INFECTION AND IMMUNITY, Jan. 1983, p. 483–486
0019-9567/83/010483-04$02.00/0

Vol. 39, No. 1

Elaboration of *Bacillus anthracis* Antigens in a New, Defined Culture Medium

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Received 17 June 1982/Accepted 7 October 1982

Improved culture conditions and a new, completely synthetic medium (R medium) were developed to facilitate the production of *Bacillus anthracis* holotoxin antigens. Levels of these antigens up to fivefold greater than the highest previously reported values were recovered with the described system. Cultures of Sterne, V770-NP1-R, and Vollum 1B strains of *B. anthracis* were monitored for growth, pH change, glucose utilization, supernatant protein concentration, lethal toxin activity, and protease activity.

The tripartite toxin of *Bacillus anthracis* is a omplex toxin or toxic measure (3) composed of three polypeptide components (2, 17): edema factor (EF; factor I), protective antigen (PA; factor II), and lethal factor (LF; factor III). None of the factors alone possesses demonstrable toxic activity (4, 9, 14); however, intravenous injection of PA plus LF kills mice (14), rats (1, 2), and guinea pigs (16), and intradermal injection of PA plus EF produces edematous lesions in the skin of guinea pigs (5) and rabbits (15). LF, PA, and possibly EF are individually immunogenic. Parenteral administration of combinations of the three factors or of LF or PA alone elicits various degrees of protection from toxin or spore challenge in experimental animal hosts (10, 17).

For studying the mechanism of action of anthrax toxin and its role in *B. anthracis* pathogenesis and as a prerequisite to vaccine development, investigators have devoted considerable attention to the production and purification of the three toxin components. Previous attempts to improve both growth media and culture conditions for production of protective antigen (11, 12, 23) or whole toxin (19, 20) have led to the development of the 1095 medium of Wright et al. (23) and the Casamino Acids medium described by Haines et al. (6). Unfortunately, rather low yields of protective antigen are produced in the 1095 medium (7), and the Casamino Acids medium is incompletely defined and contains activated charcoal powder (6). We therefore undertook to formulate a new synthetic medium (R medium) that would permit high recovery of the toxin components.

The R medium was developed by modifying the Casamino Acids (CA) (20) medium in the following ways. (i) The quantities of individual amino acids found in 3.6 g of yeast extract were substituted for 3.6 g of Casamino Acids (13); (ii) the concentration of glucose was raised from 0.2 to 0.25%; (iii) monobasic potassium phosphate was omitted, and the concentration of dibasic potassium phosphate was increased from 0.088 to 0.3% to provide increased buffering; and (iv) activated charcoal powder was omitted.

The components of the medium are as follows (milligrams per liter): L-tryptophan, 35; glycine, 65; L-cystine, 25; L-tyrosine, 144; L-lysine, 230; L-valine, 173; L-leucine, 230; L-isoleucine, 170; L-threonine, 120; L-methionine, 73; L-aspartic acid, 184; sodium L-glutamate, 612; L-proline, 43; L-histidine-hydrochloride, 55; L-arginine-hydrochloride, 125; L-phenylalanine, 125; L-serine, 235; thiamine-hydrochloride, 1.0; glucose, 2,500; $CaCl_2 \cdot 2H_2O$, 7.4; $MgSO_4 \cdot H_2O$, 9.9; $MnSO_4 \cdot H_2O$, 0.9; K_2HPO_4, 3,000; $NaHCO_3$, 8,000; uracil, 1.4; and adenine sulfate, 2.1. The pH was adjusted to 8.0 with 5.0 N NaOH before filter sterilization. The medium was filter sterilized under positive pressure and stored at 4°C in tightly capped Erlenmeyer flasks. Growth characteristics of three *B. anthracis* strains in the new, defined medium are shown in Fig. 1. The Vollum 1B strain, unlike the others, showed no lag phase; however, all three strains reached stationary phase, approximately 2×10^8 colony-forming units (CFU)/ml, after 16 to 20 h of culture. Logarithmic-phase doubling time was 35 to 40 min for the Sterne and V770-NP1-R strains and approximately 50 min for the Vollum 1B strain. The viability of the Sterne and V770-NP1-R strains decreased to about 10^7 CFU/ml between 20 and 42 h. Microscopic examination of material from the Vollum 1B culture revealed marked conversion of long chains into shorter chains and individual bacilli beginning at 24 h postinoculation. This resulted in a temporary increase in viable count followed by a decrease

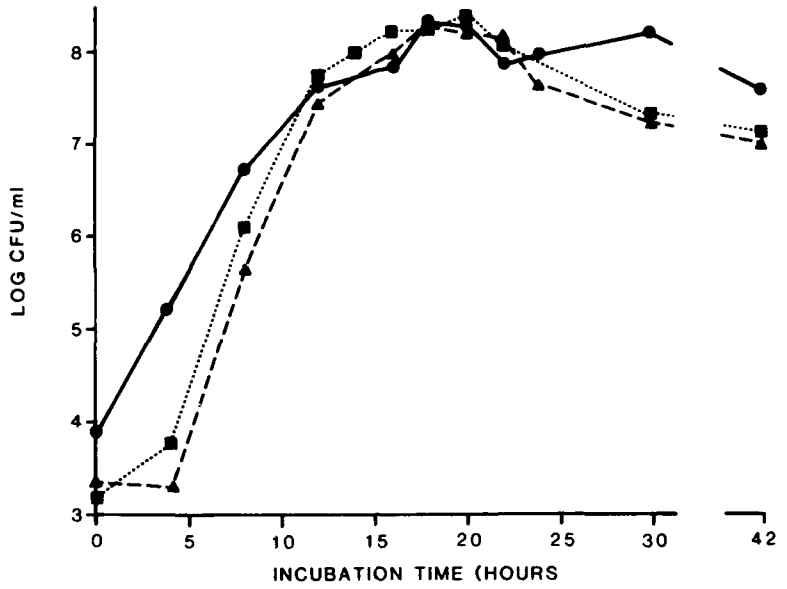

FIG. 1. Growth kinetics in the R medium for *B. anthracis* strains Sterne (▲), V770-NP1-R (■), and Vollum 1B (●). Culture protocol: blood agar plates were inoculated with *B. anthracis* and incubated for 12 to 16 h at 37°C in 5% CO_2. Individual colonies were suspended in 10 ml of sterile phosphate buffered saline to a density of approximately 10^6 CFU/ml (approximately 0.01 absorbance units of 540 nm on a Coleman Junior spectrophotometer, using a 1.0-cm light path). Erlenmeyer flasks half-filled with R medium were inoculated with 1.0 ml of the *B. anthracis* suspension per 500 ml of medium, yielding an initial concentration of approximately 2×10^3 CFU/ml. Flasks were tightly capped to prevent loss of CO_2 and then incubated in a controlled environment shaker (New Brunswick Scientific, New Brunswick, N.J.) at 37°C with 60 oscillations per min. At predetermined times the flasks were removed and gently swirled, and samples were taken.

to 4×10^7 CFU/ml at 42 h.

The rate of glucose utilization was similar in the three *B. anthracis* strains. By 16 h more than 95% of the glucose had been depleted from the medium. Concomitant with the decrease in glucose was a drop in pH from the initial of 8.1 ± 0.1 to between 7.2 and 7.4.

Concentrations of supernatant protein in the three cultures increased slowly up to 24 h. In stationary phase the protein concentrations for the V770-NP1-R, Vollum 1B, and Sterne strains were 34, 60, and 75 μg/ml, respectively. Low levels of proteolytic activity (18) were demonstrable in the culture supernatants of all three strains.

The lethality-producing activity (2) in the culture supernatants of the three strains is shown in Fig. 2. Toxin was first detectable at 12 h, when cultures were in late log phase. The Sterne, V770-NP1-R, and Vollum 1B strains showed peak toxin activities of 70, 75, and 160 toxic units (TU)/ml at 16, 22, and 20 h, respectively. Peak lethality-producing activity coincided with peak edema-producing activity titers (20) of 16 for Vollum 1B and 8 for Sterne and V770-NP1-R.

The maximum serological and biological activity levels attained are listed in Table 1. The highest PA and LF serological activity titers consistently correlated with maximum lethal toxin activity levels recovered from the three

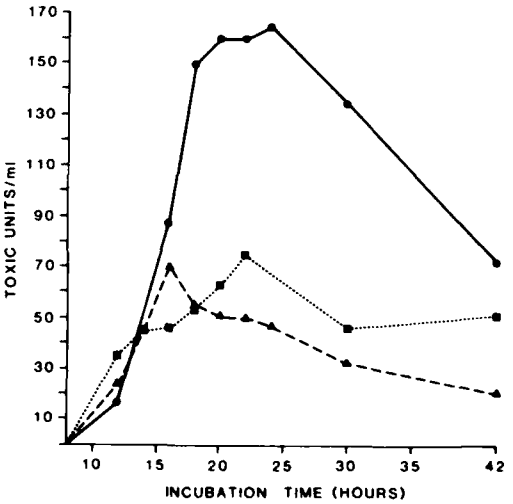

FIG. 2. Supernatant lethality-producing activity in R medium of strains Sterne (▲), V770-NP1-R (■), and Vollum 1B (●). Culture parameters were identical to those described in the legend to Fig. 1.

TABLE 1. Peak production of PA, LF, and lethal toxin in 1095, CA, and R media by *B. anthracis* strains Sterne, V770-NP1-R, and Vollum 1B[a]

Strain	Reciprocal Ouchterlony titer[b] in medium:						TU/ml of medium[c]		
	1095		CA		R				
	PA	LF	PA	LF	PA	LF	1095	CA	R
Sterne	1	—[d]	4	1	4	2	5	58	84
V770-NP1-R	1	—	1	—	4	1	23	25	79
Vollum 1B	1	—	8	2	8	2	15	143	202

[a] Culture conditions were similar to those described in the legend to Fig. 1 except that only one sample was taken from any flask.

[b] Serological activity was assayed by the double-diffusion Ouchterlony technique with antiserum possessing both PA and LF activity.

[c] Lethal toxin activity was measured by the rat lethality assay.

[d] —, None detected.

strains cultured in the three media. In the 1095 medium, all three strains produced little serologically detectable PA, no detectable LF, and very little lethal toxin. In CA medium cultures of the Sterne and Vollum 1B strains, substantially more LF, PA, and lethal toxin were recovered than in the 1095 medium, whereas there was no appreciable change in toxin antigen recovery from strain V770-NP1-R cultures. For all three strains the highest levels of PA, LF, and lethal toxin were recovered in the R medium.

Previous efforts by investigators to produce lethal anthrax toxin in vitro have resulted in culture supernatant preparations containing fewer than 40 TU/ml (1, 6, 8, 21, 22). By use of improved cultural conditions and the R medium, we are now able to recover the toxin at levels which are twofold (Sterne and V770-NP1-R strains) to fivefold (Vollum 1B strain) greater than the highest values previously reported. In addition to its ability to support elaboration of high quantities of anthrax toxin antigens, an additional advantage of the R medium is that its nutritional components are completely defined.

Johnson and Spero (7) recovered only protective antigen from cultures of *B. anthracis* V770-NP1-R in 1095 medium. We showed in these studies that very little PA or LF is recovered from cultures of strain V770-NP1-R in 1095 or CA media. In R medium, however, we recovered almost as much lethal toxin from strain V770-NP1-R cultures as from cultures of the Sterne strain. It is therefore apparent that the specific cultural environment must be taken into consideration before comparing relative toxin antigen production by strains of *B. anthracis*. The ability to recover increased quantities of anthrax toxin antigens in the defined medium described in this study will further aid investigators in their study of mechanisms of action of the toxin components and in their attempts to develop a more efficacious human vaccine.

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