STUDIES ON THE ANAEROBIC METABOLISM OF *BACILLUS ANTHRACIS* AND *BACILLUS CEREUS*[1]

MILTON PUZISS[2] AND SYDNEY C. RITTENBERG

Department of Bacteriology, University of Southern California, Los Angeles, California

Received for publication June 25, 1956

Reports have appeared in the literature for many years on the anaerobic growth of *Bacillus anthracis*. It is questionable, however, whether the early investigators achieved true anaerobic conditions in their experiments. Basset (1933) and Germanov (1931), for example, used simple "vaseline" seals on the media tubes in which they studied anaerobic growth. In rechecking their work, Pagnini (1936) found that such tubes were not under anaerobic conditions. More recently, King and Stein (1950) and other workers reported the growth of strains of *B. anthracis* in an anaerobe jar under nitrogen gas, thereby achieving true anaerobiosis.

Both *B. anthracis* and *Bacillus cereus* are known to produce acid and acetylmethylcarbinol from carbohydrates, and these metabolic activities have been used as determinative factors in their classification (Breed *et al.*, 1948; Smith *et al.*, 1952). Otherwise few clues exist as to the nature of the anaerobic metabolism of these bacilli. Consequently, during a comparative study of the metabolism of virulent and avirulent anthrax strains (Puziss, 1956), their anaerobic metabolism also was investigated. This paper presents some of the results obtained during that study.

MATERIALS AND METHODS

Cultures. The following virulent and avirulent strains of *B. anthracis* were used: Vollum, a highly virulent proteolytic strain; NP-A, a virulent nonproteolytic mutant of Vollum; R1-NP, an avirulent, rough, nonproteolytic mutant of Vollum; Weybridge, an avirulent, rough, nonproteolytic strain. These were obtained from Dr. George G. Wright, Fort Detrick, Md. *B. cereus* strains C5-25 were obtained from Dr. Walter J. Nickerson, New Jersey Agricultural Experiment Station, New Brunswick. We wish to thank them for providing these strains.

Stable stock spore suspensions of the different organisms were prepared from washed and heated spore suspensions obtained from nutrient agar Roux slants. These suspensions were used as inocula throughout this work.

Media and methods of anaerobic culture. Two media were employed; the synthetic medium of Puziss and Wright (1954) and a nonsynthetic medium in which 7.0 g of casein hydrolyzate and 0.75 g of yeast autolysate per L were substituted for the amino acids and guanine in the synthetic medium. The nonsynthetic medium gave better growth.

Twenty-five ml of media were dispensed into test tubes (20 by 175 mm) which were capped with inverted glass centrifuge tubes (28 by 75 mm). This arrangement permitted freer gas exchange in the tube than would normally be obtained with a cotton plug. The tubes of media were sterilized, inoculated with 0.1 ml of the stock spore suspension, and placed in an anaerobe jar. A tube of medium inoculated with *Clostridium sporogenes*, another inoculated with *Pseudomonas aeruginosa*, and an uninoculated tube were used as anaerobic growth controls. A tube of methylene blue-glucose-sodium hydroxide solution was included as an indicator of anaerobiosis.

The jar was evacuated with a vacuum pump and then flushed for 10 min with a stream of nitrogen, freed of oxygen by passage through an alkaline pyrogallic acid solution. This evacuation and flushing procedure was repeated three times and a positive pressure of nitrogen was left in the jar. Incubation was at 37 C.

Identification of fermentation acids. The anaerobic cultures were autoclaved at alkaline pH, acidified, and aliquots then extracted with ether for 4 hr in an extraction apparatus developed by Neish (1952). The free acids in these concentrates were identified on paper chromatographs by the method of Stark *et al.* (1951). Examination of the ether concentrates for free keto acids was done

[1] This work was supported in part by a grant-in-aid from the Permanent Science Fund of the American Academy of Arts and Sciences.

[2] Present address: Chemical Corps Biological Laboratories, Fort Detrick, Maryland.

by the method of Magasanik and Umbarger (1950). Volatile acids in the extracts were converted to their hydroxamate derivatives and identified by paper chromatography by methods described by Wolfe *et al.* (1954).

Quantitative determination of fermentation products. After the initial autoclaving the anaerobic cultures were clarified to remove cell proteins. Aliquots were then ether extracted at neutral pH, the extracts brought to a monophase with water and assayed for solvents by colorimetric methods. Lactic acid from acid ether extracts was also assayed colorimetrically, using a lithium lactate standard curve.

An aliquot of the acid ether extract was titrated for total acids. Total volatile acids were determined by titration of steam distillates obtained from the acidified and cleared culture media. The volatile acids were assayed by titration of fractions separated on a silica gel or Celite 535[3] column. Details of these procedures are described by Neish (1952).

RESULTS

The initial experiments were performed, using the synthetic medium, to investigate the ability of the organisms to grow under strict anaerobic conditions. After 72 hr incubation, during which time the methylene blue indicator remained colorless, the pH of the medium in all of the cultures except the pseudomonad and uninoculated control tubes had dropped from an initial level of 7.3 to about 5.5. No growth was observed in either of these two controls, while *C. sporogenes* grew abundantly and gave evidence of gas production. The various anthrax strains and the *B. cereus* culture grew on the bottom of the tubes in the form of large ball-like masses which disintegrated on vigorous shaking into small granular clumps or flakes. No viable counts were made, owing to the extremely tenacious nature of the chains formed during growth.

No evidence of gas formation by any of the anthrax bacilli or by *B. cereus* was detected when the growth experiments were repeated using Durham fermentation tubes. Gorrieri (1933) also commented on the absence of gas in carbohydrate fermentation by anthrax bacilli. Preliminary Voges-Proskauer tests showed considerable acetylmethylcarbinol production by anaerobically grown anthrax bacilli and *B. cereus*, but not by *C. sporogenes*.

[3] Obtained from the Johns-Manville Corporation.

TABLE 1

*Anaerobic fermentation products identified by chromatography of acid ether extracts of 72-hr-old anaerobic cultures**

	R_f Values (× 100)						
	Standards		Culture extracts of:				
Acids	Observed values	Published values	*Bacillus cereus*	*Bacillus anthracis* strains			*Clostridium sporogenes*
				Weybridge	Vollum	R1-NP	
Citric	24	26	—	—	—	—	—
Fumaric	60	63	—	—	—	—	—
Malic	41	42	—	—	—	—	—
Lactic	73	72	74	73	76	73	—
Succinic	65	66	63	62	65	64	—
Malonic	49	48	—	—	—	—	—

* Averages of several determinations.

Microscopic examination of stained smears for purity of culture failed to reveal the presence of any spores in the anthrax or *B. cereus* cultures, whereas numerous spores were observed in the *C. sporogenes* smears. Identical results were obtained when the growth periods were extended to seven days.

Chromatography of the acid ether extract of anaerobic cultures revealed the presence of two spots from all culture extracts except that of the *C. sporogenes* and uninoculated controls. These spots had R_f values corresponding to those of the lactic acid and succinic acid standards (table 1). No keto acids in the extracts could be detected on paper chromatographs.

The peculiar odor of the medium after anaerobic growth suggested the presence of volatile acids. Accordingly, the hydroxamate derivatives prepared from the acid ether extracts were chromatographed. Two spots were detected using culture extracts of the anthrax bacilli and *B. cereus*: the R_f values of these spots corresponded to the formic and acetic hydroxamate standards. The extract from the *C. sporogenes* control showed, in addition, a third spot with an R_f value equal to that of the butyric hydroxamate standard, while extracts of the uninoculated controls were free of volatile acids. These results are presented in table 2; discrepancies between the ob-

TABLE 2

*Anaerobic fermentation products identified by chromatography of hydroxamate derivatives prepared from ether extracts of 72-hr-old anaerobic culture**

	R_f Values (× 100)						
	Hydroxamate standards		Culture extracts of:				
				Bacillus anthracis strains			
	Observed values	Published values	*Bacillus cereus*	Weybridge	Vollum	R1-NP	*Clostridium sporogenes*
Formate	53	42	57	54	56	57	55
Acetate	65	50	68	65	65	64	64
Propionate	74	62	—	—	—	—	—
Butyrate	85	72	—	—	—	—	85
Valerate	92	79	—	—	—	—	—

* Averages of several determinations.

TABLE 3

*Anaerobic fermentation products in mg per 100 ml of medium, quantitative assays on 72-hr-old cultures**

Product Assayed	*Bacillus cereus*	*Bacillus anthracis* strains	
		Weybridge	Vollum
	mg/100 ml medium		
2,3-Butylene glycol	12.2	17.1	8.3
Acetylmethylcarbinol	3.6	1.8	1.7
Glycerol	1.6	1.4	2.8
Lactic acid	6.1	3.1	8.0
Succinic acid	6.3	5.6	9.5
Formic acid	3.1	6.5	5.0
Acetic acid	7.8	8.8	10.7
Butyric acid	Nil	Nil	Nil

* Averages of several determinations.

served and published R_f values can be accounted for by differences in chromatographing temperatures.

A flask of synthetic medium inoculated with the Vollum strain was incubated aerobically at 37 C for 48 hr with constant agitation on a shaker. This culture was then extracted and tested in the same manner as the anaerobic cultures. It proved to be negative for lactic, succinic, formic, and acetic acids.

Quantitative assays were made for the acids detected by chromatography and also for certain solvents in the culture extracts. The nonsynthetic casein hydrolyzate medium, altered to contain 1.6 per cent glucose, was used to increase the yield of fermentation products. No attempt was made to obtain a complete fermentation balance. Results are presented in table 3. It can be seen that in addition to the acids a considerable amount of 2,3-butylene glycol and smaller amounts of glycerol and acetylmethylcarbinol were formed.

DISCUSSION

Although there were variations in the amounts of the different fermentation products formed, no major differences were noted among the anthrax and *B. cereus* strains studied. Further, a striking similarity was found between the products observed and those found for the Ford strain of *B. subtilis* (Neish *et al.*, 1945; Neish, 1953). In addition to the products listed in table 3, the Ford strain also produced carbon dioxide, ethanol, and butyric acid. Carbon dioxide and ethanol were not determined in our work, and butyric acid was not detected as a fermentation product of the anaerobically grown bacilli. As Neish and co-workers indicated, the products of the *B. subtilis* fermentation varied qualitatively and quantitatively with such factors as pH and media composition. It is possible that alteration of fermentation conditions would similarly affect the products of the *B. anthracis* and *B. cereus* fermentations. It can be concluded that *B. anthracis* and *B. cereus* carry out a complex heterolactic fermentation related to the *B. subtilis* (Ford strain) fermentation and it is possible that further investigations would show the same characteristic for still other *Bacillus* species.

The observed absence of spores in the anthrax cultures after anaerobic growth is in agreement with the findings of other workers that free oxygen is a factor in sporulation by these organisms (Roth and Lively, 1955). The notable absence of spores in the *in situ* bacterial cells of terminal phase anthrax in animals (Wilson and Miles, 1955) may be the result of a lowered oxygen tension (or anaerobiosis) within the host's tissues which may occur during the terminal phase of the disease.

It is evident that, given the right conditions, both *B. cereus* and *B. anthracis* are capable of an active anaerobic metabolism, although *B. cereus*

has hitherto been considered primarily as an aerobic organism. The parallelism of the fermentation pattern between these two organisms lends further weight to the hypothesis of Smith and co-workers (1952) and Brown *et al.* (1955) that the anthrax bacillus is merely a variant of *B. cereus*, differing primarily in its pathogenicity.

SUMMARY

Anaerobic growth of strains of *Bacillus anthracis* and *Bacillus cereus* occurred in both synthetic and nonsynthetic glucose-containing media. No spores were formed by either organism under anaerobic conditions. Qualitative and quantitative assays showed the formation of lactic, succinic, formic, and acetic acids, acetylmethylcarbinol, 2,3-butylene glycol and glycerol as fermentation products of glucose. No essential differences in products were found between the virulent and avirulent anthrax strains grown anaerobically. The similarity of the fermentation pattern of these organisms and the Ford strain of *Bacillus subtilis* is noteworthy.

REFERENCES

BASSET, J. 1933 La Bactéridie charbonneuse en culture anaérobie. Compt. rend. soc. biol., **113**, 786–788.

BREED, R. S., MURRAY, E. G. D., AND HITCHENS, A. P. 1948 *Bergey's manual of determinative bacteriology*, 6th ed. The Williams & Wilkins Co., Baltimore, Md.

BROWN, E. R., CHERRY, W. B., MOODY, M. D., AND GORDON, M. A. 1955 The induction of motility in *Bacillus anthracis* by means of bacteriophage lysates. Significance for the relationship of *Bacillus anthracis* to *Bacillus cereus*. J. Bacteriol., **69**, 590–602.

GERMANOV, N. E. 1931 The question of assimilation of carbohydrates from synthetic medium by *Bacillus anthracis* and anthracoides bacteria. Mikrobiol. Zhur. (U.S.S.R.), **13**, 173–175.

GORRIERI, I. 1933 L'azione fermentativa del *Bacillus anthracis* e la genesi del fenomeno di Castellani. Giorn. batteriol. immunol., **10**, 926–936.

KING, H. K. AND STEIN, J. H. 1950 The non-toxicity of *Bacillus anthracis* cell material. J. Gen. Microbiol., **4**, 48–52.

MAGASANIK, B. AND UMBARGER, H. E. 1950 The separation and identification of keto acids by filter paper chromatography. J. Am. Chem. Soc., **72**, 2308–2309.

NEISH, A. C. 1952 *Analytical methods for bacterial fermentations*. Report No. 46-8-3, 2nd revision. National Research Council of Canada, Saskatoon.

NEISH, A. C. 1953 Studies on the anaerobic dissimilation of glucose by *Bacillus subtilis*. Can. J. Botany, **31**, 265–276.

NEISH, A. C., BLACKWOOD, A. C., AND LEDINGHAM, G. A. 1945 Dissimilation of glucose by *Bacillus subtilis* (Ford's strain). Can. J. Research B., **23**, 290–296.

PAGNINI, U. 1936 Sulla biologia del *B. anthracis*; A proposito dell' articolo di J. Basset. Giorn. batteriol. immunol., **16**, 876–885.

PUZISS, M. AND WRIGHT, G. G. 1954 Studies on immunity in anthrax. IV. Factors influencing elaboration of the protective antigen of *Bacillus anthracis* in chemically-defined media. J. Bacteriol., **68**, 474–482.

PUZISS, M. 1956 Studies on the metabolism of virulent and avirulent strains of *Bacillus anthracis* and of *Bacillus cereus*. Ph.D. Dissertation, University of Southern California, Los Angeles.

ROTH, N. G. AND LIVELY, D. H. 1955 Germination of spores of certain aerobic bacilli under anaerobic conditions. J. Bacteriol., **71**, 162–166.

SMITH, N. R., GORDON, R. E., AND CLARK, F. E. 1952 *Aerobic Sporeforming Bacteria*, Agr. Monograph No. 16. U.S. Dept. Agr., Washington, D. C.

STARK, J. B., GOODBAN, A. E., AND OWENS, H. S. 1951 Paper chromatography of organic acids. Anal. Chem., **23**, 413–415.

WILSON, G. S. AND MILES, A. A. 1955 *Topley and Wilson's principles of bacteriology and immunity*, 4th ed. The Williams & Wilkins Co., Baltimore, Md.

WOLFE, J. B., IVLER, D., AND RITTENBERG, S. C. 1954 Malonate decarboxylation by *Pseudomonas fluorescens*. II. Magnesium dependency and trapping of active intermediates. J. Biol. Chem., **209**, 875–883.