NUTRITIONAL REQUIREMENTS OF SOME PUTREFACTIVE ANAEROBIC BACTERIA[1, 2]

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It has been firmly established that the methods of testing the viability of bacterial spores previously exposed to heat greatly affect their apparent resistance. Curran and Evans (1937) reported that spores of several bacilli which survived severe heat treatment appeared to be more exacting in their nutritional requirements than the less resistant spores present in the population before treatment. This finding has led to the use of enrichment substances in media used for recovery of severely heated spores.

We have recently shown that the composition of complex recovery media greatly influences the apparent thermal resistance of spores of putrefactive anaerobe 3679 (Frank and Campbell, 1955).

While complex culture media are satisfactory for routine studies on spore recovery, the use of chemically defined media would permit a study and evaluation of the influence of the nutritional environment on recovery of heat-treated spores. Before such media can be formulated, however, information is needed on the nutritive requirements of the organisms concerned. With this in mind we have studied the nutritional requirements of a group of closely related putrefactive anaerobic spore-forming bacteria responsible for the spoilage of thermal processed foods. The present paper reports the results of our findings.

METHODS AND MATERIALS

Cultures. Ten strains of putrefactive anaerobe 3679 (P. A. 3679), three strains of *Clostridium parabotulinum* and one strain of *Clostridium sporogenes* were employed. The cultures were obtained from the following sources: P. A. 3679 strain A, Dr. J. C. Ayres; strain B, A. T. C. C. No. 7955; strain C, National Canners Association, Berkeley, California; strain D, Dr. O. B. Williams; strain E, Dr. W. B. Esselen; strain G, National Canners Association, Washington, D. C.; strain H, Dr. C. R. Stumbo; strain J, Dr. H. R. Curran; strain K, Dr. H. Reynolds; strain L, Dr. E. S. Wynne; *Clostridium parabotulinum* strain 172 (type A), strain 213 (type B), strain 221 (nontoxic), Dr. K. F. Meyer; *Clostridium sporogenes* strain 31G, Dr. H. A. Barker. All cultures were checked for purity upon receipt. Stock cultures were maintained as spore suspensions in liver infusion broth prepared according to Stumbo *et al.* (1950).

Materials. Pyrex glassware and deionized distilled water were used throughout. Flasks and tubes were washed with "Alconox" detergent, rinsed thoroughly in tap and distilled water and placed in a hot air oven at 200 C for 12 hours. Pipettes were cleaned by soaking in potassium dichromate cleaning solution, rinsed thoroughly in tap and distilled water and allowed to air dry.

Media. The complete chemically defined medium employed was a modification of that described by Mager *et al.* (1954) for *Clostridium parabotulinum.* The composition of the modified medium is given in table 1. This medium supported good growth of all cultures through six serial transfers.

For the determination of the nutritional requirements of the cultures, individual amino acids and vitamins were omitted from the complete medium. The various deletion media were prepared in screw cap bottles, adjusted to pH 7.4, and autoclaved at 121 C for 20 minutes. Glucose was added aseptically from a Seitz-filtered solution. The respective media were pipetted aseptically into sterile 16 × 150 mm tubes in 7.0-ml amounts. The tubes were steamed for 10 minutes, cooled to room temperature and inoculated immediately.

Inocula. Media were inoculated with 0.05 ml

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TABLE 1

Synthetic medium employed for the determination of the nutritional requirements of putrefactive anaerobic bacteria

Constituent	Amount	Constituent	Amount
	g		
L-Tryptophan	0.05	DL-Serine	0.25 g
L-Arginine	3.0	Thiamin	0.40 mg
DL-Phenylalanine	2.0	Biotin	0.5 μg
L-Tyrosine	0.25	*p*-Aminobenzoic acid	10.0 μg
DL-Valine	2.0	Nicotinic acid	1.0 mg
L-Leucine	1.5	Folic acid	10.0 μg
DL-Isoleucine	0.50	Pantothenic acid	1.0 mg
DL-Threonine	1.0	Pyridoxal	0.5 mg
DL-Methionine	0.60	Glucose	5.0 g
L-Proline	0.45	Cysteine	0.25 g
L-Histidine	0.20	Sodium thioglycolate	0.50 g
L-Glutamic acid	0.50	Salts A*	10.0 ml
L-Aspartic acid	0.45	Salts B†	1.0 ml
L-Lysine	0.60	Deionized distilled water	to 1000 ml
DL-Alanine	0.42		

* Salts A: K_2HPO_4, 25 g; KH_2PO_4, 25 g; distilled water, 250 ml.

† Salts B: $MgSO_4 \cdot 7H_2O$, 10 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $MnSO_4 \cdot 4H_2O$, 0.5 g; distilled water, 250 ml.

TABLE 2

Nutritional requirements of some putrefactive anaerobic bacteria

Amino Acids and Vitamins	Organism													
	P. A. 3679										*Clostridium parabotulinum*			*Clostridium sporogenes*
	Strain										Strain			Strain
	A	B	C	D	E	G	H	J	K	L	172	213	221	31G
Tryptophan	+	−	+	−	−	−	+	−	+	−	+	−	−	−
Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leucine	−	−	−	−	−	+	+	−	−	−	+	−	−	−
Isoleucine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Threonine	+	−	+	+	−	+	−	−	+	−	+	−	−	−
Methionine	−	−	−	+	−	−	−	−	−	−	+	−	−	−
Proline	+	+	+	+	−	−	−	−	−	−	−	−	−	−
Histidine	+	+	+	−	−	+	−	−	+	−	−	−	−	−
Glutamic acid	+	−	+	+	−	−	−	−	+	−	−	+	−	−
Aspartic acid	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Lysine	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Alanine	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Serine	+	+	+	+	+	+	+	+	+	+	−	−	−	−
Thiamin	−	+	+	+	+	+	−	+	+	−	+	−	−	−
Biotin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
p-Aminobenzoic acid	−	−	+	−	−	−	−	−	+	−	+	−	−	+
Nicotinic acid	+	−	+	−	−	−	+	−	+	−	−	−	−	+
Folic acid	−	−	+	−	+	+	−	+	−	−	−	−	−	−
Panthothenic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pyridoxal	−	−	−	−	−	−	−	−	−	−	−	−	−	−

\+ indicates requirement.

− indicates no requirement.

of 24-hour cultures grown in fluid thioglycolate medium (Difco) at 30 C. In some experiments the inoculum was washed with 0.01 M phosphate buffer, pH 7.0, containing 0.05 per cent sodium thioglycolate. In general, however, washing was omitted due to its harmful effect on viability. Carry-over of growth factors could be eliminated by serial transfers.

Incubation and estimation of growth. After inoculation the tubes were incubated at 30 C for 48 hours in the absence of oxygen (potassium carbonate-pyrogallol seal). Growth response was determined by optical density measurement at 525 mμ with a "Spectronic 20" colorimeter (Bausch and Lomb). Uninoculated media served as controls.

RESULTS AND DISCUSSION

Table 2 summarizes the nutritional requirements of the organisms studied. The data presented are representative of results obtained in at least five separate determinations. In the deletion media supporting growth, the optical density values ranged from 0.40 to 1.2, depending on the particular strain.

The data presented in table 2 show that all of the cultures studied required arginine, phenylalanine, tyrosine, valine, isoleucine, and biotin for growth. Our findings with *C. parabotulinum* (type A) and *C. sporogenes* are in essential agreement with those reported by Mager *et al.* (1954) for *C. parabotulinum* and by Shull *et al.* (1949) for *C. sporogenes.*

It is interesting to note that all strains of P. A. 3679 required serine for growth, while the *C. parabotulinum* and *C. sporogenes* cultures did not. It has been shown previously that serine is not a requirement for the latter two organisms (Mager *et al.*, 1954; Fildes and Richardson 1935; Shull *et al.*, 1949).

While P. A. 3679 is closely related to *C. sporogenes*, its taxonomic position is not clear. Gross *et al.* (1946) have reported that it is culturally and serologically distinct from a strain (Spray) of *C. sporogenes*. The finding that P. A. 3679 requires serine for growth while *C. sporogenes* does not, suggests that the serine requirement may serve as a useful character in establishing the taxonomic position of this organism. The validity of employing nutritional requirements as taxonomic characters has been discussed recently by Stanier (1953) and Knight (1955).

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SUMMARY

The nutritional requirements of a group of closely related spore-forming putrefactive anaerobes responsible for food spoilage have been determined. While the requirements varied depending on the organism, all cultures required arginine, phenylalanine, tyrosine, valine, isoleucine, and biotin. In addition, all strains of putrefactive anaerobe (P. A. 3679) required serine while *Clostridium parabotulinum* and *Clostridium sporogenes* did not. It was suggested that the requirement for serine might serve as a useful character in establishing the taxonomic position of the putrefactive anaerobes.

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