al. (5) for cholera toxin and recently by Alderete and Robertson (1) for heat-stable enterotoxin from *Escherichia coli* was used for initial determinations of the amino acid requirements for the production of PE. Nine combinations of amino acids were prepared as shown in Table 2. Each combination consisted of four amino acids with a single amino acid overlap between different combinations, plus the salts listed in Table 1. The amino acid combinations were determined empirically, and combinations which would precipitate were avoided (5). Equal quantities of individual amino acids (except tyrosine and cystine, which have low solubilities) were used, and the final amino acid concentration for each medium was 1%. There was no additional source of energy. Cultures were sampled for growth at various intervals, and protein and toxin concentrations were determined after 20 h.

Toxin levels were determined after concentrating each spent medium 5- to 10-fold by ultrafiltration. Volumes were measured before and after concentration, toxin was measured by HI in each concentrate, and values were corrected for volume changes. Each value shown in Table 2 is the mean of three experiments in which HI titers did not vary more than one serial dilution. With few exceptions the final pH values of the media ranged from 7.7 to 8.2. These values varied not more than two-tenths of a pH unit with the same medium between experiments and did not appear to affect PE synthesis. Although medium 8 contained only about 10% of the toxin produced in TSBD, it contained more toxin than any of the other synthetic media and was the only formulation where toxin could be detected before concentration. The spent media containing detectable toxin showed more protein and, except for medium 3, the highest levels of growth.

TABLE 2. *Effect of amino acid mixtures on toxin synthesis, extracellular protein, and growth*

Medium	Amino acid mixture[a]	Growth[b] (absorbance)	Extracellular protein[c] (μg/ml)	Toxin[d] (μg/ml)
1	his phe glu arg	2.7	35	0.068
2	gly leu tyr ser	0.8	19	ND[e]
3	cys ile try ala	1.3	50	0.041
4	met val thr asp	1.7	4	ND
5	his gly cys met	0	2	ND
6	phe leu ile val	1.6	13	ND
7	glu tyr tyr thr	1.6	30	ND
8[f]	arg ser ala asp	2.7	182	0.453
9	pro lys ala ile	2.8	38	0.035
TSBD		8.3	75	3.69

[a] Amino acid abbreviations from *Biological Chemistry* (14).
[b] Peak absorbance values (growth at 12 to 13 h) at 620 nm.
[c] Determined before concentrating media.
[d] By HI after concentrating spent media and correcting for volume changes.
[e] ND, Not detectable.
[f] Designated T8 medium.

A comparison of amino acids in those combinations in which toxin was found revealed alanine common to three of the four and arginine to two of the four. Other combinations, using amino acids from the four media in which toxin was detected, were prepared and tested; however, no combination could be found superior to medium 8 (henceforth designated T8 medium).

Individual amino acids from T8 medium were tested in different combinations to determine empirically which amino acids were important for PE production. The total amino acid concentration was maintained at 1%, and equal quantities of amino acids were used (Table 3). Less toxin was measured when either alanine or arginine was absent from the medium. The absence of serine in medium T8A did not affect growth or toxin production. Although the absence of aspartic acid in medium T8B did not affect toxin production, growth measurements indicated that it lengthened the lag phase by 1 to 2 h (Fig. 1).

Toxin characterization. The identity of the toxin produced in the chemically defined media with that produced from TSBD was checked by mouse lethality, ADP-ribosyl transferase activity, and antigenicity. Fivefold concentrates of T8 medium and TSBD were lethal for mice within 24 h. Concentrates of the other synthetic media (Table 2) did not cause death in mice within 5 days. ADP-ribosyl transferase activity was de-

TABLE 3. *Effect of amino acid combinations based on T8 medium*

Medium	Amino acid mixture[a]	Toxin (μg/ml)	Growth[b] (absorbance)
T8	arg ala asp ser	0.304	3.2
A	arg ala asp	0.304	2.7
B	arg ala	0.304	2.8
C	ala asp	0.152	2.8
D	arg asp	0.152	2.8
E	ala ser	ND[c]	0.6
F	arg ser	ND	0.6

[a] Equal quantities of each amino acid were added to each medium; total amino acid concentration in each medium was 1%. arg, Arginine; ala, alanine; asp, aspartic acid; ser, serine.
[b] Peak absorbance values (growth at 12 to 13 h) at 620 nm.
[c] ND, Not detectable.

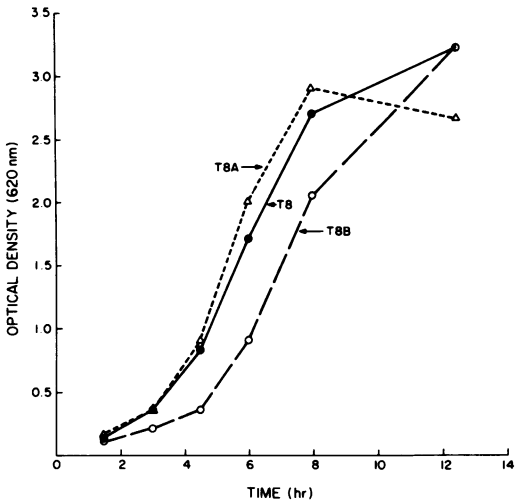

FIG. 1. *Growth of P. aeruginosa strain PA-103 on chemically defined media different only with amino acids, which totaled 1% (wt/vol) in each medium. T8 contains 12 mM arginine (arg), 24 mM serine, 28 mM alanine (ala), and 19 mM aspartic acid (asp); T8A contains 16 mM arg, 37 mM ala, and 25 mM asp; T8B contains 24 mM arg and 56 mM ala.*

termined as the difference in counts per minute between activated (treated with dithiothreitol and urea) and untreated toxin samples. Difference in counts per minute varied less than 10% between tests, and pure toxin samples were used as controls. Mean values for T8 medium and TSBD were 1.4×10^3 and 9.1×10^3 cpm, respectively. A similar difference was observed in the amounts of toxin measured by HI tests (Table 2).

Ouchterlony gel diffusion was performed using specific antiserum to PE, which was tested against samples of pure toxin, TSBD spent medium, and spent medium T8. Complete identity was observed among the samples (Fig. 2).

Glucose, glycerol, and phosphate supplements. Commercial preparations of Trypticase soy broth contain 14 mM (final concentration) each glucose and K_2HPO_4. Also, Liu (12) reported that toxin production could be increased with small amounts of glycerol. Medium T8A was selected to test the effects of varying amounts of K_2HPO_4 in the presence of glucose alone and glucose plus 1% glycerol (Table 4). Potassium chloride was added in inverse proportions to K_2HPO_4 to help balance the free potassium ions in each medium. Glucose improved toxin synthesis substantially (Table 2). As the concentration of K_2HPO_4 decreased, the quantity of toxin produced increased. Moreover, the glycerol caused a slight increase in toxin production.

The spent media from T8A contained a hemolytic factor which was observed in those preparations without K_2HPO_4. Production of this hemolytic factor was strictly dependent on the absence of small quantities of phosphate. As little as 5 mM K_2HPO_4 inhibited production, as determined by the hemolysin assay, but hemolytic activity was easily measured in media with KCl (Table 5). The hemolytic factor could be titrated linearly with both human type O and ovine erythrocytes. The significance of this factor is presently under investigation.

Adjustment of amino acid concentrations. Medium T8A with 14 mM glucose, 14

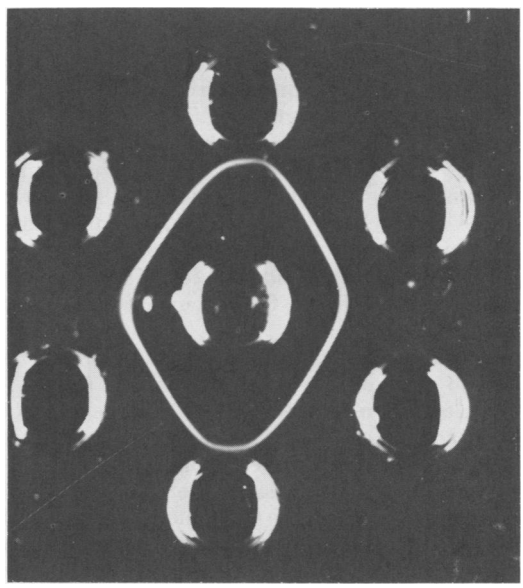

FIG. 2. *Ouchterlony gel diffusion pattern: control well, sheep antitoxin; clockwise from the top or bottom well: spent medium 8, spent TSBD medium, and pure exotoxin A (PE). The plate was prepared with 0.75% agarose and 0.2% sodium azide in normal saline.*

TABLE 4. *Effect of glucose, phosphate, and glycerol supplements on toxin production in a synthetic medium*

K_2HPO_4 (mM)	KCl (mM)	Toxin (μg/ml)	
		+ Glucose[a]	+ Glucose and glycerol[b]
0	25	0.728	ND[c]
5	20	0.624	0.936
10	15	0.520	0.624
25	0	0.416	0.624

[a] Final concentration of glucose was 2.5 g/liter.
[b] Final concentration of glycerol was 1%.
[c] ND, Not detectable because hemolysis was rapid and present in all wells.

TABLE 5. *Extracellular protein, toxin, and protease production from Pseudomonas strains on T8A synthetic medium*

Strain	Extracellular protein (μg/ml)	Toxin[a] (μg/ml)	Protease (U)
PA-103	160 (146)[b]	1.22 (4.86)	0 (0)
PAKS-1	193 (195)	0.61 (1.22)	0.59 (1.23)
PAKS-10	480 (453)	0.61 (1.22)	0.01 (0.70)
M-2	207 (252)	0.076 (0.038)	0.21 (1.44)
WR-5	239 (533)	0 (0)	0.49 (1.67)
388	153 (373)	0 (0)	0 (0.24)

[a] By HI titration.

[b] Numbers in parentheses are values determined from TSBD.

mM K_2HPO_4, and 1% glycerol was used to determine optimal concentrations of the three amino acids. The amount utilized for incorporation into protein and for CO_2 production was measured. There was less than 5% variation in values from separate experiments. The results shown in Fig. 3a and b indicated that about twice as much aspartic acid and alanine were used as arginine. After 8 h 46% of the alanine, 44% of the aspartic acid, and 23% of the arginine were metabolized to CO_2 or incorporated into protein.

Initial concentrations (0.33% of each) of the amino acids were 16 mM arginine, 37 mM alanine, and 25 mM aspartic acid. On the basis of the radioisotope studies, alanine and aspartic acid concentrations were doubled either individually or together. In each case the quantity of measurable toxin in the spent medium decreased two- to fourfold. Cultures were prepared with the concentrations of either alanine or aspartic acid, or both, reduced by half to 19 mM alanine and 13 mM aspartic acid. Higher toxin titers were found in cultures with low levels of alanine. The lower aspartic acid concentrations had no apparent effect. The medium was prepared with 13 mM aspartic acid and 11 mM arginine; the alanine concentration was reduced by serial twofold dilutions. The results are shown in Fig. 4. As alanine was reduced, there was a concomitant rise in toxin production; however, below 0.5% (6 mM) alanine, the toxin titer decreased.

The final concentrations of amino acids for T8A medium were set at 16 mM arginine, 13 mM aspartic acid, and 6 mM alanine. PE production was not affected by the addition of 14 mM K_2HPO_4 after final adjustment of the amino acid concentration. The final formulation included K_2HPO_4, glucose, and glycerol supplements. The defined medium, with the adjusted amino acid concentrations, produced yields of 25 to 50% of the exotoxin A in the spent medium when compared with TSBD. However, the amount of growth after 12 h was approximately the same as that measured with T8 medium (Table 2). HI titers for toxin ranged from 1:64 to 1:128 with synthetic media and from 1:128 to 1:512 with TSBD.

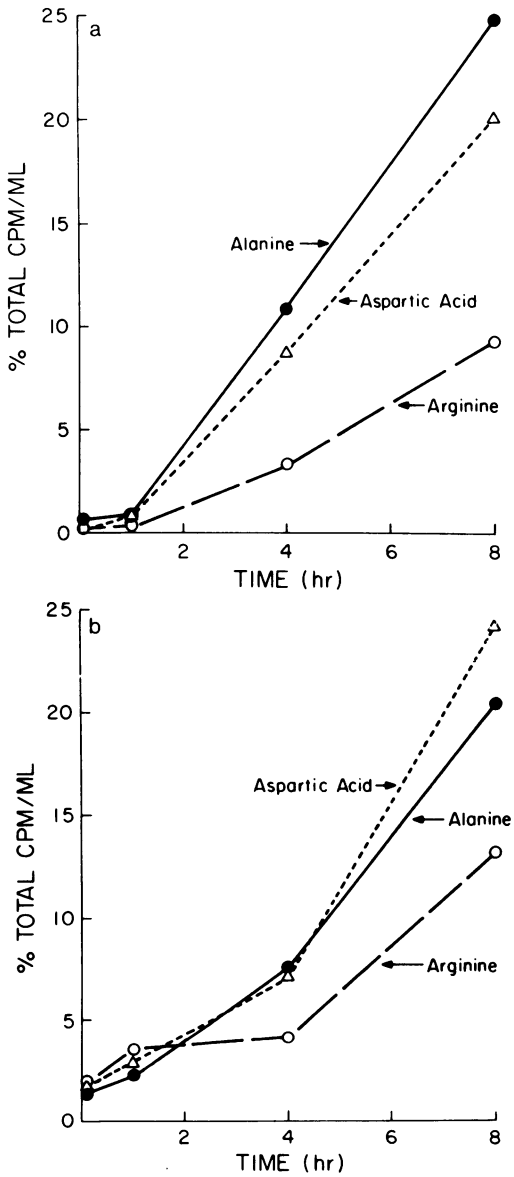

FIG. 3. *(a) Incorporation of isotopic amino acids into protein by P. aeruginosa grown in a chemically defined medium (see text). The counts per minute of the uninoculated medium per milliliter were approximately 2.0 × 10^5 for each amino acid. (b) Production of CO_2 from isotopic amino acids during growth of P. aeruginosa in a chemically defined medium (see text). These values were determined at the same time and from the corresponding samples as shown in 3(a).*

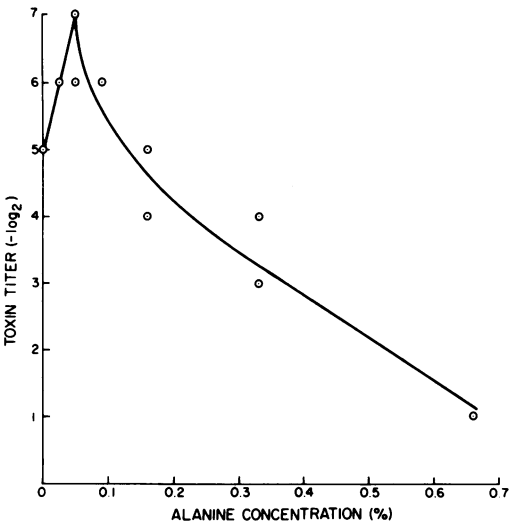

FIG. 4. *Relationship between alanine concentration and exotoxin A production (measured by HI) by P. aeruginosa in a chemically defined medium. Points represent values determined by three separate experiments. An alanine concentration of 0.05% (6 mM) was optimal for toxin synthesis.*

Tests with other *Pseudomonas* strains. The final formulation of T8A medium was used to test other strains of *Pseudomonas* as well as PA-103 (Table 5). Although quantities of exotoxin A and extracellular protein varied widely among the strains, relative proportions of toxin produced in TSBD and the synthetic medium were similar. Protease activity showed wider variation than toxin, but this was probably due to the fact that the synthetic medium was developed for optimal synthesis of exotoxin A and not protease.

DISCUSSION

A relatively simple, but completely defined medium was developed in which *P. aeruginosa* is capable of producing PE in quantities which can be easily measured. The design of this synthetic medium was based on a systematic analysis of amino acid combinations coordinated with environmental and nutrient conditions established for TSBD. Not only does the synthesis of PE require specific conditions, but it is especially sensitive to the nature and quantity of such substances as free iron (2) and alanine. Moreover, these requirements do not necessarily coincide with the growth of the organism.

Previous observations by Liu (10) using a synthetic agar medium with strains other than PA-103 indicated that alanine, aspartic acid, and glutamic acid improved synthesis of extracellular toxins (hemolysin, lecithinase, and protease). These amino acids were selected for that medium because they were present in greatest quantities in muscle extracts and other animal tissues. Later, it was shown that monosodium glutamate was an adequate replacement for alanine or aspartic acid to improve PE synthesis in TSBD (12). The results with various amino acid combinations in the present study agree with the previous findings of Liu. Alanine, aspartic acid, and arginine are important for production of PE. Arginine is apparently a suitable replacement for glutamic acid in supporting toxin production since *P. aeruginosa* has enzyme systems which allow interconversion of these compounds (7). PE production in this medium was at least three to five times higher than that previously reported by Liu using a simple salts medium with alanine, aspartic acid, and glutamic acid to culture strain PA-103 with similar growth conditions (12).

Although PE production is generally higher in the media with the highest levels of growth, the correlation between toxin production and growth is not absolute. Once medium T8A was determined, the amount of toxin produced in this medium varied almost exclusively with respect to the particular supplement added, such as glucose or glycerol, or with the concentration of alanine. However, growth in this medium, regardless of these substances, was about 30% of that in TSBD in all cases. Therefore, although the addition of glucose and glycerol approximately doubled the amount of toxin in T8A and although decreasing the alanine concentration increased toxin production, growth of *P. aeruginosa* remained essentially unchanged.

On this basis and with regard to the studies involving uptake of specific amino acids, synthesis of PE depends apparently on biochemical control mechanisms which remain to be explained. Although aspartic acid and alanine were utilized at approximately twice the rate of arginine, a simple balance of amino acid concentrations to offset these rate differences did not improve synthesis of exotoxin A. Indeed, adjusting the amino acid concentrations decreased toxin production because of the influence of high concentrations of alanine. The control system(s) responsible for PE synthesis is apparently sensitive to high concentrations of alanine, as has been established with iron (2). High iron concentrations also inhibited PE synthesis in the synthetic medium (unpublished observation). A similar phenomenon was observed with the synthesis of an extracellular hemolytic factor produced in the absence of K_2HPO_4. Small quantities of phosphate inhibited production of the hemolytic factor, which did not lose activity in a potassium phosphate buffer used for the he-

molysin assays. Under the same conditions with and without K_2HPO_4, PE production was essentially unaffected. In light of these observations, it is apparent that specific small molecules acting as catabolite repressors or other types of regulators can effectively control expression of particular extracellular proteins.

The quantity of toxin obtained from the defined medium is 25 to 50% of that measured in TSBD, which is of sufficient quantity to determine what factors affect PE synthesis using HI, ADP-ribosyl transferase activity, or mouse lethality as the assay system. The use of this defined system in future studies should help determine the effects of a variety of other materials such as carbon sources, metal ions, and coenzymes, which may either control toxin biosynthesis or act as specific precursors of exotoxin A.

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